A word from the editor

The book series “MARINE ECOLOGY – A Comprehensive Treatise on Life in Oceans and Coastal Waters” (organized and edited by Otto Kinne and contributed to by numerous outstanding experts over years) is now freely available with online Open Access.

Published in the years 1970 to 1984, the copyright for MARINE ECOLOGY was graciously transferred in 2008 by the original publisher, Wiley & Sons, to the editor.

The technical problems involved in the re-publication of the Treatise were mastered by Konstantin Kambach (Inter-Research). Unavoidably, the print quality of the final product is somewhat inferior to the original.

Otto Kinne

Oldendorf/Luhe
29.04.2008
FOREWORD

to

VOLUME III: CULTIVATION

'Cultivation' reviews the information which has accumulated on our present capacity for supporting marine micro-organisms, plants and animals under environmental and nutritive conditions which are, to a considerable degree, controlled. The volume is subdivided into three parts, containing the following chapters:

Part 1

Chapter 1: Introduction to Volume III
Chapter 2: Cultivation of Marine Organisms:
  Water-quality Management and Technology
Chapter 3: Cultivation of Micro-organisms
Chapter 4: Cultivation of Plants

Part 2

Chapter 5: Cultivation of Animals
Chapter 6: Multispecies Cultures and Microcosms
Chapter 7: Chemical Contamination of Culture Media:
  Assessment, Avoidance and Control

Part 3

Chapter 8: Diseases of Plants
Chapter 9: Diseases of Animals

We have made every effort to present comprehensive reviews, covering essential aspects of the cultivation of marine organisms. It soon became apparent, however, that only in a few cases, comparative, critical assessments of different culture methods and technologies were possible. Many publications suffer from insufficient detail, or even total lack of information regarding source, environmental history and nutrition of the organisms cultivated or the culture method employed. Exact data on environmental factors—such as light, temperature, salinity or dissolved gases—and on diet are absolute requirements for proper evaluation of the results presented. No less important are the origin of the organisms concerned, culture-water quality and technological aspects.

Culture methods are often an outcome of empiricism and intuition. A technique is tried, and if it works, the investigator sticks with it, rationalizing only afterwards the reasons for its application and success. The factors truly critical to success have rarely been pinpointed. Some portions of the reviews presented must, therefore, remain tentative, descriptive or pragmatical.
Cultivation is not an end in itself. It serves as a means to solve specific research problems. Due to the large variety of problems and the overwhelming diversity of marine life, a multitude of different culture methods have been developed. In fact, concepts, goals and techniques applied in cultivation diverge more than in other branches of marine ecology.

Most experiments conducted on marine organisms involve elements of cultivation. Micro-organisms, crustaceans, molluscs and fishes, for example, have been maintained, reared or bred in thousands of experiments. It was neither possible nor desirable to consider all publications in detail. We have attempted to settle the conflict between our intention to present comprehensive accounts and the need to avoid undue repetition by tabulating the information at hand or by referring to pertinent books or reviews.

It was considered desirable to include chapters on chemical contamination of culture media, as well as on diseases of plants and animals. There is reason to assume that contamination and disease interfere with successful cultivation more often than realized.

I acknowledge with pleasure the support, advice and criticism received from the contributors, as well as from Drs. D. F. A. ALDERDICE, J. R. BRETT, H. P. BULNHEIM, G. FERSOONE, A. GAERTNER and D. SEEERS. Additional supporters are mentioned at the end of the respective chapters. The assistance of M. BLAKE, V. CLARK, J. MARSHALL, H. L. NICHOLS, I. SCHRITT and H. WITT is deeply appreciated.

O.K.
CONTENTS
OF
VOLUME III, PART 1

Chapter 1 Introduction to Volume III

(1) History ........................................ 1
(2) Terminology ..................................... 2
(3) Goals of research cultivation and commercial cultivation ................. 6
(4) Ecological implications ................................ 7
(5) Part 1: Comments on Chapters 2 to 4 ...................................... 10
(6) Part 2: Comments on Chapters 5 to 7 ....................................... 13
(7) Part 3: Comments on Chapters 8 and 9 ..................................... 15
Literature cited .................................. 16

Chapter 2 Cultivation of Marine Organisms: Water-quality Management and Technology

(1) General aspects .................................. 19
(2) Sea water as culture medium .............................................. 20
   (a) Natural sea water .......................................... 21
   (b) Artificial sea water .......................................... 29
   (c) Conclusions .............................................. 36
(3) Culture systems .................................... 37
   (a) Open sea-water systems ..................................... 39
   (b) Semi-open sea-water systems ............................... 42
   (c) Closed sea-water systems .................................. 42
   (d) Multipurpose environmental systems ...................... 47
   (e) Conclusions .............................................. 57
(4) Culture enclosures .................................. 57
   (a) Small enclosures .......................................... 57
   (b) Medium-sized enclosures .................................. 58
   (c) Large enclosures .......................................... 68
   (d) In situ enclosures and related devices .................. 69
   (e) Conclusions .............................................. 75
(5) Life-supporting substances ............................................. 76
(6) Life-endangering substances ........................................... 79
   (a) Ectocrines .............................................. 79
(b) End products of nitrogen metabolism
   Ammonia
   Rates of ammonia excretion
   Biological consequences of ammonia accumulation
   Counteraction of NH₃ poisoning in closed culture systems
   Ammonia determination
   Nitrite and nitrate
   Other inorganic substances
   Organic substances
   Conclusions

(7) Culture-water treatment
   (a) General aspects
      Principal types of water treatment
      Disinfection and sterilization
      Sea water and other culture media
      Air
      Glassware and other equipment
      Parameters of water-quality assessment
   (b) Mechanical water treatment
      Sand-gravel filters
      Types, function and filtrant material
      Maintenance
      Rapid sand filters
      Diatomaceous-earth filters
      Disposable cartridge filters
      Other filters
   (c) Biological water treatment
      Microbial water treatment
      General aspects
      Microbial filtration in mechanical filters
      Microbial filtration in activated sludge
      Algal water treatment
   (d) Physico-chemical water treatment
      Activated carbon (charcoal) adsorption
      Foam separation
      Aeration
      Gas requirements
      Advantages of aeration
      Disadvantages of aeration
      Pretreatment of air
      Dynamics and theory of aeration
      Oxygenation
      Ozonation
      Ultra-violet irradiation
      Ion-exchange
      Other techniques
   (e) Conclusions
<table>
<thead>
<tr>
<th>CONTENTS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(8) Capacity of culture systems for supporting aquatic animals</td>
<td>166</td>
</tr>
<tr>
<td>(a) General aspects</td>
<td>166</td>
</tr>
<tr>
<td>(b) Assessment of carrying capacity</td>
<td>167</td>
</tr>
<tr>
<td>(c) Standards for closed-culture system design</td>
<td>179</td>
</tr>
<tr>
<td>(d) Relationships between metabolic byproduct concentrations and</td>
<td>179</td>
</tr>
<tr>
<td>culture-water reuse</td>
<td></td>
</tr>
<tr>
<td>(e) Conclusions</td>
<td>181</td>
</tr>
<tr>
<td>(9) Equipment used in cultivation</td>
<td>182</td>
</tr>
<tr>
<td>(a) Basic equipment</td>
<td>183</td>
</tr>
<tr>
<td>Collection of organisms</td>
<td>183</td>
</tr>
<tr>
<td>Overflow sieves</td>
<td>183</td>
</tr>
<tr>
<td>Aerators</td>
<td>183</td>
</tr>
<tr>
<td>Airlift</td>
<td>190</td>
</tr>
<tr>
<td>Float valve and constant-level siphon</td>
<td>191</td>
</tr>
<tr>
<td>Culture-tank cleaner</td>
<td>195</td>
</tr>
<tr>
<td>Flow-direction reverser</td>
<td>196</td>
</tr>
<tr>
<td>Equipment for controlling environmental factors</td>
<td>196</td>
</tr>
<tr>
<td>Light</td>
<td>197</td>
</tr>
<tr>
<td>Light and temperature</td>
<td>198</td>
</tr>
<tr>
<td>Temperature</td>
<td>199</td>
</tr>
<tr>
<td>Salinity</td>
<td>200</td>
</tr>
<tr>
<td>Water movement</td>
<td>201</td>
</tr>
<tr>
<td>Dissolved gases</td>
<td>206</td>
</tr>
<tr>
<td>Environmental gradients</td>
<td>206</td>
</tr>
<tr>
<td>Small recirculation devices</td>
<td>207</td>
</tr>
<tr>
<td>Continuous-culture devices</td>
<td>210</td>
</tr>
<tr>
<td>Chemostat</td>
<td>212</td>
</tr>
<tr>
<td>Turbidostat</td>
<td>215</td>
</tr>
<tr>
<td>(b) Specific equipment</td>
<td>218</td>
</tr>
<tr>
<td>Dialysis cultures</td>
<td>218</td>
</tr>
<tr>
<td>Accumulation and isolation of unicellular and small multicellular</td>
<td>218</td>
</tr>
<tr>
<td>organisms</td>
<td></td>
</tr>
<tr>
<td>Phytoplankton harvester</td>
<td>223</td>
</tr>
<tr>
<td>In situ sporophyte culture system</td>
<td>224</td>
</tr>
<tr>
<td>Spray apparatus for cultivating seaweeds</td>
<td>226</td>
</tr>
<tr>
<td>Apparatus for cultivating plankters</td>
<td>226</td>
</tr>
<tr>
<td>Apparatus for cultivating sessile plankton feeders</td>
<td>244</td>
</tr>
<tr>
<td>Apparatus for cultivating substrate dwellers</td>
<td>245</td>
</tr>
<tr>
<td>Apparatus for cultivating intertidal plants and animals</td>
<td>246</td>
</tr>
<tr>
<td>Holding and experimental tanks for fishes</td>
<td>253</td>
</tr>
<tr>
<td>Feeding equipment used in commercial fish culture</td>
<td>259</td>
</tr>
<tr>
<td>(c) Conclusions</td>
<td>265</td>
</tr>
<tr>
<td>Literature cited</td>
<td>268</td>
</tr>
</tbody>
</table>
Chapter 3 Cultivation of Micro-organisms

3.1 Bacteria

(1) Introduction

(2) Growth requirements
   (a) Organic nutrients
      Carbon sources
      Carbon-nitrogen sources
      Vitamins
   (b) Mineral requirements
      Nitrogen, phosphorus and sulphur
      Calcium and magnesium
      Sodium and potassium
      Iron and trace elements
      Carbon dioxide
   (c) Sea water
      Natural sea water
      Artificial sea water
      Osmotic requirements
      Particulate matter
   (d) Hydrogen-ion concentration
   (e) Composition of culture media
      Complex (oligic and meridic) media
      Defined (holitic) media
   (f) Oxygen and other electron acceptors
   (g) Light
   (h) Temperature
   (i) Hydrostatic pressure

(3) Culture methods
   (a) Basic culture techniques
      Solid media
      Liquid media
      Diagnostic media
      Adjustment of pH
      Sterilization
      Preparation of inocula
      Aerobic and anaerobic cultivation
      Measuring bacterial growth
      Maintenance of cultures
      Stock culture collections
   (b) Special culture techniques
      The chemostat
      Hydrostatic pressure cultivation
      Controlled model ecosystem (microcosms)
      The biochemical potential technique
### In situ techniques
- Submerged slide
- The peloscope
- *In situ* incubators

### Physiological types

#### (a) Heterotrophs
- Proteolytic bacteria
- Cellulose-decomposing bacteria
- Agar-digesting bacteria
- Chitin-digesting bacteria
- Lipid and hydrocarbon decomposers

#### (b) Photosynthetic bacteria
- Purple bacteria
- Green bacteria

#### (c) Chemo-autotrophic bacteria
- Sulphur bacteria
- Nitrifying bacteria
- Iron and manganese bacteria
- Hydrogen bacteria

#### (d) Actinomycetes

#### (e) Bacteriophages and bdellovibrio

### 3.2 Fungi

#### 3.21 Lower Fungi, Asco- and Deuteromycetes

1. Introduction
2. Lower fungi (Phycomycetes): Chytridiomycetes and Oomycetes
   - Baiting
   - Plating
3. Labyrinthula
4. Ascomycetes (excluding yeasts) and Deuteromycetes (Fungi imperfecti)
   - Enrichment and isolation
   - Sustenance

#### 3.22 Yeasts

1. Introduction
2. Isolation and cultivation
   - Yeasts from water samples
   - Yeasts from marine sediments
   - Yeasts from marine plants
   - Yeasts from marine animals
   - Selective cultivation
3. The importance of environmental factors in yeast cultivation
4. Cultivation of yeasts in natural sea water
(5) Some aspects of the use of yeast cultivation

Literature cited

Chapter 4 Cultivation of Plants

4.1 Unicellular Plants

(1) Introduction

(a) Importance, purpose and scope of unicellular algae cultivation
(b) Availability of unicellular algae species for cultivation

(2) Laboratory culture

(a) Technological and procedural aspects

(b) Chemical aspects

(b) Physical aspects

(3) Outdoor cultures

(a) Pool and tank cultures
(b) In situ cultures
(c) Pond and embayment cultures

(4) Harvesting

(a) Methods
(b) Yield determination
4.2 Multicellular Plants

(1) Introduction
   (a) Importance of multicellular algae for scientific purposes
   (b) Importance of multicellular algae for economic purposes
      Food for man
      Food for animals
      Medicine
      Manure
      Industrial uses
   (c) Importance of Spermatophyta (sea grasses, mangroves and salt-marsh plants)

(2) Cultivation of multicellular algae in nature and under laboratory conditions
   (a) Cultivation in nature
   (b) Cultivation under laboratory conditions
   (c) Culture media
   (d) Apparatus for cultivation and experimentation

(3) Green algae
   (a) Commercial importance
   (b) Cultivation
      Genus Monostroma
      Genus Ulva
      Genus Enteromorpha

(4) Brown algae
   (a) Commercial importance
   (b) Cultivation
      Genus Laminaria
      Genus Undaria
      Genus Macrocystis

(5) Red algae
   (a) Commercial importance
   (b) Cultivation
      Genus Porphyra
      Genus Gracilaria

(6) Multicellular algae: conclusions

(7) Sea grasses

(8) Mangroves

(9) The salt-marsh plants

(10) Spermatophyta: conclusions

Literature cited

Author Index

Taxonomic Index

Subject Index
CONTRIBUTORS
TO
VOLUME III, PART 1

Bonotto, S., Laboratoires du C.E.N./S.C.K., Boeretang 200, B-2400 Mol, Belgium.

Gundersen, K., University of Göteborg, Department of Microbiology, Botanical Institute, S-413 19 Göteborg, Sweden.


Kinne, O., Biologische Anstalt Helgoland (Zentrale), Palmville 9, 2000 Hamburg 50, Federal Republic of Germany.


Ukeles, R., National Marine Fisheries Service, Biological Laboratory, Milford, Connecticut 06460, USA.
CULTIVATION
1. INTRODUCTION TO VOLUME III

O. Kinne

(1) History

Man's efforts in cultivation have a long history. The original driving forces were hunger and search for pleasure. The first plants and animals cultivated served as sources of food and/or aesthetic satisfaction. Later, additional benefits were obtained, such as a variety of materials, cheap labour and recreational assets.

The first plants were cultivated in the pre-Neanderthal period, the first animals more than 100,000 years ago, when early man began to domesticate the dog. Cattle and sheep were presumably first cultivated in the Far East, and became important agricultural animals in Europe during the New Stone Age (neolithic culture). Horses followed around 3000 B.C., chickens 1500 B.C., and cats and fishes about 1000 B.C. Cultivation of fishes for food and as pets was practised by the ancient Chinese, Greeks and Romans. The cultivation of molluscs and crustaceans began much later. Pond cultivation commenced in many European countries during the early Middle Ages. In 1358, the first carp ponds were built in Czechoslovakia. During the following 3 centuries, a variety of ponds were developed, with special designs for holding, growing, fattening, overwintering, spawning and hatching. Actual management of fish ponds is less than a half century old, and management of individual water bodies (e.g. introduction of selected species, control of overabundant species, fertilization, controlled manipulation of water-flow dynamics) is, even today, more a goal than an achievement (Volume V).

Throughout most of its long history, cultivation has been an art rather than a science. Cultivation of aquatic organisms has received less attention and effort than that of terrestrial organisms. For a long time, the major asset of the aquatic cultivator has been a knack for growing things, a 'wet thumb', as it were. Vagueness and empiricism have dominated. In some countries, progress has been impeded by superstition and tradition. Sound system dimensioning and technological designing began only a few decades ago. Exactness of procedure and reproducibility of results are still low in many projects, both in research cultivation and commercial cultivation.

The development of facilities and equipment has long remained behind the priority placed on research itself. Technical progress has depended almost exclusively on the individual experimenter. Engineers have entered the scene only recently. To their surprise, they found little information that could be used for system design or for development of new facilities and equipment. While the make-shift period has come to an end, we are still occupied with laying the foundation for proper system designing. More information on biological functions, nutrition, multi-species cultures (Chapter 6), chemical contamination (Chapter 7) and diseases (Chapters 8, 9) is required before in-depth engineering can revolutionize the cultivation of marine organisms.
A general assessment, made in 1969, revealed that less than 2% of all known marine organisms had been cultivated through their entire life cycle under controlled environmental and nutritive conditions (Kinne, 1970). Such inadequacy has critically constricted the flow of information on life in oceans and coastal waters. Fortunately, in the last few years, many nations have increased their efforts to cultivate marine organisms. Important new information has become available on life cycles, water chemistry, management of captive sea water and nutritional requirements. As a consequence, more organisms, including some rather stenoplastic forms, can now be cultivated, and are available for ecological experimentation.

At this writing, cultivation of marine organisms is receiving unparalleled attention. It has become a focal point of marine research and one of the most intensively supported branches of marine ecology.

2. Terminology

The terminology employed in cultivation studies is not consistent. It reflects historical aspects, as well as divergencies in perspective, emphasis and aim of various schools of thought and different biological disciplines. In order to provide some degree of uniformity, a number of terms are defined or explained below, and are used as consistently as possible throughout this volume.

The term 'cultivation' comprises the provision or enhancement by man of life-supporting conditions which are, to a considerable degree, controlled. In most cases, cultivation involves intervention in natural ecological dynamics and the removal and isolation of subsystems (e.g. members of one or several species) from a more complex natural system.

Three levels of cultivation may be distinguished: maintenance (keeping alive), rearing or raising (bringing up fertilized eggs, larvae or older ontogenetic stages) and breeding (production of offspring). Rearing includes maintenance; breeding includes maintenance and rearing.

Aquaculture (commercial cultivation) is parallel in connotation to agriculture (and hence, sometimes spelled aquiculture). It involves aquafood production for commercial purposes and includes measures, such as supervision or management, adopted by man in order to obtain increased benefits from living natural resources. Aquaculture comprises two aspects: applied ecological research and profit-making considerations. We are concerned here primarily with the former. The objects of aquaculture are either marine organisms (mariculture) or limnic organisms (limniculture).

Farming of marine organisms refers to activities comparable to those of a farmer of terrestrial plants or animals.

Domestication of animals implies close relationships to man (e.g. space restriction, tameness, controlled breeding), who provides nutrients and specific living conditions, and obtains, in return, food, energy and other resources or amenities (aesthetic satisfaction); long-term domestication usually leads to genetic and non-genetic changes in functions and structures.

Stock improvement refers to controlled breeding and selection with the aim of enhancing characteristics which are useful to man (e.g. faster and more efficient growth).
growth, better taste or higher aesthetic value); it requires selection and/or genetic recombination.

Continuous cultures are characterized by continuous passage of life-supporting medium and removal of a portion of the population cultivated. In microbiology, continuous cultures have received detailed attention. Criteria for system classifica-

**HOMOGENEOUS SYSTEMS**

- Single-stage systems
  - (i) Stirred fermentor
  - (ii) Stirred fermentor with feedback

- Multi-stage systems
  - (i) Simple chain
  - (ii) Multiple substrate addition

**HETEROGENEOUS SYSTEMS**

- Single-phase system
  - (i) Pipe flow with feedback

- Multi-phase packed towers
  - (i) Liquid—liquid
  - (ii) Liquid—gas

**MIXED SYSTEM**

- Stirred tank feeding tubular reactor

Fig. 1-1: Continuous cultures: 'open' microbial culture systems. S: substrate addition; C: continuous centrifuge or settling tank. (After HERBERT, 1961; modified; reproduced by permission of Society of Chemical Industry, London.)

Batch cultures are non-continuous cultures. No medium is added and no product removed. Hence, the micro-environment tends to change during the course of the experiment.
Serial cultures involve a sequence of subcultures, each inoculated with material from its predecessors. The term serially renewed batch cultures refers to a serial culture which consists of sequential batch cultures. Routine sustenance of microbial stock cultures is usually based on serially renewed batch cultures.

**HOMOGENEOUS SYSTEMS**
1. Cellulose bag cultures
2. Stirred fermentor with 100% feed-back of cells

**HETEROGENEOUS SYSTEMS**
1. Single-phase systems
   - Pipe flow with 100% feed-back of cells
   - Partitioned tank with 100% feed-back of cells
2. Two-phase systems
   - Pellicle growth
   - Packed towers

Fig. 1-2: Continuous cultures: 'closed' microbial culture systems. S: substrate addition; C: continuous centrifuge or settling tank. (After Herbert, 1961; modified; reproduced by permission of Society of Chemical Industry, London.)

Special terms have been coined by microbiologists, botanists and invertebrate zoologists to designate cultures consisting of one or more taxonomic components. One group of terms refers to the total number of species present; another group of terms has been employed by students of nutritional requirements, who strive to isolate their test organism from all others, including bacteria (but not viruses), and who consider all other elements 'contaminants' (Table 1-1).

**Monospecific cultures** consist of one or more individuals of a single, known (taxonomically defined, but not necessarily named) species, the monobiont. Monospecific is parallel to axenic (from the Greek: α = without; xenos = foreign), a term introduced by microbiologists to indicate the absence of foreign, contaminating organisms,
i.e. of species other than the one cultivated, the axenobiont. Both terms, monospecific and axenic, imply that the cultured organism can be supported on non-living material. Apparently, BOGDANOVA (1908) was the first author to grow individuals of one species (the bluebottle fly Calliphora sp.) in the absence of organisms belonging to other species. REYNERS and co-authors (1949) and DOUGHERTY (1963) discussed and defined 'axenic', as well as related terms. From axenic, derive the terms axenity or axenicity (state of being axenic) and axenize (to render axenic). Plant pathologists use the terms axenity or axenic to designate the inability of an organism to serve as host (GAUMAN, 1946, p. 300; see also GAUMAN, 1951).

Table 1.1

<p>| Terminology of cultures consisting of one or more taxonomic components (Original) |
|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Term</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monospecific (pure)</td>
<td>1 known species</td>
</tr>
<tr>
<td>Synspecific</td>
<td>2 or more known species</td>
</tr>
<tr>
<td>dispecific</td>
<td>2 species</td>
</tr>
<tr>
<td>trispecific</td>
<td>3 species</td>
</tr>
<tr>
<td>tetraspecific</td>
<td>4 species</td>
</tr>
<tr>
<td>polyspecific</td>
<td>many species</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Term</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axenic</td>
<td>Axenobiont + 0 known species</td>
</tr>
<tr>
<td>Synxenic</td>
<td>Axenobiont + 1 or more known species</td>
</tr>
<tr>
<td>monoxenic</td>
<td>Axenobiont + 1 species</td>
</tr>
<tr>
<td>dixenic</td>
<td>Axenobiont + 2 species</td>
</tr>
<tr>
<td>trixenic</td>
<td>Axenobiont + 3 species</td>
</tr>
<tr>
<td>polyxenic</td>
<td>Axenobiont + many species</td>
</tr>
</tbody>
</table>

Synspecific cultures (from the Greek: syn = together) consist of members of two or more known species. The parallel form, synxenic, refers to cultures in which the axenobiont exists together with one or more known species. The presence of 2, 3, 4 or many species is expressed by the terms dispecific, trispecific, tetraspecific or polyspecific; the presence of 1, 2, 3 or many foreign species is expressed by the terms monoxenic, dixenic, trixenic or polyxenic. From synxenic, derive the terms synxenobiont, synxenity and synxenize, parallel to the derivatives obtained from axenic (see above).

Gnotospecific or gnotobiotic cultures (from the Greek: gnos = known; bios = life) are those in which all organisms present are known. These terms comprise monospecific or axenic, as well as synspecific or synxenic cultures. Agnotospecific or agnotobiotic cultures contain—in addition to the test organism—members of unknown species. Agnotobiotic is parallel to non-axenic, and is sometimes also referred to as xenic.
Bacteria-free cultures contain organisms cultivated in the absence of active bacterial stages.

Pure cultures consist of members of one species only; this term has, however, also been used to designate non-bacteria-free cultures of one species.

The terminology of basic media used in cultivation is listed in Table 1-2. Two basic groups of media may be distinguished: non-nutrient media (e.g., natural or artificial sea water) and nutrient media. Especially in regard to micro-organisms and plants, exact differentiation between these two groups of media is not always possible. In holidic media, all nutrients present are chemically defined. Holistic media may range from simple mineral mixtures for photo-autotrophs to elaborate mixtures for heterotrophs, containing numerous organic and inorganic components.

(3) Goals of Research Cultivation and Commercial Cultivation

Aquatic organisms are cultivated for scientific purposes and for commercial ends. The first aspect is referred to as research cultivation, the second as commercial cultivation or aquaculture. Commercial cultivation includes the home-aquarist (pet-fish) industry. While it concentrates on producing food and raw materials for human needs, as well as for aesthetic satisfaction, its progress depends upon research. This is where the two aspects overlap. Both research cultivation and commercial
cultivation root in the ecology of the species concerned. However, research cultivation and commercial cultivation are directed toward different goals.

The major goals of research cultivation are:

(i) to increase basic knowledge on living systems in the marine environment (e.g. assessment of organismic responses to environmental variation, analysis of intra- and interspecific dynamics of co-existence, investigation of life cycles, taxonomy and evolution);

(ii) to determine nutritional requirements;

(iii) to provide assay and test organisms, i.e. populations with defined genetic and environmental backgrounds, for bio-assays and for solving research problems (e.g. in genetics, biochemistry or environmental protection);

(iv) to produce food for other culture organisms;

(v) to establish environmental requirements for the continued existence of species and multispecies assemblages.

The major goals of commercial cultivation (aquaculture) are:

(i) to make profits;

(ii) to develop techniques which minimize the costs for energy, equipment, building and personnel involved in the production of food, raw materials or aesthetic satisfaction;

(iii) to modify the environmental and/or genetic background of cultured organisms, in order to enhance economical mass production of swift-growing, pleasant-tasting forms with increased tolerances to the stresses of domestication (i.e. space limitation, diseases due to crowding, water pollution), and with capacities for converting low-cost food with maximum efficiency;

(iv) to work out alternatives to sampling and hunting of sea food;

(v) to augment and manage biotic resources required by a human population which threatens to outgrow its natural endowments.

Research cultivation and commercial cultivation are usually characterized by different dimensions, and often require different techniques. We have included a brief review on aquaculture in this volume, because (i) research cultivation and commercial cultivation have a common root; (ii) both fields mutually stimulate each other; (iii) both fields are of obvious importance for the future of mankind. Scientific and technological aspects of aquaculture have been treated in preference to economic ones.

(4) Ecological Implications

For millions of years, marine organisms have been surrounded by a vast aquatorium, characterized by a higher degree of overall physico-chemical stability than most other habitats on earth. Aquatic micro-organisms, plants, invertebrates and fishes entertain immediate contact with their external medium and, through a multitude of exchange equilibria, remain captives of the properties of the surrounding water. These extremely intimate interrelationships between aquatic organisms and their environment must be of primary concern to all ecologists engaged in cultivation.

Cultivation of micro-organisms, plants and animals provides an essential avenue
for analyzing and comprehending the forces which govern life in oceans and coastal waters at the individual, population and ecosystem levels. In order to investigate details of organism-environment and organism-organism interrelations, we must learn how to sustain the organisms concerned under adequate, controlled and reproducible conditions, both environmental and nutritive. Cultivation helps to provide the data necessary for: (i) increasing our knowledge on ecological dynamics in the seas, (ii) proper management of our living resources, and (iii) developing sound measures of environmental protection.

More and more scientists, administrators and statesmen are realizing that man's fate depends on his ability to analyze, comprehend and manage essential functions and structures of natural multispecies systems. Cultivation goes a long way toward producing important information urgently required for assessing the characteristics of multispecies systems.

Cultivation is an indispensable tool, not only for the ecologist, but also for the physiologist, biochemist, geneticist, evolutionist and taxonomist. Ecologists focus attention on whole individuals, populations or multispecies assemblages. Hence, cultures of cells and tissues obtained from multicellular plants or animals are not treated here.

Experimental ecologists who employ cultivation as a means of investigating the dynamics of organismic co-existence in nature—both affected and unaffected by the activities of man—must create conditions in their experiments which allow conceptual extrapolations to the situation prevailing in unconstrained marine habitats. They must satisfy the basic, normal environmental and nutritional requirements of the organisms cultured, and at the same time, devise a manageable, controllable micro-environment. In many cases, this is a formidable task.

As has been pointed out above (p. 2), cultivation involves the removal and isolation of subsystems from more complex natural systems. While such isolation is a prerequisite for detailed analyses of organismic responses to environmental variations, as well as for assessing nutritional requirements, it tends to modify the organism-environment and organism-organism interrelationships, under which the subsystem had evolved, and into which it had been integrated.

As is well-known, isolated components do not necessarily behave in the same manner as they do when fully integrated into the next higher system, nor may the characteristics of that system be derived solely from the properties of its parts. Consequently, results obtained from cultivating organisms must be used with care when interpreting functions and structures of multispecies assemblages. During cultivation, environment and nutrition tend to deviate from the situation met in the unconstrained, natural habitat. In many cases, it is impossible to provide an exact replica of in situ conditions in the laboratory. Unsurmountable barriers of dimension, diversity and dynamics exist. If allowed to attain critical levels, the deviations may elicit responses which differ significantly from organismic performances under in situ conditions.

The experimental ecologist engaged in cultivation should never forget that his inquiry reveals, at best, the physiological potential of the living system examined. He must define the portion of this potential which is actually used under in situ conditions—the ecological potential—before his physiological findings can make ecological sense. The concepts of physiological and ecological potential ("ökologische
Existentz') have been introduced and interpreted by KINNE (1953, 1956, 1957), who pointed out that information on both potentials is needed for working out an ecological diagnosis of a population or species. Ecological diagnoses (based on genetic, physiological and ecological parameters) for all key components of an ecosystem provide a basis for analyzing the system's characteristics.

Such considerations do not pertain to commercial cultivation. The aquaculturist can modify environment, nutrition and genetic constitution of his organisms ad libitum—as long as he produces good-tasting sea food or adequate biological raw materials economically. While ecological and technological aspects of aquaculture involve research, success or failure of commercial operations depends on the profit made. Aquaculture is, first and foremost, a commercial enterprise. Considering the seemingly insatiable worldwide demand for aquafood, the most important prerequisites for successful aquaculture operations are: (i) sufficient resources of clean (unpolluted) water; (ii) low-cost real estate, labour and energy, in a suitable area with proper climate; (iii) access to marketing and transportation; (iv) ecological information on the organism cultured and technical know-how on water-quality management; (v) potent investors and able business managers.

Industrial and domestic wastes released into coastal waters may seriously interfere with cultivation. Research cultivation and commercial cultivation are incompatible with environmental degradation. Most industrial nations are aware of this conflict between waste treatment and cultivation. Solving water-pollution problems and progress in aquaculture go hand in hand. However, large fish, crustacean or mollusc farms may themselves pose a threat to coastal areas also used for fishing or recreation. Large aquaculture farms release amounts of organic waste comparable to those of an average-sized city. The result is heavy eutrophication.

Why waste a valuable fertilizer? Several marine ecologists have discussed the design of aquaculture farms based on fertilizer recycling. However, very few have progressed beyond the discussion stage. Pioneer studies on a continuous aquaculture—tertiary sewage treatment system have been conducted by RYTHER and co-authors (1972; see also RYTHER and DUNSTAN, 1971); the social, political, regulatory and marketing problems of recycling systems have been reviewed by HUGUENIN and KILDOW (1974). According to HUGUENIN (1974), the two objectives of eutrophication avoidance and maximization of aquacultural output have enough in common to make a combined system feasible, even though the optimal operation points for the two goals are not coincident. The combined aquaculture—tertiary sewage treatment system projected by RYTHER and his associates is illustrated in Fig. 1-3. Recycling requires multispecies cultures rather than single-species systems (e.g. TENORE and co-authors, 1973; TENORE and HUGUENIN, 1973).

Aquaculture has often been compared with the poultry industry—a highly sophisticated branch of agricultural enterprise. However, as WEBBER (1973) has pointed out, no aquaculture animal is yet as well understood as the chicken, nor do we have sufficient command over the aquatic environment yet. The present know-how in aquaculture does not facilitate the sustained production of aquafoods in the vertically integrated system from feed to marketing, working so smoothly in the poultry industry.

What contributions can marine ecology make to mariculture? It can help to fill the gaps in our knowledge on the ecological background of aquaculture candidate
organisms and provide a solid fundament for water-quality management (Chapter 2), disease control (Chapters 8, 9) and assessment of organism–organism relationships in multispecies cultures (Chapter 6).

![Diagram of a combined aquaculture–sewage-treatment system.](image)

Fig. 1-3: Model of a combined aquaculture–sewage-treatment system. (After HUGHENIN, 1974; reproduced by permission of the American Society of Agricultural Engineers.)

For cultivating animals, mass production of suitable food organisms is essential. While synthetic foods are easier to handle, thus far, they have supported all life functions of cultured animals in only a few cases. We still depend heavily on living foods. Numerous micro-organisms, plants and animals cultured represent potential food sources. Their suitability and usefulness as food sources or as bio-assay organisms are referred to throughout this volume.

General reviews on—or including—cultivation of marine organisms have been presented by HAGMEIER (1933), NEEDHAM (1937), BRAARUD (1961), McNEIL (1968), COSTLOW (1969), KINNE and BULNHEIM (1970), SPOTTE (1970), BARDACH and co-authors (1972), PILLAY (1972), AVAULT (1973) and STEIN (1973); see also BUZZATTI-TRAVERSO (1958). A catalogue of cultured aquatic organisms has been published by JHINOKAN and GOPALAKRISHNAN (1974) in order to assist aquaculturists. The catalogue contains brief biological data but no literature sources.

(5) Part 1: Comments on Chapters 2 to 4

Part 1 comprises accounts on water-quality management and technology, and on cultivation of bacteria and fungi, as well as unicellular and multicellular plants.
Chapter 2: Cultivation of Marine Organisms: 
Water-quality Management and Technology

There are four fundamentals to successful cultivation: the water (medium) used, the management of its life-supporting qualities, adequate nutrition of the organisms cultured and proper equipment. Concentrating on animal cultures, Chapter 2 covers three of these aspects; adequate nutrition is considered, in context with the type of organism cultured, in Chapters 3 to 5.

Sea water is an unusually complex medium. When removed from the ocean and used in sea-water systems (open, semi-open or closed) holding heterotrophs, its life-supporting capacity tends to decrease. The major alterations include increase in ammonia, organic compounds and carbon dioxide, and decrease in alkalinity, dissolved oxygen and pH. Such changes must be counteracted by mechanical, biological or physico-chemical water treatment. Several recipes for preparing artificial sea water are presented. The principal drawbacks to artificial sea water are the absence of organic substances, chelating agents and bacteria, as well as the presence of relatively large amounts of heavy metals and of other pollutants. Addition of natural sea water, organic substances, chelating agents or bacteria tends to neutralize life-endangering conditions. Nevertheless, many attempts to accommodate stenoplastic marine animals in artificial sea water have failed; in only a few cases was it possible to adequately support all life processes and all life-cycle stages of truly stenoplastic forms.

Among the life-endangering substances accumulating in culture water, \( \text{NH}_3 \) is of particular importance. \( \text{NH}_3 \) accumulation causes such detrimental effects as impairment of gas exchange, metabolic inhibition and damage to epithelia and internal tissues. \( \text{NH}_3 \) concentrations should remain below 0.001 mg l\(^{-1}\). The most important countermeasure against ammonia poisoning is biological water treatment. In addition to microbial and algal water treatment, disinfection of sea water, filtration, activated carbon adsorption, foam separation, aeration and ozonation comprise the basic techniques for water-quality management.

Many investigators have conducted experiments without ensuring that the capacity of the culture system used was sufficient to support their test organisms adequately. Although a few pioneers have developed methods for estimating the carrying capacity of a system, the picture which has thus far emerged is incomplete.

The list of equipment used for cultivating marine organisms is almost endless. Chapter 2 presents a selection of useful devices and interesting technological solutions to general problems confronting the cultivator. Additional technological details are presented in other chapters of this volume.

Chapter 3: Cultivation of Micro-organisms

Many bacteria and some fungi are easier to cultivate than ‘higher’ forms of life. However, seldom have culture conditions been provided which allow conceptual extrapolation of the results obtained to the situation in the marine environment. In most cultures, population densities and nutrient concentrations have exceeded natural values by several orders of magnitude, and the low temperatures dominating at sea have rarely been applied to micro-organism cultures. As a consequence, the
contrast between the assumed ecological role of micro-organisms and the solid facts available on the forces which govern microbial dynamics in the sea is extraordinary. There is urgent need to develop new culture methods which are adequate for tackling and solving ecological problems.

Chapter 3.1 presents examples of traditional bacteriological culture methods. However, these are considered not fully relevant to ecological research, for the reasons outlined above. A few newer techniques are discussed, in the hope of inspiring badly-needed progress. Bacterial requirements for organic compounds, minerals and oxygen are reviewed, as well as the importance of sea-water quality, pH, light, temperature, pressure and the composition of culture media. The most useful device for studying bacterial population growth under nutrient-limited conditions is the chemostat. Controlled model ecosystems and in situ techniques open up important new avenues for modern microbiological research.

Chapter 3.2 comprises two reviews on lower fungi, Ascomycetes and Deuteromycetes. Although belonging to the Ascomycetes, the yeasts are treated in a separate account, in order to fully accommodate the respective competences of the two reviewers. Numerous fungi, representing nearly all taxonomic subgroups, inhabit the sea, and, presumably, constitute essential components of the marine environment. Most of the species concerned belong to the Chytridiomycetes, Oomycetes, Ascomycetes and Deuteromycetes (Fungi imperfecti). In addition, species of Labyrinthula and Dermocystidium, as well as Althornia crouchii and Ostracoblabe implexa, have increasingly attracted attention. Fungi have been found in sediments, water samples, wood, algae, animals and calcereous materials. Apparently, only a few species are obligate or facultative parasites; most species appear to be saprophytes. Having small thalli of only one or a few cells, lower fungi are difficult to cultivate. In cultures, they grow slowly, often suffer from structural modifications and prove to be more ephemeral than Asco- or Deuteromycetes. Frequently, they display rather specific environmental and nutritional requirements. Methods of collection, enrichment, baiting, plating, isolation, sustenance and storage of fungi cultures are discussed in detail.

Chapter 4: Cultivation of Plants

Unicellular and multicellular algae constitute the fundament for the flow of energy and matter through marine ecosystems. The analysis of their environmental dependencies and metabolic performances, under controlled culture conditions, is a basic prerequisite for assessing ecological dynamics in the marine environment. Successful cultivation of algae further provides: (i) tools for investigating life cycles and physiological processes—such as photo- and RNA-synthesis, nuclear-cytoplasmatic interrelationships or membrane transport—as well as for studying nutritional requirements and morphogenetic processes; (ii) assay organisms for water-quality assessments; (iii) food for man and cultured animals; (iv) materials for industrial, medical (pharmacological) and agricultural (e.g. fertilizer, manure) uses.

Chapter 4.1 considers the principles of unicellular algae cultivation. It devotes considerable attention to procedural details (purification, media preparation, sterilization, subculturing), specialized culture types (mass, synchronous and continuous cultures), chemical aspects (nutritional requirements, culture media) and
physical aspects (illumination, temperature, salinity, pH and Eh). Aquaculture projects must give prime consideration to nutrient provision, control of predators, and economic harvesting procedures. They hold promise in areas with suitable year-round illumination, low-cost coastal land and easy access to fertilizers. In situ cultures in plastic bags and dialysis cultures offer new perspectives for studies under close-to-natural conditions.

Chapter 4.2. Cultivation of multicellular algae has elucidated the life cycles of numerous representatives and contributed significantly to our knowledge on the ecological role of benthonic forms in coastal waters. It has helped to provide important background data for commercial cultivation projects, and to explore new uses for multicellular marine algae (food, industrial raw materials, medicine, manure).

While sea-grasses, mangroves and salt-marsh plants are important for sediment stabilization and coast protection, and because they form habitats for numerous other plants and animals, only very few representatives have thus far been cultivated.

Part 2: Comments on Chapters 5 to 7

Part 2 considers animals, multispecies cultures and microcosms, as well as the chemical contamination of culture media.

Chapter 5: Cultivation of Animals

This chapter contains two accounts, one is devoted to animal research cultivation, the other to commercial cultivation. Since general principles and problems of aquatic-animal cultivation have received attention in Chapter 2, Chapter 5 concentrates on: (i) the diverging environmental and nutritional requirements of different, major animal groups and the specific techniques employed to accommodate them in cultures; (ii) a special review on axenic cultivation; (iii) a brief account on the large and fast-growing field of aquaculture.

Chapter 5.1 reviews, in considerable detail, Protozoa, Porifera, Cnidaria, Turbellaria, Rotifera, Nematoda, Bryozoa and Annelida, as well as some other groups of marine animals on which less pertinent literature is available. The information at hand on crustaceans, molluscs and fishes defeats attempts at completeness; however, the majority of studies are based on relatively few culture methods. These have been employed over and over again without significant modification. Numerous publications on crustaceans, molluscs and fishes consider predominantly commercial aspects, and hence, belong to the realm of aquaculture. Perhaps the most striking impressions conveyed by Chapter 5.1 are the wide scattering of contributions (some important older papers have appeared in difficult-to-locate, obscure publications) and the fact that many authors pursued aims other than the development of culture methods. It is primarily for these reasons that it turned out to be very difficult to present an exhaustive, critical and well-balanced review.

Axenic cultivation originated with microbiology, and due to technical problems, has remained restricted to 'lower' animals. The use of axenic cultures and of chemically defined media has permitted studies on nutritional requirements, on rates of uptake and utilization of nutrients, as well as on synthesis and excretion of metabo-
lites in osmotrophs. Only a few marine animals are in axenic culture. It is very difficult to axenize phagotrophs. None of the higher marine animals has yet been cultivated axenically.

The wholly unnatural conditions prevailing in axenic cultures have been criticized by ecologists. However, information may emerge which is, paradoxically, of ecological significance. The subchapter reviews general procedures of axenization and of preparing culture media. Gnotobiotic cultures of invertebrates and axenic cultures of protozoans, nematodes and crustaceans receive detailed attention.

Chapter 5.2 provides a summarized overview of essential aspects of the field of commercial animal cultivation, rather than an exhaustive account on the state of the art. The dominating dimension of aquaculture—profit making—cannot very well be treated in detail in a treatise on marine ecology. We have restricted ourselves to: (i) assessing the world-wide potential of commercial cultivation for producing more food and, in particular, more protein for the growing human population; (ii) areas and animals suitable for aquaculture; (iii) ecological implications; (iv) general evaluations of the present state of farming crustaceans, molluscs and fishes; and (iv) future perspectives. The home-aquarist industry receives no special attention; many good books on pet-fish culture are available in the hobby literature.

Chapter 6: Multispecies Cultures and Microcosms

Multispecies (mixed) cultures and microcosms—comprising several species that interact with each other—are essential tools for analyzing ecological dynamics in natural assemblages of micro-organisms, plants and animals. While most of our cultivation endeavours have been directed towards unispecies cultures ('contaminated' with a variety of small 'tolerated' associate organisms), single-species systems do not exist in oceans and coastal waters. The dynamics of natural multispecies systems can only be analyzed if we investigate the organizational integration of different species into a system, and if we define the forces that govern the rules of species co-existence. Experimental microcosms serve as models of natural communities and may stimulate new approaches to mariculture. Man's potential impacts on the marine environment can be studied more adequately in multispecies than in unispecies cultures.

Unfortunately, published information on microcosm cultures is rare and largely concerned with non-marine organisms. Consequently, Chapter 6 considers all microcosm studies available, whether marine, limnic or terrestrial, and is more concerned with prospects than with facts and accomplishments. Interactions of species cultured together are considered from two perspectives: nutritional and other chemical interactions and mathematical models of such interactions.

On the basis of the information at hand, the following general conclusions are drawn: (i) It is necessary to develop models in which species and environmental factors are treated as primary variables. We need to study the effects of adding, removing or exchanging species in a 'calculus of species', and to develop new methods for fitting models and ecological data. (ii) More knowledge is required on the role played by bacteria and related micro-organisms in 'cementing' an ecosystem. (iii) Chemical interactions between system components appear to be more important for system organization than is often realized.
Chapter 7: Chemical Contamination of Culture Media: Assessment, Avoidance and Control

Culture media come into contact with numerous foreign substances and materials. This may lead to chemical contamination and significantly affect success and results of culture experiments. Leaching and etching processes release substances from culture enclosures of glass or porcelain. Plastics and rubbers tend to leach substances such as plasticizers, fillers, catalysts, stabilizers, flame retarders, colouration and antistatic agents, or impurities introduced with raw materials. Most metals and alloys readily dissolve in sea water and may release toxic ions (i.e. copper, zinc, chromium). Detergents employed for cleaning glassware and equipment may interfere with cultivation in a variety of ways, and food organisms from polluted waters may introduce DDT and other detrimental substances (Chapter 5.1). In addition, chemicals used for making up culture media, radio-isotopes, and improperly prepared distilled water, as well as de-ionized water and filter materials constitute potential contamination hazards. Sea water must be carefully collected so as to avoid contamination from the ship's hull, oil, petrol or equipment. Sorption (negative contamination) to culture-enclosure walls, equipment or filter materials may diminish or remove essential substances.

The effects of chemical contamination on cultured organisms are insufficiently investigated. They include inhibition or enhancement of normal biological functions, modifications in tolerance limits to environmental stress, and structural abnormalities.

Detection of contamination is difficult. Suggestions for suitable tests (chemical determinations, bioassays) and for proper cleaning procedures are presented, as well as a list of substances and materials which are considered suitable for cultivation purposes.

(7) Part 3: Comments on Chapters 8 and 9

Chapter 8: Diseases of Plants

Organism–environment and organism–organism interrelationships are perhaps nowhere more intimate than in parasitism and pathogenicity. While disease agents may affect the distribution and abundance of marine plants, their role in marine ecology has been referred to in most texts only in passing.

From the very limited published information, the brief review allows the following conclusions: (i) Most of the numerous plant diseases, well known in terrestrial habitats, have not been recorded in oceans and coastal waters. (ii) While viruses and phages are known to invade and lyse marine bacteria, their nature, mechanism of penetration, as well as the factors controlling host susceptibility and resistance are insufficiently known. (iii) The role of bacteria as disease agents has remained uninvestigated. Only two associations of bacteria and marine plants have been labelled 'diseases': the occurrence of tumours in Chondrus crispus and in Saccorhiza bullosa and the black rot of kelp. However, no proof has been provided that bacteria do indeed cause black rot and this disease may very well be the result of high temperature. (iv) Some fungi contribute to the death of marine plants, particularly algae; other fungi may be most active as decomposers of weakened or moribund hosts.
(v) The major consequences of invasion of marine plants by other organisms are necrosis, hypertrophy, hyperplasia and cell disintegration. (vi) Parasitism or pathogenicity require fuller investigation before valid generalization can be made.

Chapter 9: Diseases of Animals

Animal diseases are a common phenomenon in oceans and coastal waters and hence, of fundamental ecological importance. A large body of information has accumulated, especially during the last few decades. However, most ecologists have neglected to consider animal diseases in context with fluctuations of organismic distributions in space and time, and with animal performances under defined environmental conditions.

Progress in animal disease investigation has been stimulated by commercial interests in the exploitation of natural seafood resources and in aquaculture. Consequently, our knowledge is grossly unbalanced in favour of commercially important species, especially molluscs, crustaceans and fishes. Paucity of information prevails in regard to most lower invertebrates which are, of course, hardly of less importance for ecological and overall economical considerations.

Chapter 9 reviews our present knowledge on diseases of marine invertebrates, fishes, birds and mammals. Wherever sufficient information is available, diseases of viral, bacterial, fungal, or protozoal etiology are described. These accounts are followed by the array of parasitic diseases, caused by metazoan parasites—helminths, parasitic crustaceans and members of other phyla. A brief section is provided on prevention and treatment of diseases of cultivated marine animals.

Literature Cited (Chapter 1)


2. CULTIVATION OF MARINE ORGANISMS: WATER-QUALITY MANAGEMENT AND TECHNOLOGY

O. KINNE

(1) General Aspects

The basic prerequisite for successful cultivation is the establishment of environmental and nutritive conditions under which relationships between organisms and environment, as well as among organisms, are sufficiently balanced and controlled within defined, life-supporting ranges. Over extended periods of time, proper levels of life-supporting conditions can only be maintained by continuous water-quality management or by regular culture-water replacement.

In culture systems, it is impossible to duplicate the physical, chemical and biological processes taking place in the gigantic water masses of the world ocean. The differences in dimensions and dynamics cannot be overcome, no matter how sophisticated our technology.

'Early aquarists considered their aquariums and water systems to be microcosms whose elements behave in the same way as they do in the whole earth's grand economy ... Standing on the shoulders of these pioneers, we now recognize many significant differences between the biornomics of aquarium systems and the sea' (Atz, 1964a, p. 10).

In large bodies of ocean water, physico-chemical water properties, as well as gradients between organisms and environment, tend to be balanced and buffered. Fluctuations are slow and usually remain well within the ranges tolerated. However, as soon as a sample of sea water is removed from the ocean, the physico-chemical properties of this 'captive' water begin to change, and its capacity to support life tends to decrease. The cultivator must reduce the speed of water degeneration and introduce means for water regeneration, i.e. of culture-water treatment (p. 100).

In small culture vessels, environmental fluctuations are rapid and often extensive. While such factors as light, temperature and salinity can be maintained at constant levels (or defined variation patterns), other environmental entities defeat attempts for exact control or make it very difficult, e.g. dissolved organic substances, composition and abundance of associated microflora and microfauna.

Improvements in water-quality management are imperative for breakthroughs in cultivation research. We must develop our facilities for: (i) fast and efficient chemical water analyses; (ii) recording and controlling biological and physico-chemical fluctuations in the culture medium; (iii) treating recirculating water; (iv) dimensioning essential parts of complex culture systems.

Bacteria require special attention. For the microbiologist, culture and nutrient
conditions comparable to situations prevailing in the sea are of fundamental importance if the results of culture experiments are to be used for comprehending and interpreting natural ecological dynamics. For botanists and zoologists, bacteria are essential as culture partners of the test organisms. Unless axenic conditions prevail, bacteria and related micro-organisms tend to establish thriving populations in culture systems. Extensive surface areas (e.g. glass, plastics, sand, gravel, living and dead organisms) and high concentrations of dissolved organic matter promote microbial growth (Volume I: ZOBELL, 1972, p. 1200, p. 1264). We must know more about the species composition and abundance of filter-bed micro-organisms; about their capacity to transform organic matter; and about the capability of different strains to release substances that affect co-existing organisms.

Progress in cultivation depends to a considerable degree on technological aspects. While some basic equipment has remained essentially unchanged over decades or even centuries, the current situation is characterized by the fact that cultivation of marine organisms is no longer the domain of a few biologists. Additional groups with varying interests have entered the scene: Applied ecologists revolutionizing the field of commercial cultivation (aquaculture); engineers with new ideas for solving technical problems; conservationists concerned with the impact of large-scale aquaculture farms on natural habitats; investors and businessmen devoted to making profit; and statesmen compelled to make additional food sources available for the growing human population. As a consequence, papers reporting on technological aspects of cultivation are being produced in numbers never known before. Close cooperation between ecologists and engineers has proven especially successful. At present, the foundations are being laid for a new branch of cultivation—eco-engineering—a growing field that will soon need to be reviewed in its own right.

Water-quality management and technology have received considerable attention in the fields of municipal and industrial waste-water treatment. While concepts and goals differ, experimental ecologists involved in culture-water management may benefit much from consulting the comprehensive literature on sanitary waste-water treatment.

This chapter deals primarily with animals. Culture-water management and technology related to micro-organisms and plants have received special attention in Chapters 3 and 4, respectively. While the home aquarist will find information of interest to him, the chapter has been written for investigators, teachers and students interested in experimental ecology. Emphasis is placed on research cultivation and on scientific aspects of commercial cultivation. We concentrate here on the topics sea water as culture medium, culture systems and -enclosures, life-supporting and -endangering substances, culture-water treatment, capacity of culture systems for supporting aquatic animals, and the equipment used.

(2) Sea Water as Culture Medium

Sea water is the cradle of life on earth. This complex medium has obtained, and is still receiving, its many constituents from geological structures eroded and bathed by water, both on continents and sea floors, from a multitude of biological and chemical processes involved in the flow of energy and matter through aquatic ecosystems, and from man's activities. Although a few elements present in nature have not yet
been demonstrated analytically in sea water, we may postulate that traces of all known natural elements occur in the sea (Volume I: KALLE, 1971).

The amounts of many elements dissolved in sea water depend upon chemical properties which control their solubility. These properties are not necessarily proportional to the quantities of elements discharged by rivers and released from the sea floors (FORCHHAMMER, 1865; GOLDBERG, 1963). The dissolved organic substances contained in sea water (Volume I: KALLE, 1972b; RHEINHEIMER, 1972; VIDAVER, 1972; and VÉHNEBRA, 1972) originate from excretion or leakage of living organisms, from dead, decaying organisms, as well as from man-made pollution of the sea. The production of dissolved organic matter receives detailed attention in Volume IV: WANGERSKY (in press). Dissolved organics play a role as growth promoter or inhibitor, inter-organism mediator, energy source, antibiotic, chelating agent, surface-active substance, toxicant or detoxicant. Presumably, the organic substances contained in sea water are of basic ecological importance in mediating interrelationships between members of an ecosystem.

In regard to cultivation, four properties of sea water are of particular importance: (i) the many different inorganic components and their relative proportions; (ii) the dissolved organic substances which are low in concentration, but often powerful in their biological effects; (iii) seasonal changes in constituents; (iv) changes in captive sea water as a function of time and of manipulation by the cultivator.

(a) Natural Sea Water

Natural sea water collected in different parts of the seas may reveal differences in its capacity to sustain life. Although this fact has been recognized for some time (e.g. WILSON, 1951; WILSON and ARMSTRONG, 1952), a detailed, critical analysis of such local differences in sea-water quality is not yet available.

Inorganic and organic constituents of natural sea water have been listed by GOLDBERG (1963, pp. 4–5) and in Volume I: COLLIER (1970, pp. 66–71) and KALLE (1971, p. 684). While the relative amounts of major elements remain quite stable, the concentrations of constituents which enter into biochemical processes may undergo marked changes. Iron or silicon, readily involved in biochemical cycles, can differ greatly from one oceanic water mass to another (GOLDBERG, 1963; see also SVERDRUP and co-authors, 1942; HARVEY, 1955; REDFIELD and co-authors, 1963; HORNE, 1969).

The chemistry of natural waters has been investigated by KEMP and others. KEMP (1971a) considers ideal fundamental equations governing the interionic equilibria in natural waters, employing the systematic approach of RICCI (1952). These equations lead to relatively simple expressions for the hydrogen-ion concentration (pH) of such waters. In later papers, alkalinity (KEMP, 1971b), carbonic acid (KEMP, 1971c), saturation pH value (KEMP, 1971d) and hardness (KEMP, 1971e) receive attention, and in a final assessment (KEMP, 1971f), a scheme is proposed for standardizing water analyses, utilizing the molar concentrations of the acids and bases considered to be dissolved in the water.

A chemical model for sea water at 25° C and 1 atm total pressure has been presented by GARRELS and THOMPSON (1962). This model has been extended or modified, for example, by WANGERSKY (1972a), WHITFIELD (1973, 1974) and PYTRO-
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

Pytkowicz and Hawley (1974). Pytkowicz and Hawley consider their model (Tables 2-1, 2-2) to represent progress because no assumptions were made regarding the activity of coefficients of ion-pairs and of bicarbonate, carbonate and sulphate ions, and because the compositional dependence of the ionic interactions was determined. However, they do not claim that their model is final.

Table 2-1

Natural sea water at 25° C. Chemical model for major components, calculated from sulphate association constants (Kester and Pytkowicz, 1939) and bicarbonate and carbonate association constants (Pytkowicz and Hawley, 1974). The constants for magnesium and calcium fluoride association are taken from Elgquist (1970). The number of significant figures is used for mass balance purposes and does not reflect the accuracy which is unknown because of the assumptions made (After Pytkowicz and Hawley, 1974; reproduced by permission of American Society of Limnology and Oceanography).

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Na⁺</th>
<th>Mg⁺⁺</th>
<th>Ca⁺⁺</th>
<th>K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Free cation</td>
<td>97.70</td>
<td>89.11</td>
<td>88.35</td>
<td>100.00</td>
</tr>
<tr>
<td>% MSO₄</td>
<td>2.25</td>
<td>10.35</td>
<td>10.87</td>
<td></td>
</tr>
<tr>
<td>% MHCO₃</td>
<td>0.05</td>
<td>0.24</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>% MCO₃</td>
<td>0.01</td>
<td>0.17</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>% MgCO₃</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MgCaCO₃</td>
<td>0.01</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MF</td>
<td>0.07</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total molality</td>
<td>0.4822</td>
<td>0.0580</td>
<td>0.0163</td>
<td>0.0162</td>
</tr>
</tbody>
</table>

Constituents | SO₄²⁻ | HCO₃⁻ | CO₃⁻⁻ | F⁻ |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Free anion</td>
<td>39.19</td>
<td>81.33</td>
<td>7.99</td>
<td>51.04</td>
</tr>
<tr>
<td>% NaX</td>
<td>37.29</td>
<td>10.73</td>
<td>15.99</td>
<td></td>
</tr>
<tr>
<td>% MgX</td>
<td>19.55</td>
<td>6.44</td>
<td>43.86</td>
<td>48.94</td>
</tr>
<tr>
<td>% CaX</td>
<td>3.97</td>
<td>1.50</td>
<td>20.96</td>
<td>2.02</td>
</tr>
<tr>
<td>% MgCO₃</td>
<td>7.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MgCaCO₃</td>
<td>3.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total molality</td>
<td>0.02906</td>
<td>0.00213</td>
<td>0.000171</td>
<td>0.000080</td>
</tr>
</tbody>
</table>

Some firms in Europe and North America offer sea salt obtained from slowly evaporated sea water. While a few components of the original sea water may be lost due to evaporation, storage and re-dissolution, the media obtained from such sea salts by adding water (distilled water, rain water, pond water, tap water, etc.) often sustain marine organisms quite well. Sea water produced from natural sea salts might be considered an intermediary between natural and artificial sea water.

Major changes that tend to occur in captive sea water used for extended periods in animal cultivation include: (i) increase in ammonia and other end products of nitrogen metabolism; (ii) increase in complex organic compounds, phosphates and carbon dioxide; (iii) decrease in alkalinity, pH and dissolved oxygen. Sea water that had been used for 20 years in the New York public aquarium revealed a 250-fold increase in nitrate content and a decrease in pH below neutrality (Townsend, 1928). Comparable data have been reported for the public aquaria at London
At Enoshima and Ueno, SAeki (1963) determined the major ions (Table 2-3); potassium and calcium were present in higher amounts, and magnesium in lower amounts than in natural sea water. Among the minor elements, phosphate had increased considerably. Alkalinity was greatly reduced. Changes in the major elements are listed in Table 2-4. The chlorinity ratio of the mean values of potassium and calcium increased 20 to 30%, that of magnesium decreased about 10%, and that of alkalinity about 50% in comparison to ocean water.

### Table 2-2

Natural sea water at 25°C. Modification of the chemical model presented in Table 2-1: an association constant for KHSO₄, based on the results of Garrels and Thompson (1962), is added to the calculations; the values in parentheses are theirs (After Pytkowicz and Hawley, 1974; reproduced by permission of American Society of Limnology and Oceanography)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Na⁺</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Free cation</td>
<td>97.71 (99)</td>
<td>89.15 (87)</td>
<td>88.39 (91)</td>
<td>98.85 (99)</td>
</tr>
<tr>
<td>% MSO₄</td>
<td>2.24 (1.2)</td>
<td>10.31 (11)</td>
<td>10.82 (8)</td>
<td>1.15 (1)</td>
</tr>
<tr>
<td>% MHC0₃</td>
<td>0.05 (0.01)</td>
<td>0.24 (1)</td>
<td>0.29 (1)</td>
<td>0.41 (0.2)</td>
</tr>
<tr>
<td>% MCO₃</td>
<td>0.01 (1)</td>
<td>0.17 (0.3)</td>
<td>0.41 (0.2)</td>
<td></td>
</tr>
<tr>
<td>% MgCO₃</td>
<td>0.03</td>
<td>0.17 (0.3)</td>
<td>0.41 (0.2)</td>
<td></td>
</tr>
<tr>
<td>% MgCaCO₃</td>
<td>0.01</td>
<td>0.17 (0.3)</td>
<td>0.41 (0.2)</td>
<td></td>
</tr>
<tr>
<td>% MF</td>
<td>0.07</td>
<td>0.42 (0.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Constituents</th>
<th>SO₄²⁻</th>
<th>HCO₃⁻</th>
<th>CO₃²⁻</th>
<th>F⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Free anion</td>
<td>39.01 (54)</td>
<td>81.33 (69)</td>
<td>7.98 (9)</td>
<td>51.03</td>
</tr>
<tr>
<td>% NaX</td>
<td>37.13 (21)</td>
<td>10.73 (8)</td>
<td>15.98 (17)</td>
<td></td>
</tr>
<tr>
<td>% MgX</td>
<td>19.47 (21.5)</td>
<td>6.45 (19)</td>
<td>43.86 (67)</td>
<td>46.95</td>
</tr>
<tr>
<td>% CaX</td>
<td>3.96 (3)</td>
<td>1.50 (4)</td>
<td>20.96 (7)</td>
<td></td>
</tr>
<tr>
<td>% KX</td>
<td>0.42 (0.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MgCO₃</td>
<td>0.07</td>
<td>7.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MgCaCO₃</td>
<td>3.82</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SAeki (1963) offers the following explanations for the changes observed: Potassium increase is due mainly to animal excretion, although small amounts are contributed by the fresh water used for replacing evaporated culture water. A significant augmentation in calcium is caused by solvation from the concrete walls or from the filter sand, as well as from neutralization by addition of lime. Decrease in magnesium is due to precipitation with phosphate. In the first few months, phosphate tends to increase to the saturation level and then to precipitate with magnesium or calcium. Nitrate increases considerably as a result of microbial nitrification in the filter bed. Alkalinity reduction is a consequence of alkali (magnesium) decrease and acidity (nitrate) increase. No significant changes were observed in regard to other inorganic constituents of the culture water.
A significant difference between captive sea water and captive fresh water seems to lie in their microbiology. The 'biological balance' in freshwater aquaria is largely based on relatively stable microbial population dynamics, even in the presence of excessive amounts of organic substances (Atz, 1964a). While the actual reason for this difference is not known, the fact may be of importance that autotrophs as well as bacteriophages and bacteria-eating protozoans are well represented in most freshwater aquaria.

The storage of natural sea water has received attention from several authors. The

### Table 2-3

Ionic composition of sea water in the public aquaria at Enoshima and Ueno (Japan) (After SAEKI, 1963; modified; reproduced by permission of the author)

<table>
<thead>
<tr>
<th>Water sample</th>
<th>Na (g l(^{-1}))</th>
<th>K (g l(^{-1}))</th>
<th>Ca (g l(^{-1}))</th>
<th>Mg (g l(^{-1}))</th>
<th>Cl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoshima 1</td>
<td>10.68</td>
<td>0.442</td>
<td>0.492</td>
<td>1.36</td>
<td>19.75</td>
</tr>
<tr>
<td>Enoshima 2</td>
<td>11.20</td>
<td>0.504</td>
<td>1.16</td>
<td>20.74</td>
<td></td>
</tr>
<tr>
<td>Ueno 1</td>
<td>11.34</td>
<td>0.504</td>
<td>1.23</td>
<td>20.20</td>
<td></td>
</tr>
<tr>
<td>Ueno 2</td>
<td>11.34</td>
<td>0.504</td>
<td>1.23</td>
<td>20.44</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Water sample</th>
<th>SO(_4) (g l(^{-1}))</th>
<th>P(_2)O(_5) - P (%)</th>
<th>Alkalinity (meq. l(^{-1}))</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoshima 1</td>
<td>2.81</td>
<td>2.48</td>
<td>1.26</td>
<td>18.6</td>
</tr>
<tr>
<td>Enoshima 2</td>
<td>2.73</td>
<td>2.38</td>
<td>1.25</td>
<td>18.8</td>
</tr>
<tr>
<td>Ueno 1</td>
<td>2.75</td>
<td>7.7</td>
<td>1.02</td>
<td>19.8</td>
</tr>
<tr>
<td>Ueno 2</td>
<td>2.74</td>
<td>5.66</td>
<td>1.86</td>
<td>19.6</td>
</tr>
</tbody>
</table>

### Table 2-4

Chlorinity ratio of major elements of the sea water in the public aquaria at Enoshima and Ueno (Japan), at London (Great Britain), and in ocean water (After SAEKI, 1963; modified; reproduced by permission of the author)

<table>
<thead>
<tr>
<th>Water sample</th>
<th>Na</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>SO(_4)</th>
<th>Alkalinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoshima 1</td>
<td>0.527</td>
<td>0.0224</td>
<td>0.0243</td>
<td>0.0672</td>
<td>0.132</td>
<td>0.038</td>
</tr>
<tr>
<td>Enoshima 2</td>
<td>—</td>
<td>0.0233</td>
<td>0.0248</td>
<td>0.0538</td>
<td>0.135</td>
<td>0.038</td>
</tr>
<tr>
<td>Ueno 1</td>
<td>0.540</td>
<td>—</td>
<td>0.0264</td>
<td>0.0522</td>
<td>0.132</td>
<td>0.0505</td>
</tr>
<tr>
<td>Ueno 2</td>
<td>0.564</td>
<td>0.0286</td>
<td>0.0394</td>
<td>0.0812</td>
<td>0.132</td>
<td>0.0970</td>
</tr>
<tr>
<td>Mean values</td>
<td>0.544</td>
<td>0.0247</td>
<td>0.0282</td>
<td>0.0586</td>
<td>0.133</td>
<td>0.0873</td>
</tr>
<tr>
<td>London*</td>
<td>0.564</td>
<td>0.0285</td>
<td>0.0615</td>
<td>0.0848</td>
<td>0.144</td>
<td>0.0668</td>
</tr>
<tr>
<td>Ocean water</td>
<td>0.566</td>
<td>0.0191</td>
<td>0.0220</td>
<td>0.0870</td>
<td>0.139</td>
<td>0.123</td>
</tr>
</tbody>
</table>

*After OLIVER (1957).
information at hand is, in some points, contradictory, in others, incomplete. Stored natural sea water is subject to changes in physico-chemical and biological properties. Degree and type of these changes depend upon pretreatment (e.g., filtration, sterilization), time span of storage, biological components present, temperature, container material, water movement and gas exchange. The resulting modifications are complex, and the processes involved are far from being adequately understood.

In sea-water samples freshly removed from the sea, life-supporting properties change as a function of time. COLLIER and Marvin (1953) detected physico-chemical changes in a sample of sea water within 30 mins after collection. In captive open-ocean water, most protozoans, invertebrates and fishes die within a short time, unless important physico-chemical and biochemical properties of the sea water are restored. Only bacteria survive in large numbers. As a consequence, the amount of organic substances and subsequently, that of microbial populations increases significantly. These changes affect the chemistry of the water.

According to MacGinitie (1947), captive sea water may be lethal to stenoplasic marine animals during the period of rapid bacterial population growth and during the phase of pronounced decline in bacterial numbers, as well as for some time thereafter. The lethal effects are assumed to result from accumulated metabolites and from the breakdown products of bacteria and dead organisms, especially phyto- and zooplankters. In addition to such effects, micro-organisms may, conceivably, use up dissolved organic substances essential to the animals cultured (amino acids, sugars, vitamins). This possibility requires critical examination.

Fluctuations in the number of bacteria as a function of storage times have been examined by several authors. At the London Aquarium (England), storage of newly collected sea water in the dark for 28 days eliminated 66% of the ordinary bacteria and 97% of the so-called 'blood-heat' organisms and bacteria of the 'human intestinal type' (Stowell and Clancey, 1927). Waksman and Carey (1935) filtered sea water through ordinary filter paper (size not stated), and stored it in the dark. At 25° and 30° C, the number of colony-forming units (CFU) increased rapidly within the first 3 days (Fig. 2-1a); at 6° C, multiplication was much slower; however, ultimately, about the same (if not higher) CFU values were obtained. Reduced concentrations of dissolved oxygen retarded bacterial growth. At 20° C, ZoBell and Anderson (1936) recorded increases in CFU to values from 2 to 100 million cells cm⁻³ within 3 to 6 days. After about 2 weeks, the initial growth phase came to an end, and CFU values decreased considerably (from a few thousand to more than 100,000 cells cm⁻³). Sea water stored for 4 years at 2° to 6° C still contained more than 200,000 CFU cm⁻³. Hanes and Fraga (1968) report that counts of Escherichia coli Type 1 and related forms decreased as a function of sea-water storage time. They used filtered, fresh domestic sewage for inoculation. Some of their curves are illustrated in Fig. 2-1b. Use of sterilized sea water reduces the variability in microbial population dynamics encountered in captive bodies of sea water (e.g., Carlucci and co-authors, 1961). Storage in the dark tends to reduce the ammonia content (Stowell, 1926b). According to Harvey (1941, 1955) and Vaccaro (1962), this may result from direct chemical oxidation, as well as from biological activities.

The diminution of CFU in stored sea water is paralleled by a reduction in the number of bacteria species. This is true both for sea water (ZoBell and Anderson, 1936) and tap water (Whipple, 1901). While some 25 to 30 species were generally
found immediately after collection, this number decreased to 9 or 10 by the time CFU values had attained a maximum, and to less than 4 or 5 species after decline from the maximum.

The initial increase in CFU is much greater in sea water stored in small (as opposed to large) containers, as well as in bottles in which the interface between water and solid substratum is increased by filling the container with glass beads (Zobell and Anderson, 1936; Lloyd, 1937). The ratio substrate-surface area to water volume and the proximity of the main water body to the substrate surface are, no doubt, major denominators of bacterial development in stored sea-water samples. Solid surface areas (and water-air interfaces) absorb and concentrate organic matter and thus make it more readily available to the micro-organisms (Volume I: Zobell, 1972, p. 1258; Gunke, 1972, p. 1535).

When stored unfiltered, offshore sea water may support only a few hundred bacteria cells cm⁻³. These low values have been attributed to the presence of bacteriophages, protozoans and other bacteria feeders (e.g. Carlucci and Pramer, 1960b); in addition, antibiotic substances may be of importance (e.g. Warsman and Hotchkiss, 1937; Volume I; Gunke, 1972, p. 1545; Fogg, 1972, pp. 1559/60; Wilber, 1972, p. 1567; this volume, Chapters 5.1, 5.11).

Limbic bacteria encounter difficulties in establishing populations in stored sea water. Differential salt tolerances (or requirements), plus the fact that sea water—especially from offshore areas—usually contains lower nutrient concentrations than river water, seem to be responsible, at least in part, for this fact. Several authors have presented evidence for inhibitory effects of sea water on non-marine bacteria (e.g. Nicati and Bertsch, 1885; Greenberg, 1956; Orlob, 1956; Carlucci and Pramer, 1959; Aubert and co-authors, 1968). The possible causes of such effects have been discussed by Moebus (1972a). Among the abiotic factors which may be
involved, salinity and heavy-metal ions rank highest (see also Volume I: MacLeod, 1971). However, seasonal variations in the bactericidal capacity of sea water (e.g. Vaccaro and co-authors, 1950; Sieburth and Pratt, 1962) indicate that biotic factors are of greater importance. Sieburth (1958, 1960, 1964, 1968), Sieburth and Pratt (1962), and Aubert and co-authors (1968) have attributed the antibacterial activity of sea water primarily to inhibitory substances released by a variety of marine organisms, especially planktonic algae. Aubert and Pesando (1969) recorded, over one year, variations in antibiotic activity of phytoplanktonic diatoms against Staphylococcus aureus 209 P. They assume variations to be due to the presence or absence of a chemical mediator, capable of blocking (at certain times of the year and under certain biological conditions) the production of antibacterial substances by the phytoplankters. In addition, phytoplankters are known to produce and to consume growth-promoting substances (e.g. amino acids, sugars, vitamins); hence, they may both support microbial growth and also act as competitors for essential nutrients. Bacterial inhibition in oceanic surface-water samples (Sieburth, 1971) suggests a source of antibacterial activity other than algae, possibly lipid or oily substances from water pollutants.

The capacity of marine micro-organisms to produce antibiotic-like substances has been demonstrated by Rosenfeld and ZoBell (1947), Greer and Meyers (1958), Baam and co-authors (1966a, b), Buck and Meyers (1966), Gauthier (1969a, b, 1970) and others. Since marine bacteria are superior to non-marine forms in competing for the nutrients available in sea water (Carlucci and Pramer, 1960a, b; Volume I: MacLeod, 1971), this capacity could significantly reduce the chances for non-marine forms to establish themselves in stored sea-water samples. In a series of papers, Moebus (1972a–e) has claimed that the amount of nutrients present in stored sea-water samples may significantly affect its bactericidal capacity. This claim deserves critical attention.

Moebus (1972b) examined 3 North Sea water samples of about 25 l each, stored at 18°C in the dark, for changes in bacterial content and antibacterial activity as a function of time, using mainly Serratia marinorubra as test bacterium. Storage caused considerable variations in antibacterial activity (Fig. 2-2). Variability in antibacterial activity is maximum in raw sea water; it decreases in filter-sterilized and even more so in autoclaved subsamples. Variations in CFU and bactericidal activity of the stored sea water are positively correlated during the first months of storage; later, their correlation becomes negative. Moebus concludes that changes in the amount of nutrients available to the bacteria (due to successive periods of bacterial growth and autolysis) are the primary cause of the changes observed in antibacterial activity of the stored sea-water samples. In another study, Moebus (1972a) inoculated freshly collected sea-water samples (raw or filter-sterilized) with resting cells of Escherichia coli, Staphylococcus aureus and S. marinorubra and recorded seasonal changes in CFU values correlated with diatom life cycles. This study and subsequent ones (Moebus, 1972c, d, e) support Moebus’ hypothesis of nutrient-dependent antibacterial activity. Variations in the amount of nutrients available to the bacteria (due to consumption as well as lysis of inactivated test bacteria) are assumed to exert more significant effects on the bactericidal capacity of sea-water samples than bactericidal metabolic products of marine bacteria (Moebus, 1972e).
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

The exact causes of increased variations in CFU and of microbial population changes in stored sea-water samples have remained largely unknown. Quantitative recordings of cells or species present seem insufficient as a tool for pinpointing the factors involved in promoting or inhibiting microbial components. A given factor may inhibit growth of species A, but not of species B; however, inhibition of A may, secondarily, promote or inhibit B. With the methods at hand, such interaction and interdependence defies detailed analyses of cause and effect.

Identical treatment of stored sea water will not necessarily lead to identical quality. In addition to treatment, the end product depends on collecting details such as season, locality, water depth and sample size. Finally, the quality of the sea water obtained after storage cannot be considered stable; it will continue to change even if more slowly and less pronouncedly.

Since several marine ecologists have reported that prolonged storage improves the capacity of sea water to support stenoplastic organisms, it has become common

![Fig. 2.2: Stored sea water. Number of colony-forming bacteria units (CFU) and antibacterial activity as a function of storage time. (a) CFU (log *Serratia marina* cells ml⁻¹) on 2216 E-agar at the beginning of each test (N₀) and after 3 days of incubation at 25°C of raw subsamples inoculated with test bacteria (N₅). (b) Antibacterial activity of autoclaved, filter-sterilized and raw subsamples, presented as log (N₅-N₀). (After Mohnus, 1972b; modified; reproduced by permission of Springer-Verlag.)](image-url)
practice in some marine laboratories to store the sea water for one or several months before using it in experiments. Sea water should be stored in the dark in glass containers (or plastic vessels that have been shown not to release significant amounts of organics). While low storage temperatures (e.g. 4° C) tend to preserve the original water quality for some time, higher temperatures (e.g. 20° C) and mild aeration (with sterilized air) assist in stabilizing bacterial population dynamics.

(h) Artificial Sea Water

It is difficult to make chemically defined artificial sea water. Even though numerous artificial sea-water recipes have been devised, tested and published, very few formulae are yet available that would adequately support all life processes of a stenoplastic marine animal. Major problems persist in regard to pollution by impurities of component salts and to administering proper qualities and quantities of trace elements and organic constituents. In all cases, the components used for making up artificial sea water must be exactly known and weighable (or they must be calibrated against primary standards). The salts used must be added in such a way that precipitation is avoided. All major salt components should be technical grade.

Considering the various recipes for artificial sea water reported in literature, four groups may be distinguished: Recipes devised: (i) for plants, i.e. with nutrients; (ii) for animals, i.e. without nutrients; (iii) for maximum resemblance to natural sea water; (iv) for maximum support (lowest mortality, fastest growth, highest rates of reproduction) of a given organism. We are concerned here primarily with recipes developed for animals; artificial sea waters for micro-organisms have been dealt with in Chapter 3, for plants in Chapter 4.

The first use of artificial sea water as a medium for culturing marine animals dates back more than a century. The first evaluation of the importance of artificial sea water for cultivation has been presented by Hoffmann (1884); the first institution to use artificial sea water with some degree of success was the public aquarium at Berlin which opened in 1869.

In the nineteenth century, a large number of formulae for artificial sea water were tested, and several recipes recommended for use. Based on these studies, firms in the USA and Europe have developed synthetic sea salts (e.g. 'Aqua Marine', 'Büsüm Meersalz', 'Instant Ocean', 'Rila Marine Mix', 'Triton Marine Salts', 'Utility Seven Seas Marine Mix'). In general, major elements (Volume I: Kallé, 1971) of artificial sea water are technical grade, minor elements analytical reagent grade. Usually, artificial sea waters require inoculation with natural sea water or with living organisms, such as a seaweed or a euryplastic animal, before they can support stenoplastic organisms. Presumably, such inoculation introduces micro-organisms, life-supporting substances (e.g. vitamins) and chelating agents that neutralize life-endangering substances (e.g. heavy metals introduced, for example, with the chemicals used for making up the artificial sea water).

A potential disadvantage of commercial sea salts is the fact that the manufacturer may change his recipe without notice or without the knowledge of the user. This has, in some cases, led to considerable problems. Of the recipes recommended for the preparation of artificial sea water, one has been presented in Volume I, p. 687. Additional recipes which have proved useful for cultivating marine animals are presented in Tables 2-5 to 2-12.
LYMAN and FLEMING's (1940) formula (Table 2-5) has been widely used for studying physico-chemical and biological phenomena (e.g. HARVEY, 1960; RILEY and SKIRROW, 1965). LYMAN and FLEMING recommend that all the chlorides listed in Table 2-5 and potassium bromide be dissolved in about 800 ml distilled water. The remaining salts should then be dissolved in 100 ml distilled water and added slowly, while stirring, in the form of a fine jet. The final mixture obtained is then filled up with distilled water to 1000 ml, left to stand for a day, and filtered. After thorough

Table 2-5
Recipe for preparation of artificial sea water of 19·00% Cl or 34·33%, S. All values expressed as ppm (After LYMAN and FLEMING, 1940; reproduced by permission of Sears Foundation for Marine Research)

<table>
<thead>
<tr>
<th>Salt</th>
<th>Mg kg⁻¹ of solution</th>
<th>Molecular wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>23,477</td>
<td>192</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>4,081</td>
<td>96</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>3,917</td>
<td>26</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1,102</td>
<td>24</td>
</tr>
<tr>
<td>KCl</td>
<td>664</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2-6
Recipe for artificial sea water (After KESTER and co-authors, 1967; reproduced by permission of American Society of Limnology and Oceanography)

A Gravimetric salts

<table>
<thead>
<tr>
<th>Salt</th>
<th>Mg kg⁻¹ of solution</th>
<th>Molecular wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>23,926</td>
<td>58·44</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>4,008</td>
<td>142·04</td>
</tr>
<tr>
<td>KCl</td>
<td>677</td>
<td>74·56</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>196</td>
<td>84·00</td>
</tr>
<tr>
<td>KBr</td>
<td>98</td>
<td>119·01</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>26</td>
<td>61·83</td>
</tr>
<tr>
<td>NaF</td>
<td>3</td>
<td>41·99</td>
</tr>
</tbody>
</table>

B Volumetric salts

<table>
<thead>
<tr>
<th>Salt</th>
<th>Mg kg⁻¹ of solution</th>
<th>Molecular wt.</th>
<th>Stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0·05327</td>
<td>203·33</td>
<td>1·0 m</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0·01033</td>
<td>147·03</td>
<td>1·0 m</td>
</tr>
<tr>
<td>SrCl₂·6H₂O</td>
<td>0·00009</td>
<td>266·64</td>
<td>0·1 m</td>
</tr>
</tbody>
</table>

C Distilled water to 1,000·000 g
aeration, the pH will be between 7·9 and 8·3. In order to bring the Lyman–Fleming artificial sea water to within 1 mg kg⁻¹ of natural sea water for all major constituents, Kester and co-authors (1967) have revised the recipe (Table 2-6). Impurities in the reagent grade salts used by them do not change the composition of the artificial sea water by more than 1 mg kg⁻¹ for all major constituents. However, impurities are important with regard to minor constituents; if reagent grade NaCl contains maximum PO₄³⁻ and Fe impurities, the artificial sea water will contain 10 times the average PO₄³⁻ and 4 times the normal Fe of natural sea water.

Hauenschild (1962) used the mixture listed in Table 2-7 for cultivating a variety of marine invertebrates. He points out that his artificial sea water supports only euryplastic forms. Stenoplastic invertebrates require addition of natural sea water.

Hückstedt’s (1963a, b) artificial sea water (Table 2-8) does not contain, aside from iodine, defined amounts of minor elements. This medium should be mixed with natural sea water for long-term cultivation of animals, unless these are extremely euryplastic.

### Table 2-7

Recipe for artificial sea water of 35·00% S. All values in mg l⁻¹  
(After Hauenschild, 1962; modified; reproduced by permission of Institut für Meereskunde, Univ. Kiel)

<table>
<thead>
<tr>
<th>Basic constituents</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>26,700</td>
</tr>
<tr>
<td>MgSO₄·(7H₂O)</td>
<td>3,200</td>
</tr>
<tr>
<td>MgCl₂·(6H₂O)</td>
<td>2,200</td>
</tr>
<tr>
<td>CaCl₂·(6H₂O)</td>
<td>1,500</td>
</tr>
<tr>
<td>KCl</td>
<td>700</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutrient salts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complexing agent</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>SrCl₂</td>
<td>3·8</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>2·3</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>0·2</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>0·8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trace elements: cations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RbCl</td>
<td>0·06</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>0·03</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>0·006</td>
</tr>
<tr>
<td>LiCl</td>
<td>0·006</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0·001</td>
</tr>
<tr>
<td>KBr</td>
<td>22</td>
</tr>
<tr>
<td>Na-silicate</td>
<td>20</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>2</td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>0·2</td>
</tr>
<tr>
<td>KI</td>
<td>0·02</td>
</tr>
</tbody>
</table>
The recipe recommended by Segedi and Kelley (1964) is based on the composition of synthetic sea water prepared by Dr. D. Backhaus of the ‘Zoologischer Garten’ at Frankfurt (FRG). Backhaus’ formula was modified by Segedi and Kelley into a 4-step preparation resulting in a final mixture with a specific gravity of 1.025. Part I of their formula consists of gross dry components (Table 2-9). After weighing, these are placed in a mixing tank with a hard stream of tap water directed into them to dissolve the chemicals. The container is then filled almost to the level which provides the desired specific gravity. Part II is dissolved in hot water and added to the Part I mix. Calcium chloride is mixed separately. Parts III and IV are stock solutions of trace elements. They are now added to the mix in proper proportions, and the total mix is brought up to the correct specific gravity with tap water.

Table 2-8
Recipe for artificial sea water. All values in mg l⁻¹ (After Hückstedt, 1963a, b; reproduced by permission of Franckh, Stuttgart)

<table>
<thead>
<tr>
<th>Component</th>
<th>Mg/l</th>
<th>NaHCO₃</th>
<th>250†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>27,650</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄ (+7H₂O)</td>
<td>6,920</td>
<td>NaBr</td>
<td>100</td>
</tr>
<tr>
<td>MgCl₂ (+6H₂O)</td>
<td>5,510</td>
<td>SrCl₂</td>
<td>15*</td>
</tr>
<tr>
<td>CaSO₄ (+2H₂O)</td>
<td>1,600*</td>
<td>KI</td>
<td>5</td>
</tr>
<tr>
<td>KCl</td>
<td>650</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* To be dissolved separately.
† To be dissolved separately and added as last component.

Table 2-9
Recipe for artificial sea water. T.g.: technical grade; A.r.: analytical reagent (After Segedi and Kelley, 1964; modified; reproduced by permission of the authors)

<table>
<thead>
<tr>
<th>Part I: Gross components</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>MgCl₂ 6H₂O</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
</tr>
<tr>
<td>KC1</td>
</tr>
<tr>
<td>NaHCO₃</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Part II: Separate mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Part III: Trace element stock solution (add 80 cm³ to final mix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium gluconate</td>
</tr>
<tr>
<td>KI</td>
</tr>
<tr>
<td>KBr</td>
</tr>
<tr>
<td>CuSO₄ 5H₂O</td>
</tr>
</tbody>
</table>

Dissolve in 2 l of distilled water

<table>
<thead>
<tr>
<th>Part IV: Trace element stock solution (add 80 cm³ to final mix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al₂(SO₄)₃</td>
</tr>
<tr>
<td>CoSO₄</td>
</tr>
<tr>
<td>RbCl</td>
</tr>
<tr>
<td>ZnSO₄ 7H₂O</td>
</tr>
</tbody>
</table>

Dissolve in 2 l of distilled water
SEGEDI and KELLEY have maintained marine invertebrates more successfully in their artificial sea water than in any other synthetic medium tested by them. Calcium gluconate supports bacterial growth during storage, unless sterile conditions prevail; however, its usefulness as a component in artificial sea water has been questioned by Goldizen (in: Conover, 1970).

ZAROGIAN and co-authors (1969) formulated an artificial sea water recipe (Table 2-10) for cultivating larvae of the American oyster *Crassostrea virginica*. They were able to induce spawning in mature *C. virginica* at 20°C; fertilized eggs reached the straight-hinge stage within 48 hrs. These larvae appeared to be identical to those allowed to develop in natural sea water of identical temperature, salinity and pH. Additional trace elements were not essential for normal development at this stage.

<table>
<thead>
<tr>
<th>Components</th>
<th>mg l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>23,500</td>
</tr>
<tr>
<td>MgCl₂·(6H₂O)</td>
<td>5,000</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>4,000</td>
</tr>
<tr>
<td>CaCl₂·(2H₂O)</td>
<td>1,100</td>
</tr>
<tr>
<td>KCl</td>
<td>700</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>200</td>
</tr>
<tr>
<td>KBr</td>
<td>100</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>30</td>
</tr>
<tr>
<td>Na₂SiO₃·(9H₂O)</td>
<td>20</td>
</tr>
<tr>
<td>SrCl₂·(6H₂O)</td>
<td>20</td>
</tr>
<tr>
<td>NaF</td>
<td>3</td>
</tr>
<tr>
<td>EDTA (tetra-Na-salt)</td>
<td>1</td>
</tr>
</tbody>
</table>

ZAROGIAN and co-authors postulate that the presence of a substance such as EDTA (tetra-Na-salt) is essential for normal larval development.

Artificial sea water for cultivating the colonial hydroid *Bougainvillia* sp. has been prepared by Tusov and Davis (1971). These authors modified a formula devised by Bruzewicz (in: Subow, 1931) (Table 2-11). The composition of commercially available sea salts is sometimes considered a trade secret. However, in the case of 'Instant Ocean', the composition has been made known (Table 2-12). A variety of marine organisms have been raised in sea water made from commercially available sea salts. Larvae of the euryplastic crab *Rhithropanopeus harrisii*, for example, grow equally well in 'Instant Ocean' and 'Utility Seven Seas Marine Mix' as in natural sea water (Sulkin and Minasian, 1973). Stenoplastic forms such as echinoderms, however, accept artificial sea water much less readily.
COURTRIGHT and co-authors (1971) formulated an artificial sea water for bioassays with embryos of *Mytilus edulis*. At a salinity of 25%, their formula (BioSea) is claimed to allow normal growth and development to the shelled veliger stage. The essential inorganic compounds of the artificial sea water are: Leslie coarse hide salt, sodium bicarbonate and potassium chloride, reagent anhydrous magnesium sulfate and reagent calcium chloride. Leslie coarse hide salt (source of NaCl) contains essential materials (L factor), thought to be organic, necessary for calcification of mussel larvae. The L factor was destroyed upon burning the salt. (Natural sea water contains 4 times as much L factor as BioSea.) Growth and development of *M. edulis* embryos and larvae were improved by adding alkaline phosphatase, carbonic anhydrase, purine (free base), DL-lysine, and DL-aspartic acid to BioSea.

<table>
<thead>
<tr>
<th>Components</th>
<th>mg l(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>25,625</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>4,006</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>3,809</td>
</tr>
<tr>
<td>CaCl(_2) (anhydrous)</td>
<td>1,132</td>
</tr>
<tr>
<td>KCl</td>
<td>723</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>202</td>
</tr>
<tr>
<td>NaBr</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 2-12
Composition of 'Instant Ocean' synthetic sea salts adjusted to specific gravity 1.025 at 15°C (33-76%, S). All values in ppm (After KING and SPOTTE, 1974; reproduced by permission of Aquarium Systems, Inc.)

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl</td>
<td>18,400</td>
</tr>
<tr>
<td>Na</td>
<td>10,220</td>
</tr>
<tr>
<td>SO(_4)</td>
<td>2,518</td>
</tr>
<tr>
<td>Mg</td>
<td>1,238</td>
</tr>
<tr>
<td>Ca</td>
<td>390</td>
</tr>
<tr>
<td>K</td>
<td>370</td>
</tr>
<tr>
<td>HCO(_3)</td>
<td>140</td>
</tr>
<tr>
<td>Br</td>
<td>65</td>
</tr>
</tbody>
</table>

* Present in trace-elements solution.
Comparison of major elements in natural sea water and 8 artificial sea-water formulae. All values expressed in g kg⁻¹, and calculated for 35.00%, salinities given in parentheses were arrived at from authors’ original recipes (Original).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity</td>
<td>35.00%</td>
<td>(34.33%⁎⁎)</td>
<td>(35.00%⁎)</td>
<td>(31.44%†)</td>
<td>(33.32%†)</td>
<td>(34.96%†)</td>
<td>(38.88%†)</td>
<td>(34.33%†)</td>
<td>(33.70%†)</td>
</tr>
<tr>
<td>Cations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>10.76</td>
<td>10.76</td>
<td>10.76</td>
<td>11.54</td>
<td>12.16</td>
<td>11.16</td>
<td>10.65</td>
<td>12.16</td>
<td>11.40</td>
</tr>
<tr>
<td>K</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>0.35</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>0.38</td>
</tr>
<tr>
<td>Mg</td>
<td>1.295</td>
<td>1.30</td>
<td>1.30</td>
<td>0.63</td>
<td>1.36</td>
<td>1.30</td>
<td>0.69</td>
<td>1.36</td>
<td>1.36</td>
</tr>
<tr>
<td>Ca</td>
<td>0.410</td>
<td>0.41</td>
<td>0.41</td>
<td>0.30</td>
<td>0.38</td>
<td>0.49</td>
<td>0.34</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>Sr</td>
<td>0.013</td>
<td>0.014</td>
<td>0.008</td>
<td>0.002</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.00</td>
<td>0.001</td>
</tr>
<tr>
<td>Anions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br</td>
<td>0.066</td>
<td>0.066</td>
<td>0.066</td>
<td>0.016</td>
<td>0.019</td>
<td>0.019</td>
<td>0.077</td>
<td>0.006</td>
<td>0.064</td>
</tr>
<tr>
<td>F</td>
<td>0.0013</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>SO₄</td>
<td>2.701</td>
<td>2.70</td>
<td>2.71</td>
<td>1.36</td>
<td>3.65</td>
<td>2.64</td>
<td>3.10</td>
<td>2.70</td>
<td>2.62</td>
</tr>
<tr>
<td>HCO₃</td>
<td>0.145</td>
<td>0.14</td>
<td>0.14</td>
<td>0.16</td>
<td>0.16</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>H₂BO₂</td>
<td>0.027</td>
<td>0.027</td>
<td>0.026</td>
<td>0.002</td>
<td>0.00</td>
<td>0.00</td>
<td>0.034</td>
<td>0.00</td>
<td>0.026</td>
</tr>
</tbody>
</table>

* Author’s information.
† Cl + Br + I.
‡ Cl + Br.
§ Temperature + density.
A comparison of the amounts of major elements contained in natural sea water and in the artificial sea waters mentioned (Table 2-13) reveals that most cations and anions are present in comparable amounts. Notable exceptions are magnesium which is about half the normal value in the recipes of Hückstedt (1963a, b) and Zaragozian and co-authors (1969) and sulphate (about half the normal amount in Hückstedt's recipe and considerably above normal in SEGDA and KELLEY's). In addition, Hückstedt's and Kester and co-authors' formulae contain much less bromide than natural sea water. Additional recipes for artificial sea water (based on McLendon, Penn, and Schmalz) have been presented by Galtsoff (1937).

For preparing large amounts of artificial sea water, a variety of mixers have been constructed, and automatic dissolvers are commercially available. Two examples are illustrated in Fig. 2-3. The major salt components should be technical grade.

Fig. 2-3: Mixer systems for preparing large amounts of artificial sea water. (Original.)

(c) Conclusions

Natural sea water is an unusually complex medium. When removed from the ocean, it undergoes a variety of changes that cannot yet be fully controlled. Major alterations, such as increase in ammonia, organic compounds and carbon dioxide, and decrease in alkalinity, pH and dissolved oxygen, can be counteracted by culture-water treatment (p. 100).

Storage of natural sea water requires careful attention. Fluctuations in bacterial numbers and species present tend to modify essential biochemical sea-water properties. In some laboratories, it has become common practice to store newly collected sea water for some time (1 to several months) before use as culture medium. For stabilizing bacterial population dynamics, the sea water is usually stored at 20° C in the dark under conditions of mild aeration with sterilized air. Identical storage procedures do not necessarily lead to an identical sea-water quality.
Artificial sea water with ratios of major elements that deviate significantly from natural sea water and those without close-to-natural amounts of minor elements (trace elements) cannot be recommended for use in ecological experiments. Principal drawbacks of artificial sea water are the absence of organic substances, chelating agents and bacteria, and the presence of relatively large amounts of heavy metals and other pollutants in the salts used. Addition of natural sea water, organic substances, chelating agents or bacteria, tend to neutralize life-endangering conditions and to initiate stabilization processes. To avoid precipitation, Ca and related substances must be dissolved separately and later thoroughly mixed with the main solution.

Dilutants used to make artificial sea water should be clean rain water collected in glass containers, or glass-distilled water. Tap water or water from an oligotrophic lake can also be used, providing the chemical composition is known. Chlorinated tap water must be aerated for 3 to 4 days before use in order to reduce its chlorine content. Demineralized water is unsuitable for use as dilutant since it contains difficult-to-pinpoint organic substances, leached from the exchange resins. Sea salts obtained by slow evaporation from ocean water may provide a useful medium when redissolved. However, several components of natural sea water (e.g. calcium) resist redissolution once evaporated to dryness. They must be added separately (see above). Artificial sea water can be conveniently prepared from stock solutions of single salts. The exact concentrations of minor elements are difficult to control. It is more correct (but, of course, very complicated) to determine minor elements analytically in the artificial sea water than to calculate their concentration on the basis of the weighed amounts added. Detergents and related substances used for cleaning glassware and other equipment may contaminate the artificial sea water (Chapter 7).

Artificial sea water should be aerated moderately and allowed to ‘age’ for several days or weeks before use (stabilization of major and minor element ratios, buffering substances and other constituents). For extended storage periods, provision must be made to avoid or reduce evaporation.

Critical assessment of the capacity of the different artificial sea waters to support marine life is not possible as the information available is insufficient. Detailed comparative tests are still to be conducted. The whole field of artificial sea-water preparation requires the attention of chemists.

(3) Culture Systems

There are two basic categories of aquatic culture systems: still-water systems and running-water systems. Numerous small organisms, such as bacteria, unicellular plants and a variety of small invertebrate animals, can be cultured successfully in still-water systems. However, many multicellular plants and animals require running water.

Among the running-water systems, three types can be distinguished: open systems, semi-open systems, and closed systems. The principles of, and the differences between, these three systems are discussed on the following pages. Of course, no experimental water system is entirely open; nor can it be completely closed; over extended periods, some replacement of culture water is unavoidable. How-
ever, the three terms characterize the essence of the respective types. A very useful
compilation of papers concerned with sea-water systems for experimental aquaria
has been edited by Clark and Clark (1964). In addition to open, semi-open and
closed sea-water systems, multipurpose environmental systems (MES) have recently
been designed and are now being tested. MES combine characteristics of the three
conventional sea-water systems, and provide maximum flexibility in terms of tech-
nical design, applicability, culture size, management and control of essential ecolo-
gical parameters.
In multi-container systems, the culture enclosures may be arranged in series or parallels (each container receiving its water supply independently). In serial assemblages or 'ladder systems' (Figs 2-4, 2-5), culture-water contamination will—at any step except the last one—automatically affect subsequent steps. Hence, for most research programmes, ladder systems cannot be recommended. These systems are used primarily for sorting, demonstration (teaching) or maintenance of stock cultures.

(a) Open Sea-water Systems

In open sea-water systems (flow-through systems), the culture medium is used only once; it is not recirculated. A continuous flow of sea water from a natural source enters the culture container and is subsequently discarded. Open systems require a permanent supply of suitable natural sea water and hence, a location close to an unpolluted shore.

Natural sea water is subject to seasonal changes in a number of physico-chemical and biological parameters which are difficult, or even impossible, to record or control. It tends to contain varying amounts of growth-inhibiting or growth-promoting substances, nutrients, disease-causing agents, pollutants, silt, undesirable organisms, etc. Hence, in open sea-water systems, medium control is often insufficient for research projects other than the study of phenomena related to seasonal changes in sea-water quality.

Most open systems are used for observation and for maintenance of living organisms (teaching, demonstration, food supply, stock cultures). In some cases, however, open systems may provide the only means for successful rearing and breeding, e.g. in situations where insufficient information is available on environmental or nutritional requirements of the animals cultivated, or in cases where commercial cultivation prevails.

Open systems may require pretreatment of the natural sea water used. Adjustments in regard to water temperature, gaseous contents and turbidity can be achieved through heat exchange, aeration, sedimentation and filtration. In addition, disinfection, and removal (or addition) of nutrients, other substances or organisms, may be desirable. For details consult the section Culture-water Treatment (p. 100).

An example of an open sea-water system is the Marine Culture Laboratory, Granite Canyon, USA (Ebert and co-authors, 1974). This system (Fig. 2-6) features a dual sea-water intake and delivery unit (Fig. 2-7). It provides pretreated (filtered, heated or chilled, ultraviolet-disinfected) and untreated (raw) sea water. The sea-water intake unit is anchored on the sea floor and houses two pumps. Dual delivery pipes lead to the main reservoir of 75,700 l. Fouling by marine organisms is minimized by monthly alternating use of the delivery lines. In the pipes not in use, settled organisms desiccate and die. Valves allow the sea water to be flushed out (avoiding contamination of laboratory water by dead organisms accumulated in shut-down pipes) and repair operations to be carried out. Filtration to approximately 15 μm is achieved in a two-stage, high-transport sand-filtration system, which requires periodic (approximately weekly) backwashing to flush out clogged matter. The filtered water normally passes directly into the laboratory; however, part of the
water may be routed into an 18,900-l reservoir which is used to supply the laboratory while the filters are backwashed. Additional filtration is provided by Filterite cartridge filters (Filterite Corporation, USA).

A second example of an open sea-water system is a facility providing water of controlled temperature and salinity (HETTLER and co-authors, 1971; see also p. 199). The facility has been used for cultivating the menhaden _Brevoortia tyrannus_. The intake, a sand-bed filtration gallery, consists of a 3800-l concrete septic tank containing 2 well screens and a sand pack. Unfortunately, filtration galleries are a known source of potential trouble; they tend to clog channels, interfere with water quality and, frequently, have parts that become anaerobic (ALDERDICE, personal communication). A hard-rubber centrifugal pump, driven by a 3-h.p. electric motor, forces water through the system at about 200 l min⁻¹. The salinity of the water entering the aquaria is recorder-controlled; the flow of sea water and fresh water into the reservoir is controlled by sensors. The water-heating unit consists of 3 heat exchangers; pumping of air to the bottom of the respective containers aids water mixing (prevention of thermal stratification) and maintains about 100% air saturation.
Additional papers on open sea-water systems have been published, for example, by HINTON (1958), LASKER and VLYMEN (1969) and HETTLER (1970).

Open culture systems are not restricted to laboratory facilities. A variety of *in situ* cages (p. 69), as well as bays, fjords and other natural water bodies—separated from the sea by fences and managed by a cultivator—qualify as open culture systems. Sessile animals can be cultured in the sea without cages or fences, e.g.

![Diagram of submersible sea-water intake unit](image)

*Fig. 2-7: Submersible sea-water intake unit with dual-pumps and pipes. The unit is designed to withstand strong surf effects. (After Ebert and co-authors, 1974; modified; reproduced by permission of the authors.)*

benthonic algae, bryozoans and bivalves. Even motile animals, such as fishes, may be fattened in the sea—without cages or fences—providing they are territorial or can be made to remain at a given locality by imprinting or behavioural re-inforcement. Automatic feeders placed at strategic localities and the provision of directional stimuli (e.g. light, sound, air-bubble curtains, electromagnetic fields) can be used as re-inforcement agents. Positive behavioural stimuli are used to attract the animals to the centre of the experimental area, negative stimuli to deter them from its borders. Such behavioural *in situ* cultivation (KINNE, 1970a) deserves more attention than hitherto received, especially in regard to commercial endeavours.
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

(b) Semi-open Sea-water Systems

Semi-open sea-water systems combine the technological principles of open and closed systems in varying degrees. Depending upon the extent of water renewal or recirculation, the characteristics of open or closed systems prevail.

Semi-open systems are used (i) in coastal areas where tidal or weather conditions make a continuous supply of natural sea water difficult or impossible; (ii) in cases where culture-water treatment (p. 100) becomes uneconomical unless part of the water is renewed. Semi-open systems are useful in public aquaria and other displays of aquatic animals, as well as in aquaculture farms, where the total water capacity of the system is insufficient for long-term support of a high "animal load" (p. 166). When new sea water becomes available, or when the water quality drops below minimum requirements, part of the recirculated water is discarded and replaced by water freshly pumped in from the sea.

(c) Closed Sea-water Systems

In closed sea-water systems, the culture water is continuously recycled. Culture-water recirculation offers better possibilities for monitoring and controlling essential environmental factors than open or semi-open systems. Closed systems are more independent of the seasonal physico-chemical and biological variations which are characteristic of natural sea waters (e.g. temperature, salinity, turbidity, organic substances, plankton components, pollution), especially in coastal areas. They can be operated far away from the sea (site independence) and with artificial sea water (sea-water supply independence).

In a typical closed sea-water system (Fig. 2-8), water flows from culture tank to filter, reservoir, header (or gravity) tank and back to the culture tank. Circulation pumps are placed after the reservoir to avoid clogging. Aerators are installed in the culture tank (and/or in other components of the system). The header tank is placed high enough for sufficient water to flow by gravity feed into all culture enclosures. Aeration of header-tank water, as well as avoidance of temperature gradients between header tank and culture tank, help to prevent gaseous sub- or supersaturation. In the reservoir—which provides an emergency water supply if the pump fails, while also acting as a settling tank—a 12- to 24-hr water supply is usually sufficient.

To prevent sudden overall pollution, to restrict the spread of disease and to facilitate individual water treatment, each culture tank must have its own recirculation system (e.g. KELLEY and MORENO, 1961; Arz, 1964a). Such individual sea-water recirculation has been employed successfully for many years in several public aquaria, for example, at Wuppertal, FRG (WIEDEMANN, 1943) and Berne, Switzerland (HEDIGER, 1944). When the systems comprise less than 4000 l, adequate water recirculation can be provided by airlifts (p. 190).

Some closed systems have no filter. For example, the Marine Biological Laboratory at Plymouth (England) operates a filterless system with special sedimentation tanks (WILSON, 1962, 1960). Filterless closed systems have a lower carrying capacity than conventional types (HERALD and co-authors, 1962), but can better support filter-feeding animals.
In closed systems, the cultivated organisms tend to modify the chemistry of the culture water. If extensive, the withdrawal (e.g. oxygen) or release (metabolic end products) of substances may approach critical levels. The most obvious changes that occur in a captive body of sea water are: (i) decrease in dissolved oxygen concentration and alkaline reserve; (ii) increase in carbon dioxide, nitrogen compounds, phosphates, dissolved organic substances and in microbial numbers; (iii) changes in pH. For details consult pp. 170 to 178.

Fig. 2-8: Closed sea-water system. Principal design. (Original.)

In the sea, a balanced integration of production, transformation and remineralization of organic substances usually prevents critical accumulation at any level of the flow of energy and matter (Volume IV). In closed systems, a comparable balance must be achieved, and then maintained artificially. Most closed systems contain representatives of only a few species, which have been isolated from a multitude of environmental and organismic interrelations. The cultivator must reconstitute acceptable environmental and nutritional conditions, and counteract detrimental influences on the culture medium. Closed sea-water systems require continuous management of the basic influences which environment and organisms exert upon each other, with the aim of stabilizing the conditions within the tolerance limits of the organisms cultivated. In essence, closed-system operation means, above all, continuous culture-water treatment (p. 100).

An example of a large closed sea-water system is the aquarium of the Marine Laboratory at Aberdeen (Scotland). This system is a research tool, but adapted to
afford as high a standard for display purposes as compatible with its primary function (Thomas, 1964). The basic design is illustrated in Fig. 2-9. Capacities of the main system components are listed in Table 2-14. The sand filter has twin sections (allowing one section to be shut off during periodic cleaning) and a layer of coarse marble chips supporting pH stability. Food is restricted, in general, to fresh squid, cut into suitable pieces and washed. The firm flesh reduces pollution from water-soluble and particulate food matter not taken up by the cultured animals. The twin sedimentation tanks are used alternately each week. Thomas has worked out a detailed checklist for routine operation (daily, weekly, monthly and quarterly maintenance procedures). The practitioner will find his checklist useful.

Fig. 2-9: Closed sea-water system, Marine Laboratory, Aberdeen, Scotland. P_1: main recirculation pump; P_2: cooling recirculation pump; S: service tank; only one of the twin sedimentation tanks is shown. (After Thomas, 1964; modified; not copyrighted)

A closed system for maintaining marine gastropods (e.g. species of Aplysia) in an inland laboratory has been designed, installed and tested by Lickey and co-authors (1970). The 7550-l system (Fig. 2-10) is completely non-metallic (reinforced concrete, fibre-glass-lined plywood, polyvinyl chloride). Filtration is achieved by disposable cartridge filters (p. 121). Sea water from an underground reservoir (6000 l) is pumped to the temperature regulation tank (TR; 500 l). From there, a pump (P_2) supplies 3 culture tanks (1, 2, 3; each 350 l), and another pump (P_3) returns the water to the reservoir. The water is filtered twice per circuit, once prior to entry (F_1), and once following exit (F_2) from the culture tanks. Alternative circulation paths provide flexibility for tank cleaning, replacement of sea water, etc. The culture water is replaced routinely once every 14 to 15 weeks. It enters each culture container at approximately 20 l min^{-1} (total turnover rate 60 l min^{-1}) from a set of small jet
Table 2-14

Capacities of main components of the closed sea-water system at the Marine Laboratory, Aberdeen (Scotland) (Based on data presented by Thomas, 1964)

<table>
<thead>
<tr>
<th>Component</th>
<th>Capacity</th>
<th>Component</th>
<th>Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 large display tanks</td>
<td>31,815 l</td>
<td>2 sedimentation tanks,</td>
<td>1,818,000 l</td>
</tr>
<tr>
<td>1.5 x 1.5 x 1.5 m</td>
<td></td>
<td>each</td>
<td></td>
</tr>
<tr>
<td>8 small display tanks</td>
<td>3,636 l</td>
<td>Main circulation pump</td>
<td>18,180 l hr⁻¹</td>
</tr>
<tr>
<td>0.9 x 0.76 x 0.76 m</td>
<td></td>
<td>rated capacity</td>
<td></td>
</tr>
<tr>
<td>Portable tanks (as required)</td>
<td>1,818 l</td>
<td>Total filter area</td>
<td>3.7 x 4.9 m</td>
</tr>
<tr>
<td>Service tank</td>
<td>2,273 l</td>
<td>Refrigerator rated capacity</td>
<td>60,000 B.t.u.</td>
</tr>
<tr>
<td>Header tank</td>
<td>1,591 l</td>
<td>Heater</td>
<td>3 kW</td>
</tr>
</tbody>
</table>

Fig. 2-10: Closed sea-water system for use in inland laboratories. 1, 2 and 3: culture tanks. Comp: compressor; F: filters; HW: hot-water heater circulating 80°C water; P: pumps; SV: solenoid valve releasing from 12 into cooling coils; TR: temperature regulation tank; V: valves. (After Lickey and co-authors, 1970; modified; reproduced by permission of Springer-Verlag.)
Closed sea-water systems can also be useful in mariculture. They may provide economic advantages in regard to control of water temperature, feeding and disease; in addition, they eliminate the competition of aquaculture farms with recreational interests for use of bays or estuaries (Epifanio and co-authors, 1973). In an interdisciplinary team (a chemist, an engineer, two biologists), these workers have developed a pilot plant culturing high animal densities. Such team interaction (Fig. 2-11) is very useful for solving the many problems that confront the modern mariculturist. The closed culture system (Fig. 2-12) was designed for raising filter-feeding molluscs from egg to market size. Its main parts are: (i) an 8-6 m² biological filter composed of a 7-6-cm deep shell layer covered by a 7-6-cm thick gravel (4-5-cm diameter) layer with a slotted fibre-glass base supported 10 cm above the bottom of an epoxy-coated concrete tank; (ii) an activated carbon trickle filter, a 240-l drum loaded with 1 mm granulated carbon; (iii) a foam separator (protein skimmer); (iv) an ultra-violet treatment unit; (v) culture tanks for clams, oysters and crabs; (vi) a biological monitoring system. Water recirculation (4-4 l min⁻¹) and aeration are affected by three 2-8-cm-diameter airlifts. The team is encouraged by the results obtained thus far and expects a marketable product 18 months after spawning.

Further papers on closed sea-water systems have been published by Sachs (1929), Breder and Howley (1931), Chin (1959), Hale (1964a), McInerney and Hoar (1964), Sarky (1965), Tigue-Ford (1967a), Sandt (1968), Valentì (1968), Balloy (1969), Goldizen (1970), Hagen (1970), Siddall (1972), King (1973), King and Kelley (1973) and King and Spotte (1974).
Recent developments in research cultivation, commercial cultivation, and environmental protection have created a need for complex culture systems that can no longer be categorized in conventional terms of open, semi-open and closed sea-water systems. The new trend favours multi-purpose systems that provide (i) maximum flexibility; (ii) maximum control over as many abiotic and biotic system parameters as possible; (iii) maximum range for experimental design—from small-scale, single-species experiments through medium-size and large-size multispecies cultures, to artificial microcosms and aquaculture ‘pilot plants’. The versatility of these systems allows the accommodation of a variety of different concepts, techniques and scientific disciplines.
Three examples of the first generation of such multipurpose environmental systems (MES) are those in Woods Hole (USA), on Helgoland (FRG) and on Texel (The Netherlands).

The MES in Woods Hole (Huguenin, 1974) was sparked by experiments (Dunstan and Menzel, 1971) which made use of secondarily treated sewage effluents as fertilizer for growing marine phytoplankton (Figs 2-13, 2-14, 2-15). Flexibility was one of the foremost design considerations. It was achieved by: (i) Burying as little piping as possible and by covering the central supply channel with removable planking; such centralization provides for easy access, facilitates cross-connections and simplifies piping modifications. (ii) Making raw sea water, filtered sea water, temperature-controlled sea water, fresh water, nutrient supply, electric power, drains and oilless low-pressure air available at many locations. (iii) Designing flow conditions and physical configuration of many major components so as to allow later variation. Even if other research objectives were to be sacrificed, this MES is so flexible that it could treat the effluents of up to 500 people and perhaps produce about 4 tons of shellfish meat annually.

The sea water of the MES in Woods Hole is drawn from 2 intakes with fibre-glass screens (2.5-cm diameter holes) in 3 to 4 m water depth. According to Huguenin (1974), sea-water wells along the shoreline would have been preferred, but geological factors precluded this possibility. Each intake is serviced by a 20-cm, high-density polyethylene line. The 2 lines are designed for alternate use to prevent growth of fouling organisms by an anaerobic stagnant-water period. The system delivers 2460 l min⁻¹ with one pump on, and 3800 l min⁻¹ with both operating. The larger diameter pipes are made of polyethylene, the smaller ones of polyvinyl chloride.
Fig. 2-14: Multipurpose environmental system, Woods Hole Oceanographic Institution. Schematic water-flow diagram. (After Huguenin, 1974; modified; reproduced by permission of the American Society of Agricultural Engineers.)
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

The settling pond (dead storage capacity: 113,600 l; live storage capacity: 56,800 l) is constructed of a plastic liner on a prepared surface; it functions both as a water-quality control device and as a potential research area.

The 6 algae ponds, each with a water surface area of ca 180 m², can be operated at water depths of 15 to 90 cm. They consist of a polyvinyl chloride plastic liner on a sand surface, with a second layer to minimize sun damage in exposed areas. Thus far, the material used has stood up to mechanical stress and solar radiation, as well as to solid freezing of the pond's water. The liners can be readily cleaned, even after being heavily fouled. Effects of long-term liner use have still to be determined. Two of the ponds are temperature controlled and capable of maintaining 16°C (even with air temperatures near -10°C).

The outdoor test area is asphalted, and provides additional space for experimentation.

The concrete raceways can be operated in series or parallel. Thus raceway length can be modified in multiples of 12 m. Five raceways are 1.2 m wide, 3 are 2.4 m wide. They can all be operated with water depths of up to 1.5 m and with individual flow rates of up to about 1900 min⁻¹.

The indoor wet laboratory area has all services overhead and distributed uniformly. The floors are pitched towards the main drain channel, and spot drains are provided so that no point is further away from a drain than about 1.5 m. HUGUENIN (1974) calls special attention to a common mistake in the design of wet laboratory facilities: the sizing of the drains for steady-state flow conditions, instead of sizing the drains for high transient flows, such as occur when the drain plugs on tanks are pulled.

Since the MES is unmanned at night and on weekends, an alarm system monitors water level, temperature, air pressure, boiler and filter malfunctions, power loss or trouble with experiments of particular importance.

The MES on Helgoland has been designed for research cultivation and small-scale experiments on commercial cultivation (Figs 2-16, 2-17, 2-18). Maximum

---

Fig. 2-15: Multipurpose environmental system, Woods Hole Oceanographic Institution. Elevations and gravity flow dynamics. (After HUGUENIN, 1974; modified; reproduced by permission of the American Society of Agricultural Engineers.)
flexibility and maximum control of essential environmental factors in 9 independent sea-water systems were major planning goals. The Helgoland MES accommodates up to 7 scientists representing the following disciplines: microbiology, protozoology, phytoplanktonology, zooplanktonology and fisheries research. The MES consists of 6 basic units (Fig. 2-16): (i) an outdoor test area of about 330 m²; (ii) an indoor test area of about 330 m²; (iii) an underground area for sedimentation tanks and sand-gravel filters; (iv) a sea-water tower with 9 separate water sections (header tanks); (v) a laboratory building with laboratories for the 5 biological disciplines mentioned, as well as 22 temperature-controlled rooms and a variety of special laboratories and equipment for efficient water-quality management; (vi) a library building with a conference room, study rooms and related services.

The sea water for the Helgoland MES is drawn at 2-50 m water depth (low tide) at a 300-m distance from the laboratory building. From the two intake pumps (Steinzeug Pumpen, Deutsche Steinzeug und Kunststoffwarenfabrik, Mannheim, FRG) P₁ and P₂, the water enters 10-cm-diameter polyethylene (along the shore) and PVC lines which can be used alternatively or in combination. Each pump delivers ca 450 l min⁻¹. In addition, sea water can be drawn from a well (P₃) immediately near the MES (not shown in Fig. 2-17). The sea water moved by Pumps 1 and 2 first enters a receiver tank. This tank is dividable into 2 chambers that can be used
alternatively during cleaning; each chamber holds 11 m$^3$ of water. From the receiver tank, the sea water flows into: (i) 4 sedimentation tanks (9 to 9$_4$), each with a total capacity of 95 m$^3$; these tanks deliver unfiltered raw sea water; (ii) 8 sand-gravel filters (1 to 8), each with a filter surface of 8 m$^2$, a maximum depth of 2.50 m, and a reservoir of 16 m$^3$ water capacity which delivers filtered sea water. The unfiltered sea water is pumped (P$_{12}$) into section 9a (11 m$^3$ total capacity, subdivisible) of the sea-water tower; the filtered sea water is pumped into sections 1a to 8a (each with a total capacity of 5-5 m$^3$) of the tower. From the tower, unfiltered or filtered sea water flows to the culture tanks located in the indoor or outdoor test areas (1b to 8b and 9b).

Fig. 2-17: Multipurpose environmental system, 'Biologische Anstalt Helgoland.' Schematic water-flow diagram. (Original.)

or in the laboratory building (9b only). The maximum difference in height between water levels in tower and culture tanks is about 10 m.

All sea-water systems of the Helgoland MES can be operated as closed, semi-open, or open systems. Part of the water can be pretreated, e.g. by zentrifiklones (p. 113), served by pumps P$_1$ or P$_2$, cartridge filters, or diatom-retaining filters. Operation of freshwater or brackish-water systems is also possible. Routine water treatment involves temperature control, aeration with oilless, filtered air, and disinfection (micro-filtration, ozonation, ultra-violet irradiation).

The indoor test area was designed to yield close-to-natural light conditions that can be modified, and that are better definable than the light conditions prevailing in most conventional laboratories. The acryl-glass-dome ceiling permits ca 67% of the
Fig. 2.18: Multipurpose environmental system, 'Biologische Anstalt Helgoland.' Side view. (Original.)
ambient light to enter the indoor test area from overhead; the light absorption properties of the acryl glass are known and have been selected for simulating natural sunlight properties. In addition to the acryl-glass-dome ceiling, a second lower ceiling of light filters can be installed. Comparison between the effects of indoor and outdoor light conditions is facilitated by culture tables which can be moved easily from indoor to outdoor areas and vice versa, and which are serviced from identical sea-water and air sources. Both indoor and outdoor test areas are supplied with 21 service points (sea water, air, electricity, drains, and one reserve line for additional services) that are evenly distributed and not further away from each other than about 4 m. The temperature of the sea water that leaves the service point can, again, be regulated by carbon heat exchanges (each with an exchange area of 0.4 m² and a maximum capacity of 1200 kcal). In addition, movable service assemblage stations allow for a maximum of versatility. The indoor test area has 2 built-in tanks (with their own water, air, and energy supplies), one of 7 m³, the other of 15 m³; the latter can be divided into two subunits.

The MES on Texel consists of (i) an indoor test area (12 × 35 × 5 m) in the aquarium building (Figs 2-19, 2-20); (ii) a circular tank (15 × 4 m) in a separate building (not yet completed); (iii) an outdoor test area (8000 m²) next to the aquarium building.

Wadden-Sea water for the MES on Texel is drawn from a small, open harbour. North-Sea water must be transported to Texel by a tanker (100 m³ capacity) owned by the institute. Wadden-Sea water can be pumped directly into the culture system in 2900-m long pipes, or stored in 3 sedimentation tanks of 300 m³ each (beneath the aquarium building).

The indoor test area is north-south oriented and is covered by a zig-zag glass roof (Fig. 2-19), designed to produce close-to-natural light conditions. The glass roof is arranged in 60° angles with side-lengths of 3.5 m. A textile ceiling produces diffuse light conditions. The total solar irradiation in the indoor test area can be reduced by curtains placed over the south-viewing roof sides. Artificial, diffuse light can be produced by fluorescent-light tubes that may be programmed for different photoperiods. The water volume in the indoor test area can be used undivided or subdivided into 5
Fig. 2-20: Multipurpose environmental system, Nederlands Instituut voor Onderzoek der Zee. Ground plan of aquarium building. (Courtesy Mr. J. W. de Boer.)
sections. Each section has identical facilities, e.g. 2 sea-water systems (Fig. 2-21), 1 dry lab, 1 chemical lab, filters, as well as provisions for aeration, ultra-violet irradiation, ozonation and temperature control. A movable working platform services the whole area and can be used for transporting heavy equipment between indoor and outdoor test areas.

Fig. 2-21: Multipurpose environmental system, 'Nederlands Instituut voor Onderzoek der Zee'. One section of sea-water system with culture tanks, filters, reservoir and header tanks. (Courtesy Mr. J. W. de Blor.)

The circular-tank system comprises a reservoir of 80 m³, 2 parallel pumps, and 20 parallel filters (2.25 m² each). In the outdoor test area are 7 ponds with sea-water supply and 5 experimental enclosures for accommodating sea birds.

None of the three MES has yet stood up to critical testing. Since design details
are not available from literature, system dimensioning and carrying capacity must be investigated for each culture system. Operational details of successful small-scale systems cannot be extrapolated, without further testing, to medium or large-scale systems. According to HUGUENIN (1974), 'debugging' of the Woods Hole MES is largely completed. In the algal ponds, dense continuous-flow phytoplankton cultures of up to $2 \times 10^6$ cells ml$^{-1}$ have been maintained for months with a daily harvest of 30% of the culture volume. Phytoplankton has been fed continuously to about 450,000 juvenile oysters and 150,000 juvenile hard clams, raised in raceways supplied with heated (15-6°C), filtered (20 μm) sea water (ratio 1 part algae: 2 parts sea water). Additional projects under way include high-density lobster-culturing experiments, attempts to cultivate marine zooplankton on a large scale basis, as well as efforts to introduce salmonid fishes as consumers into a multispecies culture assemblage.

(c) Conclusions

While it has been possible to review principal characteristics of open, semi-open and closed sea-water systems, as well as of three new multi-purpose environmental systems, critical comparison and evaluation of system design, function and efficiency is, at present, not possible. Most of the information at hand does not provide sufficiently detailed and precise data. Attempts to obtain the data desired, by sending out questionnaires, failed: The information made available did not contain adequate substance. However, it became clear that the limited publications on culture systems present a sharp contrast to the large number and variety of culture systems actually operated by marine stations and fisheries institutions.

Publication of operational details on culture systems and their ability to support marine life is encouraged. Ecologists, physiologists, biochemists and engineers should discuss concepts and experiences in depth, and work out a sound basis from which we can proceed in the future.

(4) Culture Enclosures

For lack of a better word, the term 'enclosures' is used here to comprise the large variety of tubes, dishes, tanks, ponds, etc. used for cultivating aquatic organisms. The sizes of culture enclosures range from microscopic containers with volumes of less than a drop of water, through culture tubes, dishes, finger bowls, small aquaria, etc. (up to a few litres), to raceways, ponds and oceanaria with volumes of several million litres of culture water. Large water areas used for cultivation, such as embayments, salt marshes, lagoons, bays and fjords, and large artificial habitats, may contain millions of cubic metres of water.

A number of enclosures are presented below. Additional examples of small, medium-sized and large culture enclosures are mentioned in sections of this review and in other chapters of this volume.

(a) Small Enclosures

A hanging-drop culture for observation and sustenance of micro-organisms is illustrated in Fig. 2-22, small standard culture enclosures in Fig. 2-23. Examples of frequently used types of aquaria are presented in Fig. 2-24.
(b) Medium-sized Enclosures

Medium-sized enclosures comprise, among others, fish pens, raceways and ponds. Raceways and ponds receive brief attention below.

A simple, heated, double raceway for fish culture studies (Fig. 2-25) has been developed by REED and co-authors (1973). Flexible and inexpensive in construction and operation, this raceway has a capacity of 3000 l. It consists of readily available materials. Plywood (1.9 cm), fastened by brass screws and glue, is coated with fibre-glass resin and epoxy paint. Its sides are supported by 1.27-cm steel rods, secured by 5 × 16-cm planks. Two filtration systems facilitate water management adequate for maintaining an intensive fish culture: (i) A swimming-pool pump circulates water (ca 4500 l hr⁻¹) 30 to 35 times day⁻¹ through perforated plastic carboys (Fig.
(ii) The second filter is constructed by cutting a 200-l drum lengthwise and dividing the resulting semi-circular trough into 3 compartments (Fig. 2-25b). The compartments are filled with nodules of styrofoam packing material (1), glass wool (2), and activated charcoal (3). Each hour, this filter processes approximately 1100 l. The water in the raceway is heated by a closed hot-water system.
A raceway for intensive culture of penaeid shrimp is illustrated in Fig. 2-26. Developed by Mock and co-authors (1973), the raceway is accommodated in a rectangular concrete tank ($6.9 \times 2.7 \times 1.0$ m). Encouraging results were obtained with white shrimp *Penaeus setiferus* and brown shrimp *P. aztecus*, reared at population densities up to $690$ g m$^{-2}$, through postlarval and juvenile stages. The raceway has no sand substrate and no 'ends' where the shrimp may aggregate. Water recirculation, aeration and waste processing are accomplished by airlifts. The average discharge volume per airlift is $76$ l min$^{-1}$. All airlift pipes are oriented so as to propel the water through the raceway in the pattern illustrated. The total water mass (12,000 l) flows through narrow channels at a speed of $0.6$ cm sec$^{-1}$. The dividing panels consist of 1.9-mm fibre-glass, and are supported by wooden slats. Two airlift-driven, submerged filters (9-6-l plastic buckets filled with washed oyster shells of pellet-feed size; flow rate: ca 38 l min$^{-1}$) are installed in 2 corners of the concrete tank. These filters and air stones assure water circulation also in the 'end' zones of the tank. Ninety-seven per cent of the shrimp population (averaging $80.8$ mm and $3.82$ g) survived to harvest. A total of $8.8$ kg of food (tropical marine fish food 'Tetra Marine') yielded $1.4$ kg of shrimp. Mock and co-authors consider airlifts and plant growth in the raceway essential to the high biomass sustenance obtained.

For raceways constituting part of a multipurpose environmental system, see also Figs 2-13, 2-14 and 2-15.

Ponds, the oldest culture enclosures, have remained essential tools in commercial cultivation (e.g. farming of molluscs, crustaceans or fishes). Many culture ponds could easily be adapted for use in research cultivation, e.g. for studying population dynamics or the dynamic properties of multispecies systems, such as microcosms or artificial ecosystems (Chapter 6). Hence, the construction and
operation of culture ponds is also of considerable interest to the experimental ecologist devoted to basic research.

In general, culture ponds measure from 1 to 120 m in length, 0.5 to 50 m in width and 0.5 to 3 m in depth. The most frequently encountered shapes are oblong, rectangular or oval, but square and round ponds are occasionally also used. Small ponds are easier to handle (stocking, harvesting, draining, control and treatment of diseases) and to maintain (less dam erosion by water and wind). Large ponds are hydrographically and ecologically more stable, and—due to wind action—better aerated; they involve lower construction costs per unit area. While large ponds are preferably placed at right angles to the direction of prevailing winds—to reduce dam

![Diagram of culture pond](image)

Fig. 2.26: Raceway for intensive shrimp culture. (a) Top view; (b) cut-away. (After Mock and co-authors, 1973; modified; reproduced by permission of the authors.)

eroision—the long axis of small ponds should parallel the wind for maximum aeration. Pond depth is a function of climate (cold winters) and ecological requirements of the organism cultivated. Suitably located, deep (up to 4 m) holes help to protect the cultivated organisms from cold death and also facilitate harvesting. In the absence of low-temperature stress, optimum pond depths are between 0.5 and 1.0 m for most aquatic organisms farmed.

A cement-lined fish pond of 12 to 15 m x 3 m, with varying depths, was constructed by Vincent (1908). Oval design and sloped bottom facilitate cleaning (Fig. 2.27). A gutter in the middle of the pond bottom (50 cm wide, 12 to 15 cm deep) and a circular basin (1.5 to 1.8 m in diameter, 20 to 30 m deep) serve to hold the fish when the water is drained. In the pond wall opposite the water inflow, above the gutter, is a net-covered opening, the overflow. A net-covered drainage outlet in the bottom of the large, circular basin has an opening 60 x 60 cm, in which is set a box
filled with coarse gravel, resting upon a grating. Below the grating is a smaller circular basin with an opening in the centre, leading into an outflow pipe. This design is being used with satisfactory results in several fish-culture farms in France.

Details of site selection and pond construction depend on topography, water supply, drainage, harvesting techniques, organisms cultivated, soil quality, climate, access to stock, energy, personnel, market and transportation, as well as on cost of land and construction work. Hence, only basic principles can be discussed here. For further details, consult Burrows and Chenoweth, 1955. Rust (1960, 1973),

Fig. 2.27: Cement-lined fish pond (12 x 15 x 3 m) with gutter and special drain. (After Vincent, 1908; modified; not copyrighted.)

Bennett (1962), Grizzell (1967), Iversen (1968), Wheeler (1968), Broom (1969), Dillon (1970), Bardach and co-authors (1972), Delmendo and co-authors (1972), Jhingran and co-authors (1972), Liang and Huang (1972) and Usui (1974).

The best sites for mariculture ponds are on flat coastal land, protected from floods, and with permanent access to unpolluted water of suitable temperature and salinity. The ponds must be arranged to assure easy access, at least throughout stocking, growing and harvesting seasons, and to allow control and protection from trespassers and predators. Water supply and drainage systems must allow each pond to be filled or drained independently of the others. The soil quality should allow effective water retention, as well as construction of stable levees (dams). If necessary, the soil must be compacted—or suitable soil added (see below).

Pond construction depends on local topography. With the farming concept in mind, an economically sound compromise must be sought between geological fac-
MEDIUM-SIZED ENCLOSURES

There are three basic pond types: the dugout pond, excavated below the surrounding ground level; the levee pond, erected on the ground level, and enclosed by a levee; a combination of both (Figs 2-28, 2-29). While dugout ponds require pumps for complete draining, levee ponds can be gravity drained and hence, are preferred in aquaculture. In addition, pond construction can make use of natural bottom depressions, enclosed water bodies (e.g. small lakes), or employ damming of natural water courses (barrage ponds).

Water retention and stability of pond bottom and levees are of basic importance (BARDACH and co-authors, 1972). Sandy soils, with a clay content of 25% to 75% and enough silt to fill the spaces between sand particles, are considered ideal.

In addition, the soil should be compacted. Ponds with poor soil must be sealed. Common sealants are bentonite clay (0.5 to 1.5 kg m\(^{-2}\)), salts, such as sodium chloride (0.04 to 0.17 kg m\(^{-2}\)) or polyphosphates (0.01 to 0.02 kg m\(^{-2}\)). BARDACH and co-authors recommend that the sealant be first mixed with the soil, and then compacted (Fig. 2-28) to form a blanket 15 to 30 cm deep, depending on water depth. As a rule of thumb, the soil compacts best when it is too wet to plough, but not wet enough to release water during compaction. The area near normal water-level height may be protected by a 30- to 45-cm layer of gravel. A most efficient and economical method for sealing pond bottoms and levees is the application of sheets of plastic, such as polyethylene, vinyl or butyl rubber. Structurally weak, these materials must be handled with care; however, once properly installed, plastic and rubber liners are easily kept intact and watertight. Minimum thicknesses for pond liners used over fine sand are—according to BARDACH and co-authors—2 mm for poly-
ethylene and vinyl and 4 mm for butyl rubber. Where the soil contains gravel, these dimensions should be doubled.

Plastic liners are laid in strips with a 15-cm overlap for seaming with appropriate cement or tape. Ponds to be lined must have a dry, firm surface; stony areas are cushioned with a layer of fine material (e.g. sand). Above water level, the lining is anchored by excavating a trench (30 cm wide, 20 to 25 cm deep) around the upper pond edge, and burying the ends of the liner with compacted backfill. For protection against punctures or solar radiation, pond liners are covered by at least 23 cm of fine sand (Fig. 2-29; for details see RENFRO, 1969). In Japanese eel ponds, levees are protected by boulders (Fig. 2-30a), concrete plates (Fig. 2-30b,c) or solid concrete (Fig. 2-30d). These materials prevent the eels from levee tunnelling (USUI, 1974).

Water supply is of particular importance. Usually, each pond has one main water inlet and one main water outlet. The inlet may be a pipe or a sluice (Figs 2-31 to 2-33), fed directly from the sea, a river or a supply channel. The water entry should be arranged to combine three essentials: prefiltration, aeration and directional water movement. A prefiltration barrier is required to hinder predators or unwanted competitors from entering the culture pond. SILLS (no date) recommends the use of Saran screen, style MS-904 (National Filter Media Corp., New Haven, Conn., USA), a durable and inexpensive material with 7 to 8 meshes cm⁻¹, which can be fabricated into suitable shapes. The Saran Sock Barrier can be easily attached to and removed from the inflow pipe (periodic cleaning) using a clamp, rubber band or drawstring (Fig. 2-31); for a pipe with a diameter of 15 cm, the sock should be about 2 m long, and extend well below the water surface of the pond. If the incoming water enters at high velocity, SILLS suggests the use of a floating Saran Box Barrier (Fig. 2-32), held in position by vertical poles.

In Japanese eel ponds, the water enters and is drained through sluices consisting of 3 doors (Fig. 2-33a, b). These sluices have proven very useful for a variety of pond-managing operations. The first door consists of boards, and permits the pond operator to choose whether surface or bottom water is drained. The second door is a meshed frame which prevents escape of the eels. The third door controls the amount of water entering or leaving the pond.

Drainage is achieved by a multitude of devices. For proper water exchange, faeces removal and complete drainage, the pond bottom should slope evenly toward the outlet. BARDACH and co-authors (1972) recommend the L-shaped drain-pipe design which is often employed in catfish ponds (Fig. 2-34). In a vertical position, the pipe determines maximum water-level height and acts as overflow. Rotation, made possible by a swivel joint, allows the surface level to be lowered; when the pipe is turned parallel to the pond bottom, complete drainage can be achieved. By means of an outer sleeve, aged or oxygen-deficient bottom water can be discharged during normal operations. The underdrain for shrimp ponds, its purpose, method of construction and operation received detailed attention from YAMAMOTO and co-authors (1960); see also VINCENT (1908). In a drainage sluice used in Japanese eel ponds, the water discharges through the exit pipe (Fig. 2-33). When draining the pond, eels can be harvested by opening the mesh door and by tying a bag net on to the end of the pipe. Ponds can also be drained by use of 20-cm diameter, plastic-tube siphons, set across the pond bank (Fig. 2-35). A net around the inlet end of the siphon prevents fish from escaping. A pump, applied to an air valve at the top of the siphon,
Fig. 2-30: Japanese eel pond under construction. Levee enforcement by boulders (a), by concrete plates (b, c), and by solid concrete (d). (From Usui, 1974; after Williamson; reproduced by permission of Dr. G. R. Williamson and Fishing News (Books) Ltd.)
is used to remove the air from the pipe and to initiate siphoning. Exit pipes discharge into the drainage ditch.

While most pond cultures employ water movement as a principal method for pond-water-quality management, in Japan, eel ponds are operated with stagnant water. Heavy phytoplankton growth is relied on for oxygen production and waste processing. According to Usui (1974, p. 51), typical eel ponds are of thick, soupy,
green colour' (in summer, phytoplankton development is encouraged, that of zooplankton discouraged). A healthy eel pond contains phytoplankton and zooplankton in weight proportions of about 98:2. Water-quality management in eel ponds further employs water replacement, aeration by paddle splasher; pumps drawing water from the pond bottom, and shooting it out over the surface; addition of chemicals, such as CaO to reduce acidity and iron oxide to remove hydrogen sulphide. In extreme cases of malfunctioning, the pond must be emptied, dried out, disinfected and refilled with new water.

![Fig. 2-34: Pond drainage by L-shaped pipe design. (After Bardach and co-authors, 1972; modified; reproduced by permission of Wiley-Interscience.)](image1)

![Fig. 2-35: Pond drainage via siphon tubes. In practice, two or more siphon tubes are arranged next to each other to facilitate fast drainage. (After Usui, 1974; modified; reproduced by permission of Fishing News (Books) Ltd.)](image2)

Quality control of the water supply is difficult to achieve at economic costs. However, facilities for allowing the incoming water to settle (sedimentation of silt or particulate pollutants) or to equilibrate (temperature, gaseous contents) should be allowed for in the farm design. The same holds for sufficient amounts of reserve water, especially where the total water supply is subject to considerable fluctuation in quality, and where sudden pollution cannot be ruled out. For proper culture-water treatment (p. 106), important indicators of water quality (p. 108) must be determined at suitable intervals or monitored continuously.

In temperate areas, aquaculture ponds may require insulation and/or heating in order to obtain optimum yields, and to avoid mortality due to critical cooling.
Use of waste heat (e.g. effluents from power stations) and erection of plastic-sheet greenhouses (Fig. 2-36) offer a variety of economically feasible solutions to the temperature problem.

In fish ponds it is often advantageous and customary to establish definite feeding points where automatic feeders (p. 259) are installed, or the fish are being hand fed. In the still-water Japanese eel ponds, the feeding point is located at a site where the oxygen level is high, as this encourages the eels to feed well (Usur, 1974). The food (raw fish, briefly boiled to soften the skin) is prepared in a small hut placed at the feeding point (Fig. 2-37). In eel ponds larger than 600 m², many farmers have installed a 'resting corner' (Fig. 2-38). Actually a small, well-aerated, running-water pond, the resting corner is connected with the main still-water pond by small openings; it has a water inlet and outlet, a splasher aerator, and a vertical pump (vertical water exchange plus aeration). If, at night, the oxygen level in the main pond becomes critically reduced, the eels enter the resting corner. During harvesting, when large numbers of eels are netted, the full net is dragged to the resting corner for handling and sorting.

To permit vehicle access, levee tops measure at least 3 m; about 0.6 m of free-board should be left between levee top and water surface; recommended slopes are at least 4:1 on the inside and 3:1 on the outside. Immediately after construction, levees are planted with grass to impede erosion (Bardach and co-authors, 1972). A general layout of a Danish pond farm for culturing trout is illustrated in Fig. 2-39.

(c) Large Enclosures

Large enclosures, such as embayments, bays, lagoons, fjords, tidal areas, salt marshes or artificial habitats (created by controlled flooding of large low land areas) serve, in most cases, commercial cultivation. Construction and operation of large-scale coastal mariculture plants have received attention in numerous publications (see also Chapter 5.2). They will not be treated here in detail. Major factors to be con-
sidered are the organism cultured, its market value, climate, cost of land, labour, energy and transportation, morphology of the land-water boundary, soil quality, water-flow dynamics and salinity.

(d) In situ Enclosures and Related Devices

Cages, pens, pounds and plastic bags, as well as floats, racks, rafts and related devices are used for culturing marine plants and animals in the sea. A few examples are illustrated in Figs 2-40 to 2-46. Further information on similar in situ devices can be found in Kinne and Bülneheim (1970), Bardach and co-authors (1972) and in Chapter 5.2.

An octagonal fish pound has been developed at the Nanaimo Biological Station (Canada) for rearing the sablefish Anoplopoma fimbria (Kennedy, 1968). The 8 sides and the bottom of the pound are all of netting (Fig. 2-44). A detachable top netting keeps out gulls and other potential air-breathing intruders. Each of the pound sides is 122 cm wide and 550 cm deep (the upper 61 cm are above water, the remainder under water). Floats support the frame and allow access. The pound is anchored in sheltered water. The netting can be raised by pulling on the vertical tensors (to get access to the fish) and returned to its usual shape by tightening the downhauls. In the sea, algae, detritus, etc. soon begin to settle on the netting. Hence, two nets are used alternately in the same frame; the net not in use is dried to

---

Fig. 2-37: This hut serves as feeding point in a Japanese eel pond. It is located in a well aerated, shadowed site. On the cauldron, raw fish is briefly boiled to soften its skin; with strings threaded through their eyes, the feed fish are then offered in bundles to the eels. PS: paddle splasher; open arrow: prevailing wind direction; closed arrows: water flow. (After Usui, 1974; modified; reproduced by permission of Fishing News (Books) Ltd.)
free it from the fouling materials. Of course, numerous variations of the pound are possible, and are in use in a number of marine biological institutions.

Plastic bags, suspended in the sea, are most useful tools for studying natural

Fig. 2.38: Resting corner in a large Japanese eel pond. This well-aerated (vertical pump, paddle splasher) corner serves as refugium in cases of critical reduction in water quality of the main pond, as well as for handling and sorting of netted eel masses during harvesting. (a) Photograph; (b) schematic top view. M: motor. (After Usui, 1974; modified; reproduced by permission of Fishing News (Books) Ltd.)

Fig. 2.39: Danish pond farm. 1: dam with wild-fish ladder; 2: pond; 3: screen. (From Bardach and co-authors, 1972; modified; based on Bredboll; reproduced by permission of Wiley-Interscience.)
Fig. 2.40: Examples of in situ racks, rafts and frames for cultivating benthonic algae. (a) Original; (b), (c), (d) based on Cheng, 1969; (e) based on Neushul and Haxo, 1963; (f) after Luning, 1968; reproduced by permission of Biologische Anstalt Helgoland.)
1.3 Floating net cage

(a) Small gauze float for fish larvae
(b) Floating net cage, simple design
(c) Floating net cage, double-layer design
(d) Circular floating cage for schooling fishes
(e) Hanging frames for single-oyster farming

Fig. 2-41: Examples of in situ cages and oyster frames. The small gauze float was designed for laboratory experiments, but comparable, larger floats are also used in the sea. (a) after Davis, 1970; modified; (b) to (d) after Harada, 1970; modified; (e), (f) after Fujiya, 1970; modified; (a) to (f) reproduced by permission of Biologische Anstalt Helgoland.)
plankton dynamics, as well as for analyzing the effects of pollutants on large natural plankton populations (in situ test pollution). The plastic-bag concept (Fig. 4-28) has been pioneered and discussed by Strickland and Terhune (1961: see also Goldman, 1962).

Small plastic bags (‘foil tanks’) have been used by Brockmann and co-authors (1974) for studying in situ interactions between plankton and ambient water (Fig. 2-45). The cylindrical (5 m deep, less than 1 m diameter) bags consist of a transparent combination foil with an outside layer of polyamid (30 μm) and an inside layer of polyethylene (100 μm). Both layers are supported by a metal frame attached to a buoy. A motor-driven propellor produces continuous water mixing. Brockman and co-authors conducted their experiments under natural concentrations of nutrients and at natural population densities, both in mono- and multispecies cultures. In the large South Harbour of Helgoland (southern North Sea), their bags have stood up to tides, wind and waves.

In order to accommodate at least three trophic levels—e.g. phytoplankton, zooplankton, fish—the large polythene bags developed by Parsons (unpublished) and his colleagues measure ca 10 m in diameter by 30 m in depth, and contain about 2500 tons of sea water (Fig. 2-46). Initial experiments were conducted by a team of scientists of different disciplines in Saanich Inlet (Vancouver Island, Canada), a highly productive near-shore environment. The experiments demonstrated considerable similarity of biological events when four replicate bags (scale models of ca 70 tons each) were filled simultaneously. In view of the parallelism of ecological dynamics in the four test bags and in the surrounding sea, the water bodies contained in the bags can be used as models, both of the normal ecological processes taking place in the unperturbed marine environment and of isolated ecosystems exposed to sublethal amounts of pollution or other environmental stress. Multispecies cultures and natural food-web associations can be studied here under manipulable conditions never available before. These bags also qualify as macro-enclosures for a variety of projects of research cultivation and commercial cultivation.

Fig. 2-42: In situ oyster culture (foreground) and seaweed culture (background) near Hamana, Japan. (Photograph: H. Koops.)
Fig. 2-43: In situ fish culture. (a) Small net culture (2 x 2 x 2 m deep). (b) Medium net culture (4 x 4 x 2 m deep). (c) Large net culture (6 x 6 x 2 m deep). (d) Bay area (18 ha). Separated from the sea by double nets (ca. 12 to 18 m). (e) In an artificial pool in Hamburg. (f) Near Zenzaki, Japan. (All photographs: H. Koops.)
(e) Conclusions

Size and form of culture enclosures vary considerably with the test organism and the culture goal. In addition to the well-known standard enclosure types, numerous special designs have recently been developed. A few examples are described and illustrated. Ponds, the oldest culture enclosures, are still of considerable importance both in research cultivation and in commercial cultivation. They have received more attention than any other enclosure.
Among the \textit{in situ} enclosures, large plastic bags facilitate modern research on food chains, functions and structures of ecosystems and water-pollution effects under quasi-natural conditions. They are likely to become essential tools for the experimental ecologist interested in population dynamics and in biological oceanography.

The large diversity of enclosure types and of organisms cultured, and the frequently insufficient published details on the capability of a given enclosure to support life preclude a critical evaluation of comparative efficiencies.

![Diagram of a plastic bag for in situ culture of plankton](image)

\textbf{Fig. 2-45}: Plastic bag for \textit{in situ} culture of plankton. (a) General view; (b) cross-section. (After Brockmann and co-authors, 1974; modified; reproduced by permission of Springer-Verlag.)

\section{5. Life-supporting Substances}

Sea water may contain a variety of life-supporting substances, such as dissolved organics serving as nutrients, vitamins or growth promoters. Our present knowledge regarding the ecological significance of these substances is very limited. Dissolved organics (Volume I, Chapter 10) represent prerequisites for the normal development of certain marine organisms, especially bacteria; they also appear to be important for some algae and for several animals such as cnidarians, polychaetes and related
forms. In 'higher' organisms, e.g. crustaceans, fishes or marine mammals, dissolved organic substances may serve as orientation cues or as synchronizers of biological activities (Volume II, Chapters 8 and 9).

Are dissolved organics responsible for the fact that sea water from different localities may have different potentials for supporting life? Do these substances account for, or contribute to, the 'aging' of captive sea water (p. 22)? Is our inability to provide and maintain defined amounts of these substances in culture systems the reason for the failure to cultivate certain 'delicate' marine organisms? These questions are of fundamental importance to all ecologists concerned with the cultivation of marine organisms. Concerted efforts of ecologists, physiologists and biochemists are needed to provide the answers.

After reviewing the evidence at hand for the life-supporting role of external free amino acids (FAA) dissolved in sea water, Stephens (1972) concludes that FAA can meet the nutritional requirements of some marine organisms, even when offered...
at the low concentrations prevailing in nature. In other organisms, FAA can contribute appreciable portions to the total intake of nutritive organic matter. While not all marine organisms seem to be able to take advantage of FAA as potential energy source, the capacity for taking up external amino acids and other organic substances appears to be a phenomenon of general ecological significance (e.g. Stephens and Schinske, 1958; Chapman and Taylor, 1968; Anderson and Stephens, 1969; Johannes and co-authors, 1969; Schlichter, 1971, 1973, 1974a, b, 1975; Southward and Southward, 1972, Volume II: Pandian, 1975; Volume IV).

The anthozoan Anemonia sulcata, for example, resorbs and accumulates external tritiated L-amino acids at in situ concentrations (Schlichter, 1973, 1974a, b). Resorption occurs mainly through the apical membrane of the tentacle ectoderm. Uptake rates (2-10 μg g⁻¹ wet weight hr⁻¹) depend on type and concentration of the amino acid tested. Schlichter established that the internal glycine concentration exceeds the ambient concentration by a factor of 10⁷; this finding indicated to him that the uptake represents an active metabolic process. According to Schlichter's calculations, A. sulcata satisfies a substantial portion of its metabolic requirements by FAA uptake from the ambient sea water. A. sulcata resorbs external amino acids also under anaerobic conditions, and shows appreciable independence of uptake dynamics from environmental factors (Schlichter, 1974a). Uptake of amino acids is possible against a gradient of up to 1:10⁴. Schlichter (1975) claims that A. sulcata obtains a nutritional profit by resorbing dissolved ambient glucose.

In some marine interstitial polychaetes, FAA influx is rapid, and efflux relatively independent of external amino-acid concentrations in the normal in situ range (Stephens, 1972). Influx exceeds efflux at FAA levels which seem to be characteristic of the microhabitat of the polychaetes tested. Calculations, comparing rate of uptake with energy requirements of the worms, suggest that FAA uptake represents a supplement to other food pathways. Among the environmental factors influencing the dynamics of influx versus efflux rates of FAA, as well as their assimilation by the worms, are salinity, ionic composition and FAA concentration (Stephens, 1972). Presumably, temperature and dissolved gases (especially O₂) also exert measurable effects.

In culture systems, the application of defined amounts of certain dissolved organics, that may act as life-supporting substances, is difficult. If necessary, such substances must be added to individual culture enclosures (not to the total system). Since biological or physico-chemical water treatment tends to remove the substances from the recycling sea water almost as fast as they are added, a continuous supply is necessary. Addition of significant amounts of organic matter will increase the biochemical oxygen demand (BOD; p. 108) and tend to reduce the carrying capacity (p. 167). In fact, in most sea-water systems, dangerous accumulation of organic substances seems to be of greater immediate concern than under-representation of dissolved organics.

For a number of organisms, antibiotics may act as life-supporting substances. Addition of antibiotics to the culture medium has reduced mortalities, for example, in protozoans, as well as eggs and larvae of invertebrates. Marshall and Orr (1958) have used streptomycin to prevent excessive bacterial population growth in freshly collected sea water used for cultivating copepods; for further examples consult Chapters 4.1 and 5.1. In axenic cultures (Chapter 5.11), antibiotics are necessary for
purification and for protecting monospecific cultures from bacterial 'contamination'.

While addition of antibiotics to the culture medium can suppress the development of undesired micro-organisms, possible side effects on the organisms cultured have been insufficiently investigated and cannot be ruled out. Antibiotics may be harmful to marine organisms (p. 104), and several investigators, who originally used antibiotics, have recently pointed out that they obtain comparable or even better cultures without antibiotics. Much work remains to be done before we can use antibiotics with more faith in ecological experiments.

(6) Life-endangering Substances

Substances which support life may attain life-endangering qualities if allowed to accumulate. In fact, whether a given substance exerts life-supporting or life-endangering qualities is often primarily a function of its concentration.

In principle, the substances which tend to endanger life in culture systems can be considered under two terms: contaminants and metabolites. Contaminants originate from the materials used in cultivation and from laboratory procedures, such as cleaning (detergents), heating, handling, etc.; a detailed account of chemical contamination of culture media is presented in Chapter 7 (see also Blankley, 1973). Among the metabolites, two principal groups can be distinguished: ectocrines and end products of nitrogen metabolism.

(a) Ectocrines

Ectocrines are substances released into the ambient water, which may exert potent biological effects even though present only in very low concentrations (see also Volume I, Chapter 10). They can be poisonous (e.g. the toxin of the dinoflagellate Gymnodinium veneficum) or they may critically influence biological processes. They may act as stimulants (e.g. in growth, reproduction, orientation) or as mediators of interrelationships between organisms (e.g. sex attractants). It has been pointed out (Allee, 1931; Hardy, 1936; and others) that excretions and secretions of aquatic animals may 'condition' the external medium, and thereby affect metabolism and activity, as well as distributions of co-existing forms. Ectocrines or external metabolites—including pheromones (Karlson and Lüscher, 1959; Kirschchenblatt, 1962)—received early attention from Lucas (e.g. 1947, 1961).

In cultivation, the biological effects of ectocrines can be masked or enforced in the presence of toxic end products of nitrogen metabolism (p. 80). Furthermore, production of, and responses to, ectocrines may follow normal patterns in healthy individuals only. Hence, analysis of ectocrine effects requires more critical experimentation than has been practised in the majority of pertinent studies.

The information currently available on the role of ectocrines in the cultivation of aquatic animals is insufficient for detailed evaluation. Two examples must suffice: Berrie and Visser (1963) have isolated a mono-hydroxyl-tri-carboxylic acid mono-isodecyldimethyl ester (molecular weight: 360; empirical formula: \( C_{18}H_{32}O_7 \)) released by the aquatic snail Biomphalaria sudanica. This ectocrine is lethal to the snails when added to their culture water in twice the normal concentration. Yu
(1968) extracted organic substances produced by the zebra fish *Brachydanio rerio* and the blue gourami *Trichogaster trichopterus*; these substances exert inhibiting effects on the growth rates of these freshwater fishes when added to the culture water in defined amounts.

(b) End Products of Nitrogen Metabolism

End products of nitrogen metabolism that are allowed to accumulate in the culture water may interfere with essential life processes. Hence, dangerous nitrogen compounds released from living or dead organisms or from waste food must be reduced to acceptable levels. In microcosm cultures consisting of microbial, plant and animal components, nitrogenous substances must be recycled properly in terms of routes and rates. Removal of excess nitrogen compounds from the culture water and regulation of nitrogen cycling are essential keys for successful cultivation.

Animals excrete ammonia, urea, uric acid and other nitrogenous substances—including guanine, amino acids and trimethylamine oxide—as end products of their nitrogen metabolism (Norris and Benoit, 1945; Parry, 1960; Potts, 1967; Campbell, 1970a, b; Campbell and Goldstein, 1972; Volume II: Pandian, 1975). Depending upon the major end product, the type of excretion is referred to as ammonotelic, ureotelic, uricotelic, or guanotelic. Excretion types may change during the life of the animal concerned, as a function of age, developmental stage or environmental factors (e.g. Munro, 1953; Nash and Frankhauser, 1969; Sharma, 1966; Goldstein and co-authors, 1967; Pickford and Grant, 1967; Haggag and Fouad, 1968; Goldstein, 1972). In aquatic animals with low rates of nitrogen metabolism, the bulk of nitrogenous wastes may be lost by diffusion through the body wall. However, in ‘higher’ carnivorous forms such as cephalopods, a large portion of nitrogen excretion takes place through the kidneys or their equivalents (Potts, 1967; Schmidt-Nielsen, 1972); in decapods and fishes, the gill serves as chief site of nitrogen excretion (e.g. Smith, 1929; Wood, 1968; Fromm, 1963; Goldstein and co-authors, 1964; Goldstein and Forster, 1970).

In cultivation, ammonia, nitrite, nitrate and organic compounds such as urea, uric acid, proteins and amino acids are of primary concern. Nitrogen oxides ($N_2O$, NO, $N_2O_3$, NO$_2$, $N_2O_5$) and molecular nitrogen are less important. Removal of excess nitrogen compounds and nitrogen recycling rely primarily on micro-organisms and, to a lesser degree, on algae. Both the necessary micro-organisms and the algae are accommodated in biological water-treatment units (p. 122). In addition, physico-chemical water treatment (p. 134) assists in lowering critical levels of end products of nitrogen metabolism.

The role of bacteria in the oxidation of end products of nitrogen metabolism was anticipated by Pasteur (1862), and first demonstrated in soil (Schloessing and Müntz, 1877; Müntz, 1890). Oxidation of ammonia to nitrite and nitrate was discovered by Munro (1886); Winogradsky (1882) recognized the involvement of two separate oxidation processes performed by ammonia-oxidizing and nitrite-oxidizing bacteria (see below). That biological nitrification in the sea proceeds essentially along the same lines has been established by von Brand and co-authors (1937, 1939, 1942); von Brand and Rakestraw (1940, 1941); Rakestraw and von Brand (1947); see also Sverdrup and co-authors (1942); Barnes (1957); Raymont (1963).
PRODUCTS OF NITROGEN METABOLISM

and Vaccaro (1966). These authors discovered that (i) it is possible to reproduce the complete cycle of nitrogen regeneration under laboratory conditions: (ii) dead, decomposing organisms rapidly release ammonia; (iii) the main decomposition stages are: ammonia, nitrite and nitrate. Studies by Cooper (1937), Köhl and Mann (1955, 1966a, b, 1962), Botan and co-authors (1960) and others revealed that the resulting picture is quite complex. For details consult Volume II: Schlegel (1975).

The two fundamental processes of bacterial nitrogen transformation are referred to as nitrification (oxidation of ammonia to nitrite or nitrate) and denitrification (reduction of nitrate to nitrite, or of nitrite to nitrogen or free nitrogen) (Volume II: Schlegel, 1975). Molecular nitrogen can be fixed by certain bacteria, yeasts and blue-green algae.

Ammonia, nitrite and nitrate are also utilized by a number of algae. Several phytoplankters have been shown to use ammonia in preference to nitrate. While ammonia can be metabolized directly for amino-acid synthesis through transamination, nitrite and nitrate must first be reduced. Reduction of nitrite to ammonia appears to involve photosynthetically reduced ferredoxin (Hattori and Myers, 1966). Nitrate is reduced by the light-independent enzyme nitrate reductase (Hattori, 1962a, b). The role of algae in processing nitrogenous substances in culture systems has been evaluated on pp. 129 to 134.

In oceans and coastal waters, nitrogen occurs mainly in the form of molecular nitrogen and nitrate, nitrite and ammonia. Of the 9 different oxidation states (−3 to +5), molecular nitrogen (0) attains about 20 times the amount of the total combined nitrogen. The more highly oxidized nitrate (+5) and nitrite (+3) comprise about 65% of the total combined nitrogen. Reduced nitrogen (−3 to −1), i.e. ammonia and organic nitrogen compounds, both dissolved and particulate, make up some 35% of the combined nitrogen (Vaccaro, 1966).

Ammonia

The term ‘ammonia’ is used differently in the biological literature and hence, requires definition. Ammonia is defined as the gas \( \text{NH}_3 \), ammonia nitrogen, as nitrogen combined in \( \text{NH}_3 \) or ammonium, \( \text{NH}_4^+ \). The latter combines with a number of elements, but in biological excretion, occurs as ammonium hydroxide, which dissociates in a reversible reaction into ammonia, \( \text{NH}_3 \), and water (e.g. Burrows, 1964). In most biological studies—and in the present review—the term ‘ammonia’ comprises both \( \text{NH}_3 \) and \( \text{NH}_4^+ \). Where the information at hand allows specification, differentiation is made between \( \text{NH}_3 \) and \( \text{NH}_4^+ \).

In the open oceans, the range of ammonia concentration is from 0.1 to 10 \( \mu \text{g} \) at \( 1^{-1} \). In most cases, oceanic ammonia values lie between 0.1 and 2 \( \mu \text{g} \) at \( 1^{-1} \) in the surface layer. Exact analytical methods have become available only recently, and even these are not strictly ammonia specific; hence, only a few exact measurements are at hand. Ammonia is the major end product of nitrogen metabolism in most aquatic animals (Volume II: Pandian, 1975, pp. 179–184). It is often released at high rates (Needham, 1970; Tables 2-15 to 2-18). Among the potentially life-endangering substances accumulating in the culture water, ammonia is of primary importance. In waste waters discharged into coastal waters, ammonia is often present in high concentrations, especially in effluents from municipal sewage and from coke ovens (e.g. Ellis, 1837; Grindley, 1946; Wuhrmann, 1962; Volume V).
Table 2.15
Excretory rates of ammonia (\(\mu g\) at \(NH_4^+\)—N mg\(^{-1}\) dry weight day\(^{-1}\)) by zooplankton; test temperatures: 12\(^\circ\) to 15\(^\circ\) C. The number of specimens tested is given in parentheses (After Jawed, 1973; modified; reproduced by permission of Springer-Verlag, Berlin)

<table>
<thead>
<tr>
<th>Station no.</th>
<th>Water temperature</th>
<th>Animals</th>
<th>Total biomass (mg dry weight)</th>
<th>Excretory rate ((\mu g) at (NH_4^+)—N mg(^{-1}) dry weight day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>15.2(^\circ) C</td>
<td>Mixed copepods</td>
<td>7.7</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed copepods</td>
<td>8.4</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed copepods</td>
<td>5.4</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed copepods</td>
<td>4.4</td>
<td>0.44</td>
</tr>
<tr>
<td>33</td>
<td>14.8(^\circ) C</td>
<td><em>Calanus</em> sp. (15)</td>
<td>3.2</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Calanus</em> sp. (20)</td>
<td>3.6</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed copepods</td>
<td>12.6</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed copepods</td>
<td>9.4</td>
<td>0.26</td>
</tr>
<tr>
<td>36</td>
<td>14.6(^\circ) C</td>
<td>Mixed copepods and <em>Euphausia pacifica</em> (1)</td>
<td>7.1</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>6.6</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton and <em>Euphausia pacifica</em> (1)</td>
<td>14.2</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton and <em>Euphausia pacifica</em> (1)</td>
<td>18.8</td>
<td>0.33</td>
</tr>
<tr>
<td>36</td>
<td>13.8(^\circ) C</td>
<td><em>Calanus</em> sp. (≈100) and <em>Euphausia pacifica</em> (2)</td>
<td>16.2</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Calanus</em> sp. (≈120), <em>Euphausia pacifica</em> (1) and Mixed zooplankton</td>
<td>10.8</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>8.4</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>12.5</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>14.1</td>
<td>0.36</td>
</tr>
<tr>
<td>38</td>
<td>12.8(^\circ) C</td>
<td>Mixed zooplankton</td>
<td>11.7</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>18.6</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>27.6</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>21.4</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aequorea aequorea</em> (1)</td>
<td>970.0</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aequorea aequorea</em> (1)</td>
<td>840.0</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jellyfish: <em>Phialidium</em> sp. (1)</td>
<td>360.0</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Proboscidactyla</em> sp. (1)</td>
<td>360.0</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pleurobrachia</em> sp. (1)</td>
<td>360.0</td>
<td>0.04</td>
</tr>
<tr>
<td>38</td>
<td>13.8(^\circ) C</td>
<td>Amphipod: <em>Callianopsis</em> sp. (3)</td>
<td>39.4</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>20.4</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed zooplankton</td>
<td>31.9</td>
<td>0.19</td>
</tr>
<tr>
<td>39</td>
<td>11.5(^\circ) C</td>
<td><em>Euphausia</em>: <em>Thysanoessa longipes</em> (2)</td>
<td>32.7</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Euphausiids</em>: <em>Euphausia pacifica</em> (1) and <em>Thysanoessa longipes</em> (3)</td>
<td>45.3</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Euphausiids</em>: <em>Euphausia pacifica</em> (3)</td>
<td>3.1</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Rates of ammonia excretion

Rates of ammonia excretion are documented here on the basis of a few examples. Ammonia excretion has received attention in Volume II (PANDIAN, 1975, pp. 179–184).

Most molluscs examined excrete ammonia, but of the total nitrogen excreted the ammonia proportion may sometimes be small (Potts, 1967). Within 24 hrs, and at normal temperatures, the following rates of NH₃-N release have been established: marine gastropods, 4 to 85 mg g⁻¹ total body weight (Duerre, 1968); freshwater snail Lymnaea stagnalis, 34 mg g⁻¹ total body weight (Bayne and Friedl, 1968);

Table 2-16
Ammonia (NH₃-N) excretion rate in 3 zooplankters. Darkness; 13° C (After Mayzaud, 1973; modified; reproduced by permission of Springer-Verlag, Berlin)

<table>
<thead>
<tr>
<th>Species</th>
<th>NH₃-N excretion (%) body N day⁻¹</th>
<th>NH₃-N excretion (%) body protein day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Meganyctiphanes norvegica</em></td>
<td>Winter 7.1</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>Spring 4.5</td>
<td>4.8</td>
</tr>
<tr>
<td><em>Phronima sedentaria</em></td>
<td>Winter 7.4</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>Acartia clausi 61.9</td>
<td>68.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Body nitrogen (%) dry weight</th>
<th>Total nitrogen excretion (%) body N day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Meganyctiphanes norvegica</em></td>
<td>Winter 11.47</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Spring 9.63</td>
<td>6.3</td>
</tr>
<tr>
<td><em>Phronima sedentaria</em></td>
<td>Winter 6.94</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>Acartia clausi 8.40</td>
<td>73.9</td>
</tr>
</tbody>
</table>

Lamellibranch Modiolus demissus, 42 mg g⁻¹ tissue (Lum and Hamm, 1964); oyster Crassostrea virginica, 25 mg g⁻¹ tissue (Hamm and co-authors, 1966); cephalopod Octopus dofleini, 30 µg (renal excretion) g⁻¹ tissue plus, presumably, a comparable amount of extra-renal ammonia (Potts, 1965; see also Delaunay, 1931; Emmanuel and Martin, 1956; Campbell and Bishop, 1970). All crustaceans examined are ammonotelic, with the possible exception of Cardisoma guanhumi (Perry, 1960; Hartenstein, 1970). A number of amphipods and isopods have been shown to excrete 80 to 90% of their nitrogenous end products as ammonia; in their terrestrial counterparts, the respective values range from 50 to 60% (Dresel and Moyle, 1950).

In cultivated Calanus helgolandicus, nearly 90% of the nitrogen excreted is in the form of ammonia (Corner and co-authors, 1972). The soluble portions are shown in Table 2-17. The amounts of excreted ammonia and total soluble phosphorus increase significantly in fed individuals.
Zooplankters sampled off the coasts of Washington and Oregon (USA) were found to excrete NH$_4$-N at rates varying from 0.16 to 0.60 µg at mg$^{-1}$ dry weight day$^{-1}$ (experimental temperatures: 12° to 15° C); in jellyfishes, the rates varied from 0.02 to 0.06 (Jawed, 1973; Table 2-15; see also Conover and Corner, 1968; Jawed, 1969). The amounts of NH$_4$-N released by the zooplankters *Meganycytiphanes norvegica, Phronima sedentaria, Acartia clausi* and *Sagitta setosa* are listed in Table 2-18. Mayzaud (1973) calculated the protein carbon equivalent to respiratory and excretory catabolism, using atomic O:N ratios. While the main nitrogenous product excreted is ammonia, significant amounts of organic nitrogen compounds are also present. Starved copepods catabolized more protein carbon than would be accounted for by the respiratory oxygen utilized. Further information on rates of ammonia excretion by zooplankters has been presented by Corner and Newell (1967), Corner and Corner (1968) and Butler and co-authors (1970).

Rates of ammonia excretion tend to vary as a function of age (life-cycle stage), temperature, salinity (Emerson, 1969), ambient ammonia levels, and season. Seasonal variations have been reported, for example, for *Meganycytiphanes norvegica* (Mayzaud, 1973; see also Conover and Corner, 1968). Comparable seasonal fluctuations occur in other aquatic invertebrates, e.g. in the freshwater snail *Lanistes baltemia* (Haggag and Fotad, 1968): In April, 95% of the total-N was NH$_4$-N, and no urea-N was excreted. In May, 31% NH$_4$-N and 40% urea-N were produced. During June, July and August, *L. baltemia* proved to be ureotelic, excreting from 47 to 62% urea-N; NH$_4$-N ranged from 13 to 24% of the total-N. In September, similar results were obtained as in April. However, in October, no NH$_4$-N was excreted, and urea-N accounted for 82% of the total-N released. Such variations in ammonia excretion may be related to seasonal fluctuations in life-cycle stage (age), irradiance, temperature or nutrition.

**Table 2-17**

<table>
<thead>
<tr>
<th>Quantity released (µg animal$^{-1}$ day$^{-1}$)</th>
<th>Ammonia nitrogen</th>
<th>Total N</th>
<th>'Non-ammonia nitrogen'</th>
<th>Total P</th>
<th>N : P ratio (by weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfed</td>
<td>0.68 ± 0.01</td>
<td>1.02 ± 0.02</td>
<td>0.15 ± 0.013</td>
<td>0.139 ± 0.006</td>
<td>7.61 ± 0.36</td>
</tr>
<tr>
<td>Fed</td>
<td>1.21 ± 0.04</td>
<td>1.34 ± 0.05</td>
<td>0.13 ± 0.017</td>
<td>0.185 ± 0.010</td>
<td>7.48 ± 0.34</td>
</tr>
<tr>
<td><em>P</em></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&gt;0.25</td>
<td>&lt;0.001</td>
<td>&gt;0.75</td>
</tr>
<tr>
<td>Increase (%)</td>
<td>36.4 ± 4.1</td>
<td>31.3 ± 5.0</td>
<td>Nil</td>
<td>33.6 ± 5.3</td>
<td>Nil</td>
</tr>
</tbody>
</table>

*P* > 0.25

**Table 2-17**

Amounts of soluble nitrogen and phosphorus released by fed (diatom *Biddulphia sinensis*) and unfed *Calanus helgolandicus* 10° C; dim light. Mean values and standard errors based on 24 determinations for fed and 12 for unfed copepods. *P* values indicate statistical significance of differences between value pairs (After Corner and co-authors, 1972; reproduced by permission of Marine Biological Association of the U.K.)
In a number of freshwater fishes, ammonia-N accounts for 80% of the total nitrogen excreted; urea-N makes up most of the remainder (Smith, 1929). According to Baldwin (1949), about 25 to 50% of the nitrogenous compounds excreted by fishes is ammonia; among the remainder, urea, creatine, and amino acids usually predominate. A 29.7-g bluegill sunfish Lepomis macrochirus excretes 7.18 mg N day⁻¹. This is about $3\frac{1}{2}$ times as much as a warm-blooded animal produces (Gerking, 1955). Growing rainbow trout Salmo gairdnerii excrete 17 mg ammonia-N kg⁻¹ body weight hr⁻¹ (Shirahata, 1964; see also Liao and Mayo, 1972).

Table 2-18
Ammonia excretion rates and atomic O:N ratios in 4 zooplankters. Darkness; 13° C (After Mayzaud, 1973; modified; reproduced by permission of Springer-Verlag, Berlin)

<table>
<thead>
<tr>
<th>Species</th>
<th>NH₃—N excretion (µg at N mg⁻¹ dry weight day⁻¹)</th>
<th>Total nitrogen excretion (µg at N mg⁻¹ dry weight day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Meganyciphanes norvegica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>0.57</td>
<td>0.66</td>
</tr>
<tr>
<td>Spring</td>
<td>0.23</td>
<td>0.34</td>
</tr>
<tr>
<td>Phronima sedentaria</td>
<td>0.36</td>
<td>0.74</td>
</tr>
<tr>
<td>Acartia clausi</td>
<td>3.71</td>
<td>4.43</td>
</tr>
<tr>
<td>Sagitta setosa</td>
<td>3.61</td>
<td>5.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Respiration (µg at O₂ mg⁻¹ dry weight day⁻¹)</th>
<th>Atomic ratio O:N/NH₃</th>
<th>Atomic ratio O:N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Meganyciphanes norvegica</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>2.72</td>
<td>4.77</td>
<td>4.12</td>
</tr>
<tr>
<td>Spring</td>
<td>2.79</td>
<td>12.13</td>
<td>8.20</td>
</tr>
<tr>
<td>Phronima sedentaria</td>
<td>1.54</td>
<td>4.27</td>
<td>2.08</td>
</tr>
<tr>
<td>Acartia clausi</td>
<td>5.97</td>
<td>1.61</td>
<td>1.34</td>
</tr>
<tr>
<td>Sagitta setosa</td>
<td>6.33</td>
<td>1.76</td>
<td>1.25</td>
</tr>
</tbody>
</table>

In marine teleosts, ammonia is the predominant form of nitrogenous waste product (Delaunay, 1929; Wood, 1958; de Voogt, 1968). Nitrogen metabolism in fishes has been discussed by Goldstein and Forster (1970). Nitrogen excretion in amphibians has been reviewed by Balinsky (1970), in reptiles by Coulson and Hernandez (1970), and in mammals by Berg and Kolemberander (1970); see also Campbell and Goldstein (1972).

Concentrations of ammonia and other nitrogenous compounds in blood or coelomic fluids of aquatic animals have been determined in a number of cases (e.g. Delaunay, 1913, 1927, 1931; Myers, 1920; Strunk, 1930; Florkin and Houet, 1939; Florkin and Renward, 1939; Florkin and Frappez, 1940; Bahl, 1946;
ABDEL-FATTAEI, 1967; DE JORGE and co-authors, 1965, 1966; POTTS, 1965, 1967; DE JORGE and SAWAYA, 1967; GIFFORD, 1968; HARTENSTEIN, 1968; FLORKIN, 1969; NEEDHAM, 1970). The values reported vary between 0.005 and 1.5 mg NH$_3$-N ml$^{-1}$. They may turn out to be of significance for assessing rates of ammonia excretion and tolerances to ammonia concentrations present in the culture water. In rainbow trout *Salmo gairdnerii* exposed to solutions of ammonium chloride containing 0, 1, 3, 5 or 8 mg total ammonia ml$^{-1}$, a direct correlation was observed between blood ammonia (both total ammonia and NH$_3$) and total ambient ammonia after 24-hr periods (FROMM and GILLETTE, 1968). In *S. gairdnerii* exposed to 0 to 1 mg total ammonia ml$^{-1}$, mean NH$_3$-N blood levels ranged from 0.6 to 1.3 mg ml$^{-1}$. Daily excretion rates of ammonia showed an inverse relationship with starting ambient ammonia levels. Since blood ammonia concentrations always exceeded ambient ammonia levels, accumulation of ammonia in the blood must have resulted from excretory inhibition, rather than from ammonia uptake against the concentration gradient. Decrease in ammonia excretion rates concurrent with increase in ambient ammonia suggests damage to the excretory mechanism.

**Biological consequences of ammonia accumulation**

Accumulation of ammonia in the culture water produces a variety of detrimental biological consequences and remains a major cause of functional and structural disorders both in research cultivation and in commercial cultivation. Harmless at very low levels, increasing concentrations of ammonia rapidly become dangerous to aquatic animals, especially if allowed to persist over some weeks. This section considers: (i) the factors which determine proportion, distribution and relative importance of NH$_3$ and NH$_4^+$; (ii) the kinds of functional and structural disorders caused. Most of the information available is concerned with freshwater fishes. However, there is reason to assume that the biological consequences of ammonia accumulation involve phenomena which are not species specific; comparable functions and structures seem to be affected similarly in limnic and marine forms, even in different taxonomic groups.

The biological consequences of ammonia accumulation depend on the proportion and distribution of NH$_3$ and NH$_4^+$. The proportion of NH$_3$ and NH$_4^+$ is determined primarily by the pH of the fluid. At any given pH, the NH$_3$ portion of the total ammonia concentration is a function of the dissociation constant ($K$) of the molecule. The negative logarithm of $K$ ($pK$) varies with temperature and type of fluid. If $pK$, pH, total ammonia concentration, temperature and type of fluid are known, the proportion of NH$_3$ and NH$_4^+$ can be calculated by applying the law of mass action. Such calculations (Table 2-19) are necessary, because most methods of ammonia determination (p. 92) are based on microdiffusion, and give only total concentrations. As a rule of thumb, a one-unit increase in pH, e.g. from 7.5 to 8.5, produces approximately a 10-fold increase in the percentage of NH$_3$.

The distribution of NH$_3$ and NH$_4^+$ in biological systems depends on the differential permeability of cellular barriers to these two forms. In general, cell membranes are readily permeable to NH$_3$, but relatively impermeable to NH$_4^+$. Since the degree of ammonia toxicity depends on the amount of ammonia which enters the cell, NH$_3$ is the primary denominator of ammonia damage.

The influence of pH on the relative distribution of NH$_3$ and NH$_4^+$ on both sides
of a cellular barrier (cell membrane) is illustrated schematically in Fig. 2-47. Excess H\(^+\) attracts NH\(_3\). On both sides of the cellular barrier, NH\(_3\) and NH\(_4^+\) concentrations establish an equilibrium which depends essentially upon the pH on each side. Distortions in the pH gradient between extra- and intracellular fluids cause a redistribution of the two forms of ammonia. In experiments, changes in pH on both sides of cellular barriers can easily be introduced (Warren, 1962). Since H\(^+\) pene-

Table 2-19

Percentage of NH\(_3\) in solution of ammonium hydroxide as a function of pH and temperature (Prepared by J. W. Elliot after Wurmann and Woker (1949); reproduced by permission of Verlag Birkhauser, Basel)

| Temperature (°C) | pH  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.0</td>
<td>7.5</td>
<td>7.6</td>
<td>7.7</td>
<td>7.8</td>
<td>7.9</td>
<td>8.0</td>
<td>8.1</td>
</tr>
<tr>
<td>10</td>
<td>0.3</td>
<td>1.1</td>
<td>1.4</td>
<td>1.8</td>
<td>2.2</td>
<td>2.6</td>
<td>3.0</td>
<td>3.6</td>
</tr>
<tr>
<td>15</td>
<td>0.4</td>
<td>1.3</td>
<td>1.6</td>
<td>2.1</td>
<td>2.6</td>
<td>3.3</td>
<td>3.9</td>
<td>4.7</td>
</tr>
<tr>
<td>20</td>
<td>0.5</td>
<td>1.5</td>
<td>1.9</td>
<td>2.4</td>
<td>3.0</td>
<td>3.8</td>
<td>4.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>

| Temperature (°C) | pH  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.2</td>
<td>8.3</td>
<td>8.4</td>
<td>8.5</td>
<td>8.8</td>
<td>9.0</td>
<td>9.6</td>
</tr>
<tr>
<td>10</td>
<td>5.7</td>
<td>7.1</td>
<td>8.9</td>
<td>11.1</td>
<td>20.3</td>
<td>29.1</td>
<td>57.6</td>
</tr>
<tr>
<td>15</td>
<td>6.5</td>
<td>8.0</td>
<td>9.9</td>
<td>12.3</td>
<td>22.1</td>
<td>32.3</td>
<td>59.8</td>
</tr>
<tr>
<td>20</td>
<td>7.3</td>
<td>9.1</td>
<td>11.2</td>
<td>13.7</td>
<td>24.2</td>
<td>35.8</td>
<td>62.1</td>
</tr>
</tbody>
</table>

Derivation formula: \(\frac{(\text{NH}_4^+)(\text{OH}^-)}{\text{NH}_3\cdot\text{H}_2\text{O}} = K_b\) where \(K_b = \text{dissociation constant of aqueous ammonia}\) \(K_w = \text{ionization constant of H}_2\text{O}\)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>(K_b)</th>
<th>(K_w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.570 \times 10^{-5}</td>
<td>14.6346</td>
</tr>
<tr>
<td>15</td>
<td>1.652 \times 10^{-5}</td>
<td>14.3463</td>
</tr>
<tr>
<td>20</td>
<td>1.710 \times 10^{-5}</td>
<td>14.1669</td>
</tr>
</tbody>
</table>

The toxicity of ammonia has been studied by numerous biologists, veterinarians and physicians for more than 70 years. Ammonia toxicity is a universal phenomenon which gains in importance as the pollution of air, water and land continues. Unfortunately, progress in the investigation of ammonia poisoning is slow and suffers from insufficient interdisciplinary cooperation. There is an almost complete lack of
cross communication among botanists, ichthyologists, parasitologists, veterinarians, physiologists and physicians (Warren, 1962).

That ammonia pollution of rivers and coastal waters affects fishes more seriously at high than at low pH values has been known for several decades (e.g. Ellis, 1937; Grindley, 1946; Wurmann and co-authors, 1947). Rainbow trout Salmo gairdnerii can survive a 10-fold higher concentration of ammonium chloride at pH 7.0 than at pH 8.0 (Downing and Merkens, 1955). The degree of NH₃ toxicity depends also upon the amount of dissolved oxygen present. At 19.8°C, the median survival periods of S. gairdnerii increase in three concentrations of NH₃ (0.86, 1.38, 1.96 ppm NH₃—N l⁻¹) with increasing concentrations of dissolved oxygen (between 1.5 and 8.5 ppm l⁻¹). In 0.86 ppm NH₃—N, for example, median survival periods extend from about 10 mins at 1.6 ppm O₂ to about 1000 mins at 8.5 ppm O₂ (Fig. 2-48). Comparable responses were obtained by Merkens and Downing (1957) in the freshwater fishes Perca fluviatilis (perch) and Rutilus rutilus (roach), while Gobio gobio (gudgeon) showed no significant changes in survival periods at the two oxygen concentrations tested (53.4% and 96.7% air saturation; Fig. 2-49). Increased carbon dioxide levels tend to augment NH₃ toxicity in S. gairdnerii (Lloyd and Herbert, 1960), and increased, but still sublethal, levels of ambient ammonia may cause diuresis in the same species (Lloyd and Orr, 1969).

Biological consequences of ammonia accumulation are difficult to assess in exact quantitative terms. Differences in size and shape of culture enclosure, culture-water

---

Fig. 2-47: Ammonia exchange across a cellular barrier. (a) Cell membrane permeability is low for NH₄⁺, but high for NH₃; (b) excess H⁺ (side with lower pH) traps the biologically dangerous NH₃. (After Warren and Schenker, 1962; modified; reproduced by permission of American Physiological Society.)
Fig. 2-48: *Salmo gairdneri*. Ammonia toxicity as a function of \( \text{NH}_3-N \) and dissolved oxygen. Open circles: pH 7-0; filled circles: pH 8-0. Experimental temperature: 19-8°C; fresh water. (After Downing and Merkens, 1955; modified. Crown Copyright. Reproduced by permission of the Controller of H.M. Stationery Office.)

![Graph showing ammonia toxicity vs dissolved oxygen](image)

quality, water-treatment efficiency, animal load (p. 166), rates of water replacement or recirculation, oxygen and carbon-dioxide concentrations, temperature, pH, exposure time, method of ammonia determination, etc. complicate comparisons or even make them impossible.

Earlier studies were primarily concerned with lethal ammonia limits (e.g. Phillips and co-authors, 1949; Brockway, 1950; Downing and Merkens, 1955;
Merkens and Downing, 1957; Lloyd and Herbert, 1960). The last two authors studied Salmo gairdnerii and found the lethal NH$_3$ level to be 0.84 mg l$^{-1}$ (normal temperature, oxygen and carbon-dioxide levels; pH 8.2). At 10°C, 18 mg NH$_4^+$ l$^{-1}$ would be necessary to produce a lethal effect. Such high ammonia concentration is unlikely to occur in salmon and trout-rearing ponds, unless the water is re-used without filtration (Burrows, 1964).

Sublethal NH$_3$ effects cause hyperexcitability (Fromm and Gillette, 1968; see also Wurmann and Woker, 1949; McCay and Vars, 1950); reductions in growth rate (e.g. Brockway, 1950; Kawamoto and co-authors, 1967; Kawamoto, 1958, 1961); reduced gas exchange due to gill-surface-area diminution (Burrows, 1964; Fromm and Gillette, 1968); decrease in blood-cell number (Reichenbach-Klinke, 1967); reduced oxygen levels in the blood (Phillips and co-authors, 1949, 1950; Brockway, 1950); reduced physical performance (Thomas and co-authors, 1964); reduced disease resistance (Burrows, 1964); structural modifications in various organs, including the liver parenchyma (Reichenbach-Klinke, 1967).

In the public aquarium of the 'Institut für Meereskunde' in Kiel (FRG), annual average NH$_3$-N values of 0.06 mg l$^{-1}$ have been tolerated by a large variety of North Sea and Baltic Sea fishes without apparent harm (Trekel, personal communication). At temperatures between 9.3°C and 11.8°C, the average salinities ranged from 30.7 to 35.2‰ for the North Sea fishes and from 15.8 to 21.3‰ for the Baltic Sea fishes. According to Kawamoto (1961), the carp Cyprinus carpio still grows at total ammonia concentrations which inhibit growth in Salmo gairdnerii. However, at 0.3 mg NH$_4$Cl l$^{-1}$, carp decreased in average wet weight from 2.80 to 2.77 g over a test period of 91 days (temperature about 20°C; pH close to 7). During the same time, the controls increased in average individual wet weight from 2.74 to 3.13 g. The carp survived in 1.2 mg NH$_4$Cl l$^{-1}$, but Kawamoto assumes that they would die from respiratory failure at still higher concentrations.

Ambient concentrations of up to 10 mg NH$_3$ l$^{-1}$ have been reported by Fromm and Gillette (1968) to exert no significant effect on the ability of Salmo gairdnerii haemoglobin to combine with oxygen under in vitro conditions (erythrocytes suspended in Ringer's solution); however, decrease in ammonia excretion rates, in the presence of increased ambient ammonia concentrations, appear to be indicative of beginning damage to the excretory apparatus. In S. gairdnerii exposed to ambient ammonia levels of about 1 mg l$^{-1}$, blood oxygen content decreased to about 14% of the normal value (Brockway, 1950); at the same time, blood carbon dioxide increased about 15%. S. gairdnerii placed in 1.6 to 4.3 mg NH$_3$ l$^{-1}$ for 24 hrs exhibited increased oxygen consumption (53 to 106% above normal resting level; Fromm, unpublished, in: Fromm and Gillette, 1968). There can hardly be any doubt that critically increased ambient ammonia levels tend to deform gas-exchange equilibria between organism and environment.

Reichenbach-Klinke (1967) studied the toxicity of ammonia in 240 individuals of the freshwater fish species Squale cephalus, Carassius auratus, C. carassius, Gobio gobio, Esoc lucius, Cyprinus carpio, Salmo gairdnerii, Rutulus rutulus and Tinca tinca. In each experiment (1 week; minimum food ration), 8 individuals were used; all experiments were repeated. They were conducted in 50-l glass aquaria at 16°C to 17°C; pH 8.4 to 8.6, and NH$_4^+$ concentrations between 1 and 4 mg NH$_4^+$ l$^{-1}$ (0.1 to 0.4 mg NH$_3$ l$^{-1}$). Ammonia affects blood cells and gills. At first, blood-cell
number decreases (presumably as a function of abnormal concentration of blood cells in gills and skin); it then increases as in an overall inflammation process. Concentrations of 3 mg NH$_4^+$ l$^{-1}$ (0.27 mg NH$_3$ l$^{-1}$) and above cause swelling of cells (gills, liver), clubbing of gill filaments and hyperplasia. At the same time, the number of erythrocytes decreases. These severe modifications of the blood system tend to become irreversible after a short time. Damage to the liver parenchyma and blood components soon attains lethal levels. Reichenbach-Klinke concludes that ammonia toxicity manifests itself through cell damage with hyperplasia and partial degeneration, paralleled by decomposition of blood elements.

Freshly hatched fishes exhibit the lowest degree of tolerance to ammonia. Salmo gairdnerii fry suffer irreversible blood damage at 0.27 mg NH$_3$ l$^{-1}$ (Reichenbach-Klinke, 1964, 1965, 1967). It is to be expected, therefore, that in cultivation projects which include breeding, permissible NH$_3$ concentrations must remain significantly below the levels tolerated by adult individuals. In German rivers, the ammonia concentration is steadily increasing. Reichenbach-Klinke (1967) measured 0.25 mg NH$_3$ l$^{-1}$ in the River Salzach, 0.126 mg l$^{-1}$ in the Isarkanal, 2.35 mg l$^{-1}$ in the Speichersee of the Isarkanal, and 2 to 3 mg l$^{-1}$ in the Goldach. In the Rhine, 0.93 mg l$^{-1}$ ammonia was determined near Emmerich and 50 mg l$^{-1}$ in the Emscher (Wasserwirtschaftsverwaltung, 1959). All these values are critical at alkaline pH values and high temperatures (see also Doudoroff and Katz, 1950).

Burrows (1964) examined sublethal NH$_3$ effects in freshwater cultivation ponds containing fingerling chinook salmon Oncorhynchus ishawytchea at temperatures of 8° and 14° C and a pH of 7-8. Excretion patterns and composition of excretory products were found to vary with population density. At stocking rates of less than 2.3 kg per 3.8 m$^3$ min$^{-1}$ of water inflow, urea is the principal end product; above this level, ammonia becomes dominant. In continuous exposure for 6 weeks, NH$_3$ concentrations as low as 0.006 mg l$^{-1}$ can produce extensive hyperplasia of the gill epithelium. Prolonged, but intermittent exposure to NH$_3$ causes reduced growth rates and reduced physical stamina. Burrows postulates that continuous NH$_3$ exposure is the precursor of bacterial gill disease. In each experiment, Burrows subjected four lots of fingerlings to four levels of introduced ammonium hydroxide (0.0, 0.3, 0.5, 0.7 mg l$^{-1}$). Calculated exposure to NH$_3$ was 0.006 (8° C) and 0.008 (14° C) mg l$^{-1}$ at the 0.3 mg l$^{-1}$ ammonium-hydroxide level; 0.010 and 0.012 mg l$^{-1}$ at the 0.5 mg l$^{-1}$ level; 0.014 and 0.018 mg l$^{-1}$ at the 0.7 level. The highest level approximates the maximum concentration encountered in rearing ponds during afternoon surges. The degree of proliferation and clubbing of gill filaments was progressive during the first 4 weeks; thereafter, no measurable increase was discernible. There was no significant mortality during the experiments, nor during a following 3-week period. The potential to recover from severe gill proliferation is limited. Transfer to normal culture water, and inspection after 3 weeks, revealed no recovery from extensive proliferation in the 6°-C fish; the larger 14°-C fish recovered to some extent (in the first experiment, the proliferated areas had consolidated; in the second, they had not).

Several pollutants (phenols, metal salts, detergents) amplify detrimental ammonia effects, as does oxygen deficiency. Tissues with low concentrations of dissolved oxygen are penetrated more easily by NH$_3$ and hence, are more readily damaged. Compounds with a high affinity for iron tend to affect (or even block) respiratory
functions. \( \text{H}_2\text{S} \), for example, passes cell barriers 100 times faster than \( \text{O}_2 \) and inactivates the iron in haemoglobin, thereby reducing the oxygen saturation capacity of the tissue. As a consequence, \( \text{NH}_3 \) permeates more readily.

Wolf (1954, 1957) assumes that increased levels of ammonia and urea represent a cause of the blue-sac disease (exophthalmos; popeye) in fishes raised in freshwater hatcheries. Cultivation of salmon eggs and fry in hatchery water with added ammonia and urea (beginning of experiment: 2 mg l\(^{-1}\), after 7 days: 12 mg l\(^{-1}\)) at 13° C yielded increased maximum disease incidence (about 85%) over that in controls (15 to 50%).

Recent experiments, mainly on crustaceans and fishes, confirm the possibility that, in freshwater animals, ammonotelism may be related to the maintenance of the acid-base balance and to cation conservation (Maetz, 1972). The evidence available is, however, mainly indirect. Addition of ammonia to the culture medium results in an inhibition of sodium absorption, while injection of ammonia produces increased rates of sodium uptake, in accordance with the \( \text{Na}^+ / \text{NH}_4^+ \) exchange model of Maetz. No stoichiometric relationship between rates of ammonia excretion and of sodium absorption has been observed. In some cases, the rate of ammonia excretion is much higher than that of sodium absorption, while in others the reverse situation occurs. Increase in protein metabolism at inadequate ambient oxygen concentrations has been indicated in the fish Tilapia mossambica (Kutty, 1972).

**Counteraction of NH\(_3\) poisoning in closed culture systems**

The key to counteracting \( \text{NH}_3 \) poisoning in closed systems is biological water treatment (p. 122). Mechanical (p. 112) and physico-chemical (p. 134) water treatment are less efficient, but provide welcome additional means for reducing dangerously high \( \text{NH}_3 \) levels.

The susceptibility to \( \text{NH}_3 \) poisoning is reduced when high turnover rates of the culture water are employed, when dissolved oxygen levels are maintained near saturation and when the animal load is kept below the carrying capacity of the system (p. 166). Accumulation of waste food must be avoided. In situations of sudden critical overload, replacement of part of the culture water, recirculation through additional biological water-treatment units and (increased) foam separation are recommended.

\( \text{pH} \) values are of special importance for counteracting or reducing detrimental \( \text{NH}_3 \) effects. \( \text{pH} \) values in excess of 8.2 are not necessarily desirable in sea-water systems. On the other hand, \( \text{pH} \) values below 7.6 must be avoided. The nitrate and phosphorus components of animal excreta accumulate in the culture water as nitrogen and phosphate, which precipitate with calcium or magnesium (Saeki, 1958). As calcium and magnesium ions are eliminated, the culture water loses excess bases and becomes acidic. Low \( \text{pH} \) suppresses bacterial ammonia oxidation and renders the water unsuitable for marine animals. Saeki recommends addition of calcium oxide or magnesia for counteracting \( \text{pH} \) values that are too low. The maintenance of adequate \( \text{pH} \) values has received special attention on pp. 108 and 109.

**Ammonia determination**

A number of methods for determining ammonia in sea water and fresh water have been described, e.g. Wattenberg (1929), Wirth and Robinson (1933), Krogh...

Within the last few years, electrodes for measuring NH₃ and NH₄⁺ (by adding base), nitrate (by adding strong reducing agents) and organic nitrogen (after Kjeldahl digestion) have become available. The ammonia electrode model 95-10 (Orion Research Inc., Cambridge, USA), for example, is a gas-sensing electrode which measures samples of 1.0 ml, is sensitive down to 20 ppb, operates in sea and brine water, is portable, interference free (except for volatile amines) and requires less than 1 min for one determination. SERNA and co-authors (1973) tested the electrode under sea-water conditions and found it suitable for routine ammonia monitoring in closed systems, as well as for detecting rapid water-quality changes. About 5 mins were needed for each sample analysis. For recording ammonia, nitrite, nitrate, phosphate, Kjeldahl-nitrogen, iron, silicate and carbohydrates, ALBRECHT and OVERBECk (1969) have used an Auto Analyzer. The flow diagram for determining ammonia is illustrated in Fig. 2-50.

**Nitrite and Nitrate**

Near the surface of the open oceans, the usual concentration range of nitrite is about 0.01 to 4 µg at l⁻¹; that of nitrate about 0.01 to 40 µg at l⁻¹. In most cases,
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

Nitrite values range from 0.1 to 2 μg at 1⁻¹; nitrate values, from 0.1 to 20 μg at 1⁻¹. Both nitrite and nitrate tend to attain much higher values in closed sea-water systems.

Studying the nitrogen cycle (Volume II: Schlegel, 1975) in small aquaria (8 to 16 l), Kühl and Mann (1955, 1956a, b) found comparable conditions in sea and fresh water. However, in sea water, ammonification was slower, nitrite and nitrate pro-

![Fig. 2-51: Oxidation of nitrogenous compounds (5 g meat of *Mytilus edulis*) in aerated (solid lines) and non-aerated (broken lines) sea water (32.3% S). Right: water temperature, pH, alkalinity and O₂ saturation. (After Kühl and Mann, 1956; modified; reproduced by permission of Dr. B. V. Junk Publishers.)](image)

duction started later and nitrification ended later. In spite of slow nitrification, the amounts of nitrite and nitrate were higher in sea water. In one of their experiments, Kühl and Mann (1956b) placed meat of *Mytilus edulis* (5 g) in an 8-l glass aquarium (32.3% S) without bottom substrate and without animals or plants. The meat was allowed to decompose in aerated and in non-aerated water; ammonia (NH₄⁺), nitrite and nitrate, water temperature, pH, alkalinity, and oxygen saturation were determined once a day. Some of the results obtained are illustrated in Fig. 2-51. Aquaria containing metabolizing animals instead of dead organic matter yielded
END PRODUCTS OF NITROGEN METABOLISM

comparable values. In the presence of living plants, most of the nitrate was assimilated. In the public aquarium at Kiel (FRG), average annual NO$_2$–N levels of 0.18 mg l$^{-1}$ and NO$_3$–N levels of 2.28 mg l$^{-1}$ have caused no detectable detrimental long-term effects among a large variety of marine fishes and invertebrates (TREKEL, personal communication).

Carlucci and co-authors (1970) studied three sources of biological nitrite production in sea water: (i) decomposition of faecal pellets of the copepod *Calanus helgolandicus*; (ii) decomposition of cells of *Amphidinium carterae* (dinoflagellate), *Cyclotella nana* (diatom) and *Monochrysis lutheri* (chrysomonad); (iii) excretion by

Table 2-20

Decomposition of faecal pellets (*Calanus helgolandicus*) during 6 weeks of incubation in the dark at 20° C; oxidation of nitrogenous substances released. Control values subtracted from reported amounts. All values expressed as $\mu$g at N l$^{-1}$ (After Carlucci and co-authors, 1970; modified; reproduced by permission of Springer-Verlag)

<table>
<thead>
<tr>
<th>Material</th>
<th>Beginning of experiment</th>
<th>End of 2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_3$–N</td>
<td>NO$_2$–N</td>
</tr>
<tr>
<td>Faecal pellets</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Faecal pellets + 132P</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Faecal pellets + <em>Nitrosocystis oceanus</em></td>
<td>0.3</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>End of 4 weeks</th>
<th>End of 6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_3$–N</td>
<td>NO$_2$–N</td>
</tr>
<tr>
<td>Faecal pellets</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Faecal pellets + 132P</td>
<td>1.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Faecal pellets + <em>Nitrososibis oceanus</em></td>
<td>0.7</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Skeletonema costatum*. All tests were made at 20° C in non-aerated water, (i) and (ii) in the dark, (iii) in continuous light (approx. 0.01 langley min$^{-1}$). Analyses for NH$_3$–N, NO$_2$–N and NO$_3$–N were performed using methods described by Strickland and Parsons (1968). Copepod pellets released ammonia and nitrite less readily than the decomposing marine phytoplankton. At the end of 2 weeks, the faecal pellets of *C. helgolandicus* released 0.6 $\mu$g at NH$_3$–N l$^{-1}$ into the culture water; this amount increased to 0.8 $\mu$g at l$^{-1}$ after 4 weeks, and remained at that level for the duration of the experiment (Table 2-20). Nitrite production was low, even after addition of bacteria (pure cultures of ammonia oxidizers '132P' or *Nitrosocystis oceanus*). No nitrate was observed when a nitrite-oxidizer was added. During
the decomposition of algal cells (Table 2-21), the highest amount of ammonia was recorded after 8 weeks of incubation. In the presence of N. oceanus, the rate of nitrite production was 0.2 μg at N 1−1 week−1. The rate constant k was 0.1 day−1. CARLUCCI and STRICKLAND (1968) obtained a k of 0.05 day−1 for various nitrifying bacteria with substrate concentrations of 1.5 to 15 μg at (NH₄)₂SO₄—N 1−1. For physiological mechanisms of bacterial nitrification, consult Volume II: SCHLEGEL (1975).

_Skeletonema costatum_ was found by CARLUCCI and co-authors (1970) to excrete nitrite when high concentrations of nitrate were present. N-starved and non-N-starved _S. costatum_ released nitrite at 150 and 50 μg at NO₃—N 1−1, respectively. As nitrate concentrations decreased to lower levels, the nitrite was assimilated. In contrast to previous findings (VACCARO and RYTHER, 1960), nitrite production did not increase under conditions of reduced irradiance. VACCARO and RYTHER cultivated the flagellate *Isochrysis* sp., an unidentified centric diatom (*Lauderia* sp.? and the diatom _S. costatum_. They point out that the assimilation of nitrate-N by marine phytoplankton is often accompanied by the production of significant amounts of extracellular nitrite. In their cultures, the highest nitrite concentrations were produced at reduced irradiance levels by cells recovering from nitrogen deficiency. Within the range of 0 to 1300 foot-candles, the amount of nitrite-N produced varied inversely with the degree of irradiance.

Attempts to demonstrate non-autotrophic nitrification by seven open-ocean bacteria proved unsuccessful; hence, CARLUCCI and co-authors (1970) consider that heterotrophic nitrification in the sea is probably of little ecological importance (see also Volume II: SCHLEGEL, 1975). Non-biological processes hardly participate in the production of nitrite. Photo-oxidation of ammonia is insignificant in the sea (HAMILTON, 1964) and probably also in culture containers. According to HAMILTON, photoreduction of nitrate to nitrite is more likely, but also of doubtful ecological importance. Under conditions normally found in oceans, rates of bacterial nitrite production are very low (WATSON, 1965; CARLUCCI and STRICKLAND, 1968).

**Table 2-21**

Decomposition of algal cells (*Amphidinium carterae, Cyclotella nana, Monochrysis lutheri*) during 12 weeks of incubation in the dark at 20° C; oxidation of nitrogenous substances released. Control values subtracted from reported amounts. All values expressed as μg at N 1−1 (After CARLUCCI and co-authors, 1970; modified; reproduced by permission of Springer-Verlag)

<table>
<thead>
<tr>
<th>Material</th>
<th>Nitrogenous substance</th>
<th>Beginning of experiment</th>
<th>After number of weeks indicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algae + heterotrophic</td>
<td>NH₃—N</td>
<td>0</td>
<td>3.9 4.7 5.2 4.7 2.7</td>
</tr>
<tr>
<td>Bacteria</td>
<td>NO₃—N</td>
<td>0</td>
<td>0.4 0.3 0.3 0.3 0.5</td>
</tr>
<tr>
<td>Algae + heterotrophic</td>
<td>NH₃—N</td>
<td>0</td>
<td>4.6 6.2 7.0 5.2 3.8</td>
</tr>
<tr>
<td>Bacteria + <em>Nitrosocystis</em></td>
<td>NO₂—N</td>
<td>0</td>
<td>0.4 0.8 1.2 1.6 2.0</td>
</tr>
<tr>
<td>oceanus</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Nitrite and nitrate accumulations in culture systems are less dangerous to marine animals than NH₃ accumulation. According to Kelley (1965), nitrate is less toxic than ammonia by about three orders of magnitude. However, very high nitrate levels may interfere with respiratory processes, especially in invertebrates (e.g. Oliver, 1957; Hirayama, 1966c; Kumatani and Isogi, 1969). At nitrate levels of 75 to 100 mg l⁻¹, hermit crabs showed discomfort; 250 to 350 mg l⁻¹ are lethal to many fishes (Degraaf, 1964).

In closed sea-water systems, nitrite and nitrate poisoning can be avoided or adequately counteracted by biological water treatment (p. 122). A properly designed and correctly operated mechanico-biological filter tends to stabilize these substances within ranges observed in natural sea waters (e.g. Seffge, 1941; Trama, 1954; Atz, 1964a). In closed sea-water systems, nitrite levels should not exceed 0.1 mg l⁻¹, and nitrate levels should not be in excess of 20.0 mg l⁻¹ (King and Spotte, 1974). Accumulation of nitrate causes undesirable pH decrease by replacement of carbonate and bicarbonate ions, as well as nitric acid formation (Hongo, 1934). By adding lactate or tartrate to the water, the subsequent development of denitrifying bacteria brought about fast removal of nitrites and nitrates in the culture water.

Methods for determining nitrite and nitrate in sea and fresh water have been discussed and evaluated by Barnes (1959) and Strickland and Parsons (1968).

**Other Inorganic Substances**

Decomposition of uneaten food and of dead organisms tends to modify the normal ratios of all dissolved substances. Except for phosphates, reports on dangerous accumulations of inorganic substances—other than ammonia, nitrite and nitrate—have not come to the reviewer's attention. Phosphate accumulation is much less dangerous to cultivated marine animals than that of ammonia.

According to Goldizen (1970), phosphate levels are not known to be toxic in semi-open systems in the presence of a calcareous filter bed. The phosphate precipitates as the calcium salt. This process, however, can occlude buffering surfaces and hence, cause a reduction in pH. Avoidance of such undesirable side effects necessitates, at intervals, stirring of the filter bed. Excess phosphate also results in insoluble precipitations with calcium and magnesium, leading to a reduction in these ions and, ultimately, in alkalinity (Saei, 1963).

**Organic Substances**

Even very low concentrations of dissolved organic substances may have important biological consequences. More knowledge on origin, ecological significance and breakdown of organic substances dissolved in sea water is a fundamental prerequisite for progress in cultivation and in the analysis of marine ecological dynamics (Volume IV). In ocean waters, most organic substances (Volume I, Chapter 10, p. 1527) occur in concentrations in the order of 10⁻⁷ to 10⁻¹⁰ M. Few analytical techniques are sensitive enough for exact qualitative and quantitative analysis of such small amounts. In the open oceans, dissolved organic substances attain concentrations between 10⁻⁶ and 10⁻⁸ volume density (Volume I: Kalle, 1972b). In the Ara-
bian Sea, the amounts of dissolved C vary from 1.00 mg l$^{-1}$ at 1 m depth to 0.50 mg l$^{-1}$ at 250 m (MENZEL, 1964). In unpolluted marine environments, AZAM and HOLM-HANSEN (1973) recorded organic compounds in concentrations in the order of $10^{-4}$ to $10^{-9}$ M. Vitamin concentrations appear to range several orders of magnitude lower ($10^{-10}$ to $10^{-13}$ volume density). Kalle (1972b) assumes that the ratio: amount of organic substances in sediments to those dissolved in sea water to those particulate suspended in sea water to those in living organisms is about 1000: 100: 10: 1.

While certain organic substances promote growth or initiate reproduction, accumulation beyond a critical level has been reported to inhibit growth (e.g. BRCOWAY, 1950; KAWAMOTO, 1961; ATZ, 1964b; KUWATANI and ISOGAI, 1969). In closed sea-water systems, organic substances accumulate and tend to attain concentrations many times higher than in natural sea water. After months or years, the accumulation of organic substances may cause the culture water to acquire a yellowish tint. The substances responsible for such discoloration are collectively referred to as 'Gelbstoffe' (Kalle, 1966; Volume IV: WANGERSKY, in press).

In cultivation studies, organic nitrogen compounds have thus far received more attention than other organic substances. Formation and excretion of organic nitrogen compounds in the animal kingdom have been treated in a number of reviews (e.g. CAMPBELL, 1970a, b; CAMPBELL and GOLDSTEIN, 1972; Volume II: PANDIAN, 1975). These compounds are considered less life endangering than inorganic nitrogen-containing substances, especially NH$_3$. Over a period of 3 weeks, urea levels of 0.2, 0.3 and 0.4 ppm did not result in visible damage to the gill epithelium of fingerling chinook salmon Oncorhynchus tshawytscha maintained in a rearing pond (BURROWS, 1964). As 0.4 ppm exceeds the maximum urea level usually encountered in rearing ponds, and as the test period was beyond that at which NH$_3$ produces visible evidence of gill damage (p. 91), BURROWS concluded that ammonia, not urea, acted as a limiting toxicant.

MITAMURA and SAIJO (1976) suggest that the photosynthetic activities of phytoplankters constitute one of the major routes of urea decomposition at sea. Urea is assumed to play a significant role in the nitrogen nutrition of coastal phytoplankton (see also EPPLEY and co-authors, 1971; SAIJO and MITAMURA, 1971; MCCARTHY and EPPLEY, 1972; MCCARTHY, 1972; ANTIA and co-authors, unpublished). While algal water-treatment units (p. 131) may help to reduce urea levels and those of other organic nitrogen compounds in cultivation systems, the effectiveness of this process for research cultivation and its feasibility for commercial cultivation (mariculture farms) remain to be investigated.

(c) Conclusions

In contrast to life-supporting substances, considerable information is available on substances which endanger life in culture systems—contaminants (Chapter 7) and metabolites. Among the metabolites, the role of ectocrines in cultivation remains to be investigated; the potential danger of end products of nitrogen metabolism has been stressed and evaluated in numerous papers. Ammonia ranks as the most dangerous excretory product. Most aquatic animals release ammonia, often at high rates. The primary denominator of ammonia damage is NH$_3$. 

NH₃ accumulation readily causes detrimental effects to basic functions and structures of the animals cultured: impairment of gas exchange and inhibition of fundamental metabolic processes soon cause reductions in (i) growth rate, (ii) physical performance, and (iii) tolerance to environmental stress (including tolerance to pollutants) and diseases. Prolonged exposure to critically high NH₃ levels results in structural damage, e.g. in epithelia and internal tissues. At critical NH₃ concentrations, detrimental effects are augmented by high ambient pH values, high temperatures, low levels of oxygen and the presence of water pollutants. Damage increases with exposure time, and rapidly becomes irreversible. The biological consequences of NH₃ accumulation do not seem to be species specific (as is the case with some organic metabolites).

In culture systems, the NH₃—N concentration should be 0.001 mg l⁻¹ or less; even 0.001 mg l⁻¹ may, conceivably, interfere with normal life processes in stenoplasic marine forms. Spotte (1970) recommends that the total measurable ammonia level should be less than 0.1 mg l⁻¹. In commercial culture plants, higher NH₃ levels are acceptable than in ecological experiments. For salmonid fishes, Liao and Mayo (1972) have proposed the criteria for ammonia levels listed in Table 2-22. Further research on NH₃ levels acceptable or desirable for different species to be maintained, reared or bred in culture systems is urgently required.

### Table 2-22
Criteria for ammonia levels (mg l⁻¹) in salmonid culture systems (After Liao and Mayo, 1972; reproduced by permission of Elsevier Scientific Publishing Company)

<table>
<thead>
<tr>
<th>Level</th>
<th>NH₃</th>
<th>NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum</td>
<td>0</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Desirable</td>
<td>&lt;0.006</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Acceptable for short periods (e.g. 14 days)</td>
<td>&lt;0.025</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>Beginning of lethal range</td>
<td>0.08</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The most important measure for counteracting ammonia poisoning of the culture water is biological water treatment (p. 122). Additional measures include: (i) adequate aeration; (ii) avoidance of excessive animal loads and waste-food accumulation; (iii) mechanical (p. 112) and physico-chemical (p. 134) water treatment; (iv) high turnover rates of the culture water; (v) maintenance of pH values between 7.8 and 8.2.

Nitrite and nitrate accumulations in culture systems are less dangerous to marine animals than NH₃ accumulation. Nitrite levels should remain below 0.1 mg l⁻¹, nitrate levels below 10.0 mg l⁻¹. Evidence presented by Liao and Mayo (1972), Smith (1972) and Williams (1972) supports the view that, in the presence of other metabolic end products, nitrite levels in excess of 0.2 mg l⁻¹ lead to anoxia and heavy mortalities in fishes. Liao and Mayo assume that NO₂⁻, together with other
metabolites, causes methaemoglobinemia. In the presence of nitrite, conversion of fish haemoglobin to methaemoglobin tends to retard the oxygen transfer mechanism.

Among other inorganic substances, phosphates may attain critical levels, but phosphate accumulation in culture systems is much less dangerous than that of NH₃. The potential effects of organic substances require investigation.

(7) Culture-water Treatment

(a) General Aspects

Principal Types of Water Treatment

In closed sea-water systems, certain substances tend to accumulate and to deform the normal organism-environment and organism-organism relationships. Hence, closed-system operation requires water treatment, i.e. counteraction of culture-water pollution due to organismic activities and to the food substances added. Related problems of chemical contamination of the culture water from materials used in cultivation receive attention in Chapter 7.

The term ‘water treatment’ comprises planned changes conferred upon a managed body of water. In cultivation, such changes aim at improvements of water characteristics for adequate sustenance of the organisms cultured. In addition to treating the culture water, all equipment used must be carefully cleaned, and should not release critical amounts of potentially life-endangering substances into the culture water. Fixation substances, such as formalin or alcohol, detergents and other water pollutants, gasoline, and tobacco smoke must be banned from culture rooms.

Three types of water treatment can be distinguished: (i) Pretreatment of water before use in a culture system. This may include, for example, precleaning, prefiltration, sedimentation, disinfection or sterilization, addition or removal of certain substances, aeration (including degassing), and adjustments in regard to temperature, salinity, gaseous contents, etc. (ii) Reconditioning treatment of water recirculating in a culture system. This comprises a variety of mechanical, biological and physico-chemical methods. (iii) Post-treatment of culture water before its release. This involves processes similar to municipal waste-water treatment. Principal water-treatment steps in open, semi-open and closed culture systems are illustrated in Fig. 2-52.

The present chapter focuses primarily on reconditioning water treatment. Pretreatment is referred to under various headings. Post-treatment is not covered in depth although it is of considerable importance for environmental protection, particularly in areas with large-scale aquaculture farms. Processing of large volumes of waste water is considered along with other problems of water pollution and environmental protection in Volume V.

Reconditioning water treatment is based on water movement. We consider the various methods applied under the headings Mechanical Water Treatment (p. 112), Biological Water Treatment (p. 122) and Physico-chemical Water Treatment (p. 134).
These three groups of water-treatment procedures have several characteristics in common, and clear conceptional distinctions are not possible. However, didactically, the subdivisions chosen are convenient.

Open culture system (flow-through principle)

Semi-open culture system (partial recirculation)

Closed culture system (total recirculation)

![Diagram](image)

Fig. 2-52: Principal steps of water treatment in open, semi-open and closed culture systems. Dashed lines indicate alternative routes. (Original.)

**Disinfection and Sterilization**

While certain micro-organisms are essential for culture-water treatment (p. 100), others are dangerous to the cultured plants or animals (diseases: Chapters 8 and 9). In axenic cultures (Chapter 5.11), bacterial cells must be banned entirely. Inhibition of micro-organisms involves reduction in activities and numbers (suppression); elimination involves destruction or removal. Inhibition or elimination are achieved by measures collectively referred to as disinfection or sterilization (see also Chapters 3.1, 3.2, 4.1, 5.11).

The term disinfection implies partial sterilization, i.e. suppression of contaminating micro-organisms to levels acceptable to the cultivator. A well-known disinfection procedure, pasteurization (after Louis Pasteur, French bacteriologist, 1822–1895), involves careful treatment of heat-labile materials at 60° to 85° C. Depending on the degree of heat lability, the material is usually pasteurized at 60° to 65°C for 30 mins or at 85° C for 1 to 2 mins.
CULTIVATION OF MARINE ORGANISMS (O. KINNE)

The term sterilization denotes the complete reproductive inactivation of all micro-organisms present either by destruction or removal. A fractional sterilization process, tyndallization (named after its inventor), consists of steam heating at 80° to 100° C for 30 mins on 3 successive days, with intermediate storage of the treated material at normal temperatures. During tyndallization, vegetative cells and some spores are killed at the first heating; more resistant spores, which germinate subsequently, are destroyed during the second or third heating. Tyndallization is impractical for sterilizing sea water or other culture media which can be treated in the autoclave (see below). It is useful, however, in the preparation of media not sufficiently resistant to high-temperature treatment (e.g. serum media).

This section briefly considers disinfection and sterilization of sea water and other culture media, of air, as well as of glassware and other equipment.

Sea water and other culture media

Sea water and other culture media can be disinfected or sterilized by microfiltration, irradiation, heat (e.g. autoclave) or chemicals (e.g. ozone, chlorine, antibiotics). In cultivation, microfiltration, ozonation, chlorination and axenization with antibiotics are predominantly employed for sterilization. Ultra-violet irradiation results in disinfection rather than in sterilization. Inhibition, destruction, or removal of micro-organisms in liquids has received attention from McCulloch (1945), Frobisher (1957), Clifton (1958), Millipore Filter Corp. (1964), Pelczar and Reid (1963), Hamilton and Carlucci (1966), Mulvany (1969), Sykes (1969), Perry (1970), Hamilton (1973), Daubner and Peter (1974).

Microfiltration is the most elegant method for sterilizing heat-labile liquids such as sea water. Its usefulness in cultivation is, however, limited to the treatment of small fluid volumes. While a large variety of microfilters are available, only membrane filters are both truly reliable and economic. Absolute retention and high porosity make membrane filters the first-choice equipment for sterilizing small quantities of culture medium. They are widely used in microbiology for removing or collecting bacteria, especially where bacterial population densities are relatively low (Mulvany, 1969; Gunkel and Rheinheimer, 1972; Chapter 3). For sterile filtration, pore sizes near 0.22 μm are used, as all known bacteria are larger than 0.22 μm* and hence, will be physically retained on the filter surface. Although mechanical sieving is essential for micro-organism retention, electrostatic and adsorptive processes are also involved in membrane filtration. Providing the membrane filter assembly has been sterilized properly (autoclaving at 121° C for 10 to 45 mins, depending on the size of the filter holder), no bacterial growth has ever been reported after proper Millipore-membrane filtration (Mulvany, 1969). Virus retention requires filter-pore sizes significantly below 0.22 μm.

There are two types of membrane filters: Millipore (Fig. 2-53) and Nucleopore. Millipore filters employ porous cellulose-ester discs of different mean pore-size ranges. Nucleopore filters are produced by exposing a thin polycarbonate film to charged particles in a nuclear reactor, followed by an etching process. During filtration*

* However, the existence of smaller bacteria in sea water cannot yet be ruled out with certainty. Theoretically, very small bacteria could pass the filter and escape subsequent detection by current microbiological methods. This possibility requires critical examination.
tion, no differences were observed in the heat stability or flow rate for either type of membrane filter. However, a certain advantage of the Nucleopore filter lies in the lesser variation in pore size.

While filter sterilization enables heat-labile medium components to be preserved, filtration may yield a poorer culture medium (for example, for algae) than the same formulation autoclaved (Droop, 1969). There are, according to Droop, two possible reasons for this, both concerning the iron supply. (i) In mildly alkaline alga media, most of the iron is colloidal, even when chelated. Such iron is liable to be removed by filtration, as are phosphorus and humic colloids. (ii) In the autoclave, the reducing conditions ensure that at least some of the iron is returned to the ferrous state. Droop found that fastidious species often do better in pasteurized media. This applies particularly to Pringsheim's biphasic soil-water media, some of whose physical properties are destroyed by autoclaving (Pringsheim, 1946), as well as to media containing full-strength sea water, which may precipitate heavily.

Irradiation, with ultra-violet or electromagnetic γ and X-rays and particulate cathode rays can suppress or kill micro-organisms. Ultra-violet irradiation can be conveniently applied to running sea water while it passes through a disinfecting unit (p. 161). The responses of marine organisms to ionizing radiation (γ-rays, X-rays) have been reviewed in Volume I: Chipman (1972).

Application of heat, especially if combined with elevated pressures, is a universal principle for destroying micro-organisms. In the most commonly used apparatus, the autoclave (Fig. 2-54), pressurized-stream sterilization is carried out in such a way that the whole material (liquids or solids) is in contact with the saturated steam. The autoclaving procedure depends on the liquid volume treated. Normally, autoclaving lasts 30 mins at 115° C and a steam pressure of 0.7 atm, or 20 mins at 120° C and 1 atm.
Application of chemicals for disinfecting or sterilizing sea water and other culture media is largely restricted to ozone, chlorine and antibiotics. Ozonation is used for treating considerable quantities of culture water. It has been employed in open, semi-open, and closed sea-water systems (for details consult p. 154). Chlorination (see also p. 107) of large sea-water volumes is applied in zoos and oceanaria for protecting captive marine mammals such as pinnipeds or odontocetes from infectious diseases. The chlorine concentrations used for such purposes are usually similar to those employed in swimming pools. Antibiotics, e.g. penicillin, streptomycin, chloramphenicol, neomycin or chlorotetacyclins, are applied for rendering a culture axenic, and for protecting early stages—more rarely, also adults—of marine organisms which are particularly susceptible to microbial infection. Axenizing cultures of algae (Chapter 4.1) or invertebrates (Chapter 5.11) involves transferring the cultured organisms—at 1 to 3 day intervals—through several culture media containing suitable types and concentrations of antibiotics.

The significance of antibiotics for inhibition of micro-organisms is referred to repeatedly in this Volume, especially in Chapters 3.1, 4.1, 5.1 and 5.11. In most cases, antibiotics repress rather than destroy microbial populations.

Several organisms that reportedly require antibiotic treatment for survival under laboratory conditions (e.g. copepods, larvae of decapods) have later been cultured successfully in the absence of antibiotics as well. The importance of antibiotics
for cultivating marine organisms may have been somewhat overestimated; it requires critical re-assessment. Physiologically, the effects of antibiotics on the organisms cultivated are poorly understood. The possibility that antibiotics modify, or even damage, the treated organism cannot yet be ruled out with certainty.

**Air**

In cultures requiring aeration, micro-organisms carried into the culture water by the streaming air may act as contaminants. Where such contamination must be avoided, sterilization of air becomes necessary. The flow of air entering the culture vessel can be disinfected or sterilized either by heat or by filtration. In general, the simplicity, efficiency and reliability of modern air filtration makes this process superior to heat sterilization.

One design for heat sterilization has been worked out by Elsworth and co-authors (1961). Fig. 2-55 illustrates a stainless steel, 100-l min⁻¹ sterilizing unit. The time-temperature relationship obtained in this unit for reducing the survival of incoming micro-organism spores to 10⁻¹³% is shown in Fig. 2-56. At 350°C, an exposure period of 1 sec is required; at 200°C, about 16 secs. For further details on heat sterilization of air, consult Bruch (1901) and Elsworth (1969).

Filtration of micro-organisms is facilitated by the fact that they rarely inhabit the air in the naked state. They are almost invariably attached to dust particles, fibrous material or mucoid substances. Hence, the size of the actual particle to be filtered tends to be considerably larger than the micro-organism itself (Sykes,
For air sterilization, porous ceramics, sintered glass or metals, porous membranes and beds of fibrous materials are used (Elsworth, 1969). Two basic classes of air filters can be distinguished: membrane filters and fibrous filters. A glass-fibre-paper filter is shown in Fig. 2-57.

For laboratory purposes, Elsworth (1969) recommends fibrous materials, such as non-absorbent cotton wool, vegetable fibre and glass-fibre papers, slag-wool mat and fibre-glass mat. The filter efficiency of cotton wool is low because of its loose fibre diameter (17 μm). Fibre papers require special skills in mounting, in order to prevent leakage around the edges. However, a punctured fibre-paper filter can be highly efficient at low air velocities. When making individual filters from standard laboratory items, the best choices for filter material are slag wool or fibre-glass. The material used should be reproducible in quality and steam-sterilizable.

An inexpensive and reliable bacterial filter can be easily constructed from glass tubing (2.5 x 30 cm), tightly packed with sterilized cotton wool (Droop, 1969). The cotton wool should be kept dry, with a coil of resistance wire wound around the filter (6 to 10 W at 6 V). One such filter can serve many small cultures. Silicone rubber is a most suitable material for tubing (non-toxic and heat sterilizable). Preceding use, the whole filter must be sterilized and the clipped ends of any idle line must be immersed in 70% alcohol. For further information on air sterilization by filtration, the reader is referred to Elsworth’s (1969) review.
Glassware and other equipment

Glassware and much of the other equipment used for cultivating micro-organisms, plants or animals can be conveniently autoclaved. Heat-labile materials are pasteurized or are subjected to irradiation or ozonation. Chlorination, as well as treatment with 70% alcohol (acidified with a mineral acid to pH 2 for maximum efficiency), ethylene oxide or formaldehyde, must be followed by thorough rinsing.

Chloride is used primarily in the form of hypochlorite. A hypochlorite solution containing 1000 ppm of available chlorine will kill, according to SYKES (1969), 99% of a suspension of *Bacillus subtilis* spores at normal temperatures and pH 11.3 within 70 mins.

![Fig. 2-57: Glass fibre-paper filter for sterilization of air. This unit is used for effluent air treatment. 1: catchpot on culture vessel; 2, 3: valves; 4: inlet tube for laboratory air; 5: expansion chamber; 6: filter element; 7: exhaust fan. (After Elsworth, 1969; modified; reproduced by permission of Academic Press.)](image)

Inactivation of viruses attached to the equipment used for cultivation has received little attention. The literature reviewed by PAVONI and co-authors (1972) reveals that chlorine can inactivate up to 99% of the original PFU (plaque forming units) present. A residual chlorine content of 2.7 mg l⁻¹, after a contact time of 1 hr, destroyed T₂ virus with 99% efficiency. Type 9 echovirus was reduced by 99% in 30 mins, and by 99-3% in 6 hrs with 3.6 mg chlorine l⁻¹ (SHUVAL and co-authors, 1967). Comparable data have been obtained with T₂ bacteriophage by BURNS and SPROUL (1966) and others. PAVONI and co-authors conclude that, at present, only chlorination and the activated sludge process (p. 127) permit firm estimates of virus inactivation (90–99% for chlorination, 90% for activated sludge treatment).

Parameters of Water-quality Assessment

Assessment of culture-water quality is based on continuous recording and controlling of (i) environmental and nutritive conditions required by the organisms cultivated, (ii) essential life-supporting (p. 76) and life-endangering (p. 79) substances contained in the culture medium. The complexity and multiplicity of interactions between organism and culture environment makes specific measurements often very difficult.
For several life-supporting and life-endangering substances, suitable recording techniques are not yet available. In practice, convenient-to-measure environmental entities dominate, e.g. temperature, salinity (alkalinity), oxygen, carbon dioxide, turbidity and pH. Measurement methods and ecological significance of most of these and related factors have been reviewed in Volume I; pH values receive further attention below. Of particular importance are the end products of nitrogen metabolism, especially NH₃. The ecological significance of end products of nitrogen metabolism has been documented on pp. 80 to 98. For assessing the transformation dynamics of organic matter in a given culture system, collective parameters have been introduced, such as redox potential, biochemical oxygen demand (BOD), oxygen consumption of filter-bed micro-organisms (OCF), chemical oxygen demand (COD) and total organic carbon (TOC). These parameters assess gross amounts of degradable organic matter in terms of the resulting oxygen consumption. They account neither for specific substances nor for undegradable compounds. The total amount of organic wastes accumulated in a culture system, both in the form of dissolved and particulate substances, has also been estimated as oxidizable matter (OM) or filterable substance (FS).

The importance of pH values for successful cultivation has been emphasized in Chapters 3, 4 and 5. In oceans and coastal waters, pH values usually range from 7.8 to 8.2. Especially in closed sea-water systems, the pH tends to decrease critically in the presence of high animal loads (p. 166), excessive amounts of waste food, or insufficient mechanical, biological, or physico-chemical water treatment (pp. 134–166).

The procedures for maintaining adequate pH values in closed sea-water systems include filtration, aeration, ozonation, foam separation, and replacement of portions of the culture medium (see the sections on mechanical, biological and physico-chemical water treatment). In public sea-water aquaria, three methods of maintaining normal pH values have been used: (i) At the Plymouth Aquarium (England), Ca(OH)₂ is periodically added to the reservoir (BROWN, 1929; ATKINS, 1931; WILSON, 1932, 1960). (ii) At the New York Aquarium, NaHCO₃ is added continuously to the circulating water (BREDER and HOWLEY, 1931). (iii) At the aquarium of Amsterdam, London, Ueno and New York, bivalve shells, marble chips, coral sand, or calcite are kept in constant contact with the culture water (SUNIER, 1951; OLIVER, 1957; SAeki, 1958). BREDER (1934) was among the first investigators who stressed the importance of calcium ion for successful cultivation of marine fishes.

The pH effects on microbial oxidation in municipal waste-water treatment plants have been reviewed by ECKENFELDER and O'CONNOR (1961). In fresh water and low-salinity systems, the optimum pH range for activated sludge (p. 127) is pH 7.0 to 7.5 (KREFER and MEISER, 1951). While biological water treatment remains satisfactory between pH 6.0 and 9.0, its efficiency becomes reduced to about 60% at pH 4.0, and to about 50% at pH 10.0. Rapid pH changes can be detrimental to the microbial populations in the filter bed and may reduce their metabolic rates by as much as 75%.

In sea water out of contact with the air-sea surface, pH control is effected through ion-pairing equilibria, involving all major ions, rather than through reaction series between water and carbon dioxide (WANGERSKY, 1972a, b). Ion-pairing functions as a pH-stat, not as a buffer, and displays considerable resistance to change.
Wangersky concludes that the pH is ultimately maintained through regulation of the major ion composition (perhaps by the heterogenous silicate mechanism proposed by Sillen, 1961). While Pytkowicz (1972) agrees that the formation of ion-pairs in sea water influences the concentrations of carbonate and bicarbonate ions (and hence, the pH and indirectly the buffer capacity), he stresses that sea-water buffering is primarily due to the classic reaction,

\[ 2 \text{HCO}_3^- \text{, total} \rightleftharpoons \text{H}_2\text{CO}_3 + \text{CO}_3^{2-} \text{, total} \]

Whitfield (1974) calculated the contributions of ion-pair formations to the buffer capacity of the carbon dioxide system in sea water. He showed that ion-pair formations make the major contribution to the equilibrium buffer capacity at pH values above 8. The indifference of the pH to large changes in solution composition, however, is attributed by Whitfield not to a pH-statting effect, but to the fact that metal-carbonate ion-pairs form only a small proportion of the total cation concentration under normal conditions. The difference between this view and that of Pytkowicz is considered by Whitfield to be purely one of semantics. The effect of temperature on the pH of sea water has been studied by Gieskes (1969, 1970) and Ben-Yaacov (1970).

The redox potential or reduction-oxygenation potential (also known as electrode potential or reduction potential) is based on the fact that, whenever a substance in a culture system is oxidized (electron loss), another substance must become reduced (electron gain). The relation between reduction and oxidation may be written as (Jacob, 1970):

Reduced form \( \rightleftharpoons \) oxidized form + electron(s)

During cultivation, the redox potential tends to become more negative as a function of time. This tendency must be compensated for by adding oxidants, e.g. air, oxygen or ozone. A review on redox-potential measurements, especially in biological wastewater treatment, has been presented by Dirasian (1968).

The redox potential or reduction-oxygenation potential (also known as electrode potential or reduction potential) is based on the fact that, whenever a substance in a culture system is oxidized (electron loss), another substance must become reduced (electron gain). The relation between reduction and oxidation may be written as (Jacob, 1970):

Reduced form \( \rightleftharpoons \) oxidized form + electron(s)

During cultivation, the redox potential tends to become more negative as a function of time. This tendency must be compensated for by adding oxidants, e.g. air, oxygen or ozone. A review on redox-potential measurements, especially in biological wastewater treatment, has been presented by Dirasian (1968).

The redox potential or reduction-oxygenation potential (also known as electrode potential or reduction potential) is based on the fact that, whenever a substance in a culture system is oxidized (electron loss), another substance must become reduced (electron gain). The relation between reduction and oxidation may be written as (Jacob, 1970):

Reduced form \( \rightleftharpoons \) oxidized form + electron(s)

During cultivation, the redox potential tends to become more negative as a function of time. This tendency must be compensated for by adding oxidants, e.g. air, oxygen or ozone. A review on redox-potential measurements, especially in biological wastewater treatment, has been presented by Dirasian (1968).

The redox potential or reduction-oxygenation potential (also known as electrode potential or reduction potential) is based on the fact that, whenever a substance in a culture system is oxidized (electron loss), another substance must become reduced (electron gain). The relation between reduction and oxidation may be written as (Jacob, 1970):

Reduced form \( \rightleftharpoons \) oxidized form + electron(s)

During cultivation, the redox potential tends to become more negative as a function of time. This tendency must be compensated for by adding oxidants, e.g. air, oxygen or ozone. A review on redox-potential measurements, especially in biological wastewater treatment, has been presented by Dirasian (1968).

The redox potential or reduction-oxygenation potential (also known as electrode potential or reduction potential) is based on the fact that, whenever a substance in a culture system is oxidized (electron loss), another substance must become reduced (electron gain). The relation between reduction and oxidation may be written as (Jacob, 1970):

Reduced form \( \rightleftharpoons \) oxidized form + electron(s)

During cultivation, the redox potential tends to become more negative as a function of time. This tendency must be compensated for by adding oxidants, e.g. air, oxygen or ozone. A review on redox-potential measurements, especially in biological wastewater treatment, has been presented by Dirasian (1968).
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

Table 2-23

Standard redox potentials ($E_o'$) of redox dyes at pH 7 in a 50% reduced and 50% oxidized state (After JACOB, 1970; reproduced by permission of Academic Press)

<table>
<thead>
<tr>
<th>Dye</th>
<th>$E_o'$ (mV)*</th>
<th>Dye</th>
<th>$E_o'$ (mV)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol-$m$-sulphonate indo-</td>
<td>273</td>
<td>Azure I</td>
<td>11</td>
</tr>
<tr>
<td>2,6-dibromophenol</td>
<td></td>
<td>Methylene blue</td>
<td>11</td>
</tr>
<tr>
<td>$m$-Chlorophenol indo-2,6-</td>
<td>264</td>
<td>Toluidine blue</td>
<td>-11</td>
</tr>
<tr>
<td>dichlorophenol</td>
<td></td>
<td>Janus green (blue-green to red,</td>
<td>-35</td>
</tr>
<tr>
<td>Phenol-$m$-sulphonate</td>
<td>250</td>
<td>irreversible)</td>
<td></td>
</tr>
<tr>
<td>indophenol</td>
<td></td>
<td>Cibra scarlet sulphonate</td>
<td>-36</td>
</tr>
<tr>
<td>$m$-Bromophenol indophenol</td>
<td>248</td>
<td>Indigo tetrasulphonate</td>
<td>-46</td>
</tr>
<tr>
<td>Phenol-$o$-sulphonate indo-2,6-</td>
<td>242</td>
<td>Resorcinol</td>
<td>-81</td>
</tr>
<tr>
<td>dibromophenol</td>
<td></td>
<td>Methyl capri blue</td>
<td>-80</td>
</tr>
<tr>
<td>$o$-Chlorophenol indophenol</td>
<td>233</td>
<td>Ethyl capri blue (nitrate)</td>
<td>-72</td>
</tr>
<tr>
<td>o-Bromophenol indophenol</td>
<td>230</td>
<td>Indigo trisulphonate</td>
<td>-81</td>
</tr>
<tr>
<td>Phenol indophenol</td>
<td>227</td>
<td>Indigo disulphonate</td>
<td>-125</td>
</tr>
<tr>
<td>Bindscheller's green</td>
<td>224</td>
<td>Gallophenine</td>
<td>-142†</td>
</tr>
<tr>
<td>Phenol blue</td>
<td>224</td>
<td>Nile blue</td>
<td>-142</td>
</tr>
<tr>
<td>2,6-Dichlorophenol indo-$o$-</td>
<td>219</td>
<td>Indigo monosulphonate</td>
<td>-160</td>
</tr>
<tr>
<td>chlorophenol</td>
<td></td>
<td>Cresyl violet</td>
<td>-167</td>
</tr>
<tr>
<td>Phenol indo-2,6-dibromophenol</td>
<td>218</td>
<td>Brilliant alizarin blue</td>
<td>-173‡</td>
</tr>
<tr>
<td>2,6-Dichlorophenol indo-$o$-</td>
<td>217</td>
<td>2-Methyl-3-hydroxy-</td>
<td></td>
</tr>
<tr>
<td>chlorophenol</td>
<td>208</td>
<td>1,4-naphthoquinone</td>
<td>-180</td>
</tr>
<tr>
<td>$m$-Cresol indophenol</td>
<td>191</td>
<td>Neutral blue</td>
<td>-192</td>
</tr>
<tr>
<td>o-Cresol indophenol</td>
<td>181</td>
<td>1,5-Anthraquinone sulphate</td>
<td>-200</td>
</tr>
<tr>
<td>2,6-Dichlorophenol indo-o-cresol</td>
<td>174</td>
<td>$\beta$-Anthraquinone sulphate</td>
<td>-250</td>
</tr>
<tr>
<td>Thymol indophenol</td>
<td>159</td>
<td>Phenosafranine</td>
<td>-262</td>
</tr>
<tr>
<td>2,6-Dibromophenol indoguaiacol</td>
<td>125</td>
<td>Tetraethylphenosafranine</td>
<td>-264</td>
</tr>
<tr>
<td>$m$-Toluylene diamine indophenol</td>
<td>123</td>
<td>Janus green (red-colourless)</td>
<td>-258</td>
</tr>
<tr>
<td>1-Naphthol-2-sulphonate indo-</td>
<td>119</td>
<td>Dimethylphenosafranine</td>
<td>-260</td>
</tr>
<tr>
<td>indophenol</td>
<td>115</td>
<td>Tetramethylphenosafranine</td>
<td>-273</td>
</tr>
<tr>
<td>Thionine</td>
<td>62</td>
<td>Resinduline G 2</td>
<td>-281</td>
</tr>
<tr>
<td>Toluylene blue</td>
<td>56</td>
<td>Safranine-T</td>
<td>-289</td>
</tr>
<tr>
<td>Thionine</td>
<td>47</td>
<td>Induline scarlet</td>
<td>-299</td>
</tr>
<tr>
<td>Cresyl blue</td>
<td>21</td>
<td>Neutral red</td>
<td>-325</td>
</tr>
<tr>
<td>Galloacyanine</td>
<td></td>
<td>Neutral violet</td>
<td>-340</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Benzyl viologens</td>
<td>-359</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rosinduline sulphonate</td>
<td>-385</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standard hydrogen electrode</td>
<td>-421</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standard oxygen electrode</td>
<td>810</td>
</tr>
</tbody>
</table>

* $E_o'$ according to CLARK (various publications) and WURMSEY (1940).
† Except for these values, which are at 25°C, all values are at 30°C.

Waste-water treatment. In this context, BOD expresses the amount of oxygen utilized by micro-organisms as they transform degradable carbon and nitrogen compounds. The routine BOD test is standardized for 5 days of incubation. However, the 5-day BOD, or BOD$_5$, usually measures only 70 or 80% of the biochemical oxygen demand of the sample (ANONYMOUS, 1969).

Microbial oxidation of waste-waters proceeds in two steps: oxidation of carbonaceous matter and oxidation of nitrogenous substances. The first step is usually
characterized by a rate directly proportional to the concentration of the organic matter. According to Eckfenlder and O'Connor (1961), the commonly accepted sequence of microbial oxidation comprises: (i) rapid bacterial population growth by assimilation of organic matter; (ii) reduced rate of subsequent oxidation. The first reaction is usually completed after 12 to 60 hrs. Most practitioners use the first-order reaction to determine the rate of BOD, employing the equation:

\[
\frac{dL}{dt} = -K_1 L
\]  

(1)

where \( L \) = concentration of the substance reacting, \( K_1 \) = reaction constant. Equation (1) may be integrated to give:

\[
\log \frac{L_t}{L_0} = K_1 t
\]  

(2)

where \( L_0 \) = initial concentration of organic matter or ultimate oxygen demand; \( L_t \) = concentration of organic matter remaining at the end of time \( t \); \( K_1 \) = reaction coefficient to base \( e \). Equation (2) refers to the organic matter measured in terms of oxygen remaining at the end of a given period. It follows that the organic matter oxidized (or the oxygen used) is equal to:

\[
Y = L_0 - L_t
\]  

(3)

Substituting Equation (3) in Equation (2) and rearranging terms, we obtain:

\[
Y = L_0 (1 - 10^{-K_1 t})
\]  

(4)

where \( Y \) = BOD acted upon in time \( t \).
The influence of temperature on the reaction can be characterized by the coefficient $k_1$ as follows:

$$k_1 = k_{20} \times 1.047^T$$

A common range of $k_1$ values is $0.10$ to $0.30$ day$^{-1}$ for municipal sewage and many industrial wastes (ECKENFELD and O'CONNOR, 1961).

The methods employed for measuring BOD have been reviewed by MONTGOMERY (1967). He concluded that no single rapid respirometric procedure can yet be recommended that would adequately cover all functions of the standard 5-day dilution test. On the other hand, the BOD$_5$ test has two major disadvantages: (i) the BOD-values become available only after 5 days; (ii) the conditions prevailing in this bottle test may be different from those prevailing in the culture or the waste water examined. These shortcomings have prompted several investigators to develop better respirometric techniques. BRIDIE (1969), for example, has devised an electrolytic respirometer that provides automatic and continuous recording. BRIDIE'S respirometer is claimed to be independent of ambient temperature, barometric pressure and the solubility of oxygen in water; it is applicable to a wide range of organic-matter concentrations. Another example of a recent development in automatic, continuous BOD recording is the Sapromat (e.g. HALA'SZ, 1972).

The oxygen consumption of filter-bed micro-organisms (OCF) estimates—similar to COD—the intensity of microbial degradation. OCF, however, is specifically restricted to filter-bed micro-organisms, excluding the activities of microbial components in other parts of the culture system.

The chemical oxygen demand (COD) comprises the oxygen consumption of both bio-degradable and of non-biodegradable (but oxidizable) compounds. Hence, it gives higher oxygen values than the BOD test.

The total organic carbon (TOC) is determined by methods of rapid carbon combustion and measurement of the resulting amount of carbon dioxide by infrared spectroscopy.

(b) Mechanical Water Treatment

Mechanical water treatment aims at removing excessive suspended particulate and colloidal substances from the recycling culture water. The principal methods available are sedimentation, centrifugation and filtration. Gravity, pressure gradients, centrifugal force and electrostatic energy constitute the major forces involved. While sedimentation (settling) is a function of size and specific density of the suspended particles, filtration efficiency depends primarily on filter-pore size. Small-sized or low-density particles resist settling, unless the water is centrifuged.

Sedimentation is accomplished by settlers or by detention, e.g. in open clarifiers or lagoons. The design of sedimentation tanks has been reviewed by CULP and CULP (1971). These authors have considered settling devices for use in waste-water treatment. Their account also provides interesting information for the cultivator of aquatic organisms. A design of a settler employing a multitude of steeply inclined 5-cm square tubes in an all-plastic module is illustrated in Fig. 2-59 (see also HANSEN and CULP, 1967; LIVINGSTON, 1969; MCMICHEL, 1969; YAO, 1970).
recirculation systems, sedimentation tanks should precede the mechanical filter. Accumulations of sedimented particulate and colloidal substances must be removed periodically.

Fig. 2-59: Steeply inclined tube settler. Plastic channels are installed at a 60° inclination between thin PVC sheets. (Courtesy Neptune Microfloc, Inc.)

Centrifugation has, thus far, received little attention, less perhaps than it may deserve. Tests conducted by the reviewer indicate the usefulness of centrifugation for reducing high loads of suspended particles from the culture water without affecting other components. However, continuous centrifugation requires considerable energy and hence, involves higher operational costs than other methods of mechanical water treatment. The tests were performed with 'Zentriklones' (Albert Pfuhl-Apparatebau, Darmstadt and Deutsche Steinzeug- und Kunststoffwarenfabrik, Mannheim-Friedrichsfeld, FRG). Zentriklones are used in the chemical industry, i.e. for separation, classification and concentration of suspended particles, as well as for purification of particle-loaded waste waters. Zentriklone types \( Z = 1620 \quad \text{and} \quad Z = 32 \) proved to be useful for cleaning turbid sea water. Zentriklones consist of porcelain or other sea-water inert material; they are cone-shaped and employ the whirlpool principle. Turbid water is pumped at \( 2.8-3.0 \text{ atm}^* \) into the upper part of the cone and forced into an orbit that narrows progressively as the fluid moves down. Centrifugal force drives the heavier components of the treated fluid outwardly and discharges them through a throttle valve. For maximum cleaning efficiency, several Zentriklones can be operated in series.

Mechanical filtration involves trapping of larger particulates and colloids, and electrostatic adsorption of small-sized suspended matter by the surface structures of the filter material. In mechanical filters, a variety of filter materials are used, for

\* atm = pressure (in atm) in excess of that of the ambient atmosphere.
example, sand, gravel, glass- or Perlon wool, ceramic materials, diatomaceous earth, porous plastics or filter paper. Particles sedimenting on the filter surface reduce the pore size and thus act as additional filter material. Depending upon the size of the pores, septa or interstices of the filter material and the filtration pressure, the particles removed from the recycling culture water may be as small as 20 to 40 μm.

In recent years, aquarium filtration techniques have undergone considerable development and a large variety of filtration devices have become available. This is especially true with regard to the home aquarist (pet-fish) industry which produces each year an impressive number of new filters, filter masses (mostly consisting of glass- or Perlon wool, sand or charcoal), and sophisticated control systems, combining filtration, aeration, ozonation, water propulsion, temperature regulation and pH control. It is impossible to deal with these devices in detail. On a larger scale, mechanical filtration of culture water has recently also been employed in limniculture for increased commercial fish production (e.g. Greene, 1971; Knösche, 1973).

Organic particles and colloids, trapped or adsorbed by the filter material, accumulate near the filter surface, and provide food for a variety of micro-organisms. If operated continuously, the filter surface supports microbial populations which then turn the unit into a combined mechanical and biological filter. In general, filtration capacity increases with the size of the filter surface area. Filtration efficiency is augmented by small pore sizes and, to some extent, by increasing depth of the filter material (see below). Four major types of mechanical water treatment have received detailed attention: sand–gravel filters, rapid sand filters, diatomaceous-earth filters, and disposable cartridge filters.

Sand-Gravel Filters

Types, function and filtrant material

Numerous types of sand–gravel filters have been tested and described. It is neither possible nor desirable to provide here a complete documentation. Details of filter design depend upon the size of the culture system, the animals to be cultivated and the cultivation goal: maintenance, rearing or breeding. Sand–gravel filters usually consist of several layers of increasing grain size. The culture water is forced through these layers by airlifts, gravity or pumps.

Sand–gravel filters may either be installed within the culture tank (inside filter, Figs 2-60, 2-61) or outside the culture tank (outside filter, Fig. 2-62). A large variety of both inside and outside filters have been designed and tested. Many designs can easily be constructed in laboratory workshops, and numerous models are commercially available.

In sea-water systems with total water volumes in excess of 10,000 l, sand–gravel filters require attention from engineers, and usually constitute part of the laboratory building. A typical design, used at the ‘Biologische Anstalt Helgoland’ (FRG), is illustrated in Fig. 2-63. The filter consists of three main layers: crystal sand (ca 25 cm deep), pea gravel (ca 25 cm) and chestnut gravel (ca 35 cm). Two additional, transitory layers of 5-cm and 10-cm depth, respectively, are useful in preventing crystal sand and pea gravel from sinking into the next lower layer. The chestnut gravel rests on a base of stones, facilitating easy water drainage. Since filtration takes
place primarily in the uppermost centimetres of the crystal sand, all deeper layers serve, essentially, the function of supporting the crystal-sand layer, and allowing increasingly free flow to the filtered water. Gravel layers and the base may, therefore, be substituted by other suitable supports.

![Diagram of sand-gravel filters](image)

**Fig. 2-60**: Inside sand-gravel filters for small and medium-sized culture systems. Some frequently used, airlift-operated designs. (Original.)

![Diagram of airlift filters](image)

**Fig. 2-61**: Inside filters. Left: Coarse-sand corner filter with 2 walls of the lift tube cemented to the tank and with perforated bottom plate. Right. Three-layered sand-gravel funnel filter, with extra deep layers for intensive filtration of a relatively small water body. (Based on HAGEMEIR, 1933; FLÜCHTER, 1964, and others.)

The filter pressure increases with the difference between water-level heights in filter and water-exit pipe. The filter resistance depends on pore size and depth of the filter material. Both filter pressure and filter resistance are the main denominators of filtration rate (1 min⁻¹) or speed (cm sec⁻¹). These two factors...
influence the efficiency of the filtration process, that is, the percent removal of undesired culture-water components. Maintenance of a close-to-constant filtration efficiency requires automatic feedback adjustments. In cases of sudden filter-resistance increase, e.g. due to epidemic bivalve spawning and subsequent reduction in pore size by sexual products accumulating on the filter surface, the water level in

![Diagram of an outside sand-gravel filter, typical design.](Image)

Fig. 2-62: Outside sand-gravel filter, typical design. (Original.)

the filter will tend to rise. Should the resulting augmentation of filter pressure be insufficient for restoring a normal filtration rate, lowering of the exit-pipe opening (e.g. via swimmer-operated rotation of a swivel-jointed pipe, or via a control valve in the lower part of the pipe) will adjust filtration dynamics within a wide range.

The capacity of a mechanical filter depends upon the filtrant material used. Primary denominators are grain size, grain shape, secondary reduction of interstices between adjacent sand grains due to sedimentation, size of the filter's surface area, turnover rate of the culture water, proper packing of sand and gravel layers, and depth of the sand and gravel layers.
Grain sizes (average grain diameters) most often used are: 1 to 2 mm for crystal sand, 2 to 5 mm for coarse sand, and 5 to 15 mm for gravel. In closed systems with a total water volume of more than 10,000 l, additional layers may be useful, e.g. 1 to 2 mm, 2 to 5 mm, 5 to 8 mm (pea gravel), 8 to 16 mm, 16 to 30 mm (chestnut gravel). Proper grain-size ranges are obtained from suitable sand or gravel material by employing a series of sieves with different known mesh sizes. For exact grain-size determination, commercially produced grain-size selectors are available. For each filter layer, deviations from the main grain size chosen must be kept to a minimum. Uneven grain-size distribution and uneven packing result in uneven water flow. Filter areas with larger and more continuous pore systems (interstitial spaces) permit more water to pass than others; they allow the circulating water to bypass properly packed filter parts, and to carry suspended particles deep into the filter bed. Uneven water flow reduces both filter capacity (functional filter space) and efficiency (degree of suspended-matter removal).

Grain shape affects the filtration process mechanically and electrostatically. Angular, rough-shaped grains tend to form a more effective trapping system and to keep detritus particles from working too deep into the filter layers. At the same time, they increase the electrostatic surface-adsorption potential and thus enhance filtration.

Secondary reduction of interstices results from small-sized suspended material retained between adjacent sand grains. A silt layer forms on top of the filter surface that increases the capacity for retaining small particles. Consequently, old filter beds yield clearer water than new ones. In well-balanced systems, accumulation of
detritus near the filter surface and microbial degradation attain a quasi-equilibrium. Where sedimentation significantly outweighs microbial oxidation, the accumulated material must be removed (periodic filter cleaning; backwashing, p. 119).

The size of the filter's surface area is of particular importance, since mechanical filtration occurs in the uppermost millimetres or centimetres of the crystal-sand layer. Increasing surface-area size augments the rate of water recirculation. As a general rule, the surface area of the filter bed should be equal to, or larger than, the total area of the culture containers served.

Turnover rate—the amount of culture water recirculated through the filter per unit time—must be sufficient to keep the dissolved oxygen concentration in the filter bed at a level that supports adequate bacterial activities. At the same time, disturbances due to excessive water movement must be avoided. As a rule of thumb, in closed systems with a total water volume of more than 1000 l, and with a normal animal load and temperature, turnover rate should be about 50 to 80 l m$^{-2}$ of filter-bed surface min$^{-1}$; in closed systems which serve for maintenance (display), 10 to 30 l m$^{-2}$ min$^{-1}$ may suffice.

Proper packing of filter layers is necessary, in order to assure maximum use of the total filtration area available. Uneven compactness of sand or gravel, or uneven thickness of the layers may distort the vertical water flow. Reduction in compactness or layer depth leads to areas of increased water passage (channelling) and decreased filtration efficiency.

The depth of the sand and gravel layers depends upon the size of the system, water-level difference (for exerting the necessary filtration pressure) and the cultivation goal. Small systems require less deep layers than larger ones. In general, the sand layer should have a minimum depth of at least 2 cm.

**Maintenance**

The maintenance of sand-gravel filters requires careful attention. If detritus sedimentation significantly exceeds microbial degradation, mud mats form on top of the filter bed. After some time, the mud mats tend to develop a rigid, crusty surface. Heavy mat formation reduces turnover rate, carrying capacity, and the total filter-bed volume. Cracks occur in the filter surface, especially near the walls. In these cracks, water flow is accelerated, bringing about increased local sedimentation. Heavy sedimentation along the cracks indicates the need for cleaning and, in extreme cases, for renewal of the crystal-sand layer (SPORTE, 1970). Periodical stirring of the uppermost 1 or 2 cm of the filter-bed surface helps to prevent excessive detritus accumulation. The detritus thus stirred up may be siphoned off or filtered by auxiliary devices.

SPORTE (1970) recommends cleaning when any of the following conditions appear: (i) formation of a mud mat; (ii) heavy accumulations of detritus in the corners and along the walls of the filter; (iii) reduced flow rate; (iv) reduction of dissolved oxygen concentration in the filter effluent below air saturation. While stirring the filter bed, water circulation should be interrupted. Small filter beds can be stirred by hand, larger ones with plastic rakes. Devices which combine gentle surface raking and siphoning of turbid water can easily be constructed, and the swimming-pool industry offers a large variety of equipment that can be modified to suit cultivation
requirements. Applied at intervals (e.g. weekly), filter-surface cleaning helps to maintain a balanced water quality in closed systems.

Renewal of the uppermost sand layer is usually not necessary more often than once or twice a year (the uppermost 10 to 15 cm are sliced off, discarded, and replaced by washed new sand of appropriate grain size). Such renewal is acceptable only in maintenance cultures. It requires interrupting the circulation for hours and may cause significant changes in biological and chemical parameters that are difficult to assess. Changes are slow during progressive clogging, but abrupt upon sand exchange. Rearing of stenoplastic forms, analyses of metabolic performance or breeding experiments require maximum stability of the system. Hence, heavy mud accumulation must be avoided by employing a proper cleaning schedule, or by subdividing the sand-gravel filter into small units which can be handled and renewed individually. A sand-gravel filter, subdivided into 32 units (plastic buckets with perforated bottom; KESSELER, unpublished) has been installed at the ‘Litoral-station’ of the ‘Biologische Anstalt Helgoland’ (List on Sylt, FRG). Servicing 1 unit day~1 facilitates continuous, sequential cleaning without suddenly affecting overall filter characteristics.

Rapid Sand Filters

Rapid sand filters employ pressure or vacuum techniques. They are powered by mechanical pumps and attain turnover rates several times higher than those in sand-gravel filters. Pressure-operated filters need less space, are cheaper to install, and require less maintenance than vacuum filters. Both have the drawback that their working parts are buried underneath the sand and gravel (SPOTTE, 1970). The degree of water clarity attained is practically the same as in sand-gravel filters. The advantage of rapid sand filters lies in the fast turnover which reduces turbidity more quickly. Their main disadvantage—which weighs rather heavily in cultivation—is insufficient maintenance of microbial populations.

Rapid sand filters usually contain 4 grades of silica plus a top layer of sand (decreasing grain sizes, from cobblestones at the bottom to crystal sand at the surface). In recent years, anthracite coal has been used as surface filtrant with sand underneath. Anthracite helps to achieve deeper penetration of suspended particles, compensating for the relatively small surface area of the filter and thus increasing the efficiency. For culture systems with water volumes in excess of 40,000 l, SPOTTE (1970) recommends the use of sand-vacuum filters, in which the water is removed from underneath the gravel bed, thus creating a partial vacuum (Fig. 2-6). Backwashing is achieved by temporary reversal of the water flow (backwashing). Since detritus accumulates through a considerable depth of the filter bed, surface stirring would be insufficient. The filter is backwashed whenever the accumulating detritus critically reduces the water flow. This is indicated by gauges. Backwashing lifts gravel, sand grains and detritus. Being lighter, the detritus rises above the filter layers and is forced out through the overflow. During reversed water flow, gravel and sand grains are maintained in brief suspension. Later, they settle back into the original graded layers. Frequent backwashing interferes with the establishment of microbial populations (SPOTTÉ, 1970).
Diatomaceous earth Filters

Diatomaceous-earth (DE) filtration employs a thin layer of graded skeletal remains of diatoms as a filter bed (Fig. 2-65). The DE layer is kept in position against a porous sleeve by pressure or vacuum. This technology is expensive, and it requires more maintenance time than other filters. It is practical only in closed systems exceeding 40,000 l calling for a high degree of water clarity (Fig. 2-66). DE filters remove suspended particles as small as 0.1 μm (SPOTTE, 1970).

DE filters consist of a porous central core (usually of rigid polypropylene) and a filter sleeve (preferably a thin, tightly woven polypropylene cloth fitting over the central core). Central core and filter sleeve make up the filter element. The sleeve supports a layer of diatomaceous earth, through which the water enters the central core. The layer of diatomaceous earth protects the sleeve from becoming clogged with colloids, and traps suspended particles. Examples of DE vacuum filters and DE pressure filters have been described and illustrated by SPOTTE (1970), who also offers a trouble-shooting guide for clogged filter sleeves.

Cleaning of DE filters requires interruption of circulation and isolation of filter water from culture water. The old filter cake is backflushed to waste, along with accumulated colloidal matter. DE is added to the filter water by a body-coat feeder (Fig. 2-66), and internal filter recycling is continued until the new layer of diatomaceous earth (precoat) becomes attached to the filter elements (Fig. 2-65).
containing freshly added DE is milky. Precoating is completed when this water turns clear. In general, culture water should be used for precoating. Normal filtration is resumed by diverting the recycling filter water back into the culture containers.

DE vacuum filters are superior to DE pressure filters because the filter elements can be more easily inspected and the working parts are better accessible. Routine checks of pressure filters require shutting down the unit, draining it and opening the pressure tank. The operating cost of a DE filter can be reduced by connecting it in series after a rapid sand filter. Such an arrangement minimizes the load of particulates on the filter elements, and prolongs the cycle run (Spotte, 1970).

![Diagram of Diatomaceous Earth Filter](image.png)

**Fig. 2-65:** Diatomaceous-earth filter. Magnified view of a layer of diatomaceous earth (precoat) forming on the filter-sleeve fibres. (After Spotte, 1970; modified; reproduced by permission of Wiley, New York.)

**Disposable Cartridge Filters**

Cartridge filters have been used in sea-water systems by a number of investigators. They are space saving, easy-to-handle, and efficient in removing suspended matter. However, they cannot support the microbial populations required for biological water treatment (p. 122).

In their inland recirculating sea-water aquarium (p. 45), Lickey and co-authors (1970) employed two pairs of cylindrical cartridge filters connected in tandem (2-stage filtration). The primary filter in each assembly is loaded with large-pore, viscoso-rayon cartridges to remove coarse particulate matter; the secondary filter is loaded with 5-μm-pore cellulose cartridges. Filter housings consist of high-impact acrylic and styrene plastic. The cartridges require replacement once a month.
Self-cleaning filter drums covered with filter tissues of 30 to 200-μm mesh size ('Mikrosiebtrommelfilter') have been used in commercial fish cultivation plants by Kvasnicka (1960) and other investigators. A filter drum with 1 m² active filter surface area was tested in a carp (Cyprinus carpio) plant by Knösche (1969), who obtained the following results with a 'Dederon' tissue Nr. 100 of 200-μm mesh size: water passage more than 33 m³ hr⁻¹; average cleaning efficiency, 81% of 50-μm-tissue-filterable matter; use of rinsing water, 1 to 1.7%.

In 'Filterpressen-Schichtenfilter' (Schenk-Filterbau GmbH, Schwäbisch Gmünd, FRG), the medium to be filtered is pumped through a pressure equalizer into a series of adjacent filter frames with different, decreasing pore sizes. The filter frames must be cleaned regularly. 'Filterpressen-Schichtenfilter' may be used for rapid, fine filtration, e.g. in sea-water intakes, for retaining nanoplankters. Information on long-term performances in sea-water systems is not yet available.

(c) Biological Water Treatment

In oceans and coastal waters, inorganic and organic substances are recycled through integrated, balanced interactions of micro-organisms, plants and animals. In closed systems, marine organisms are bound to die, unless essential portions of such interactions are reconstituted. Their lives depend upon continuous and efficient removal of potentially dangerous substances as well as on the addition of vital components (e.g. nutrients, vitamins, growth-promoting substances). Aspects of cycles and balances of living systems in oceans and coastal waters are treated in Volumes I, IV and V. We restrict ourselves here to problems immediately pertinent to biological filtration in culture systems supporting marine animals.
BIOLOGICAL WATER TREATMENT

Biological water treatment is based on the activities of micro-organisms and autotrophic plants. Micro-organisms decompose and use the organic matter added to the culture water by its inhabitants and by waste food. They also produce life-supporting substances (e.g. vitamins, growth-promoting substances) required to keep other cultured organisms healthy. Plants assist in avoiding dangerous accumulation of metabolic products, and may add vital substances to the system. Filter-bed micro-organisms can decompose organic matter effectively only in the presence of sufficient oxygen. Hence, in the absence of oxygen-producing plants, proper aeration is an important prerequisite for biological water treatment. Micro-organisms and plants involved in biological water treatment require different environmental conditions and hence, are usually separated spatially. Consequently, we have subdivided this section into Microbial water treatment and Algal water treatment.

Microbial Water Treatment

General aspects

Culture systems support a variety of micro-organisms. These thrive on organic materials released by the organisms cultivated and on excess food materials. In filtered closed systems, microbial populations develop especially in filter areas where organic materials become trapped and absorbed, and where large surface areas are available for attachment. The micro-organisms decompose and convert proteins, fats, hydrocarbons, as well as simpler organic and inorganic compounds, and thus purify the culture water; at the same time, they provide nutrients for algal growth. Of particular importance to the cultivator of aquatic animals is the ability of bacteria to decompose end products of nitrogen metabolism—especially NH$_3$ (p. 81). In culture systems, decomposition processes of micro-organisms are collectively referred to also as 'microbial water treatment' or 'microbial filtration'.

The information available on the physiological mechanisms involved in microbial decomposition has been reviewed in Volume II: SCHLEGEL (1975). Trophical aspects of microbial activities in the sea are dealt with in Volume IV. Since NH$_3$ is highly toxic (p. 86), its rapid and efficient oxidation by microbial nitrifiers is essential. There are 5 species of ammonia oxidizers (Nitrosomonas europaea, Nitrosocystis javanensis, Nitrosospira brieriensis and Nitrosolobus multiformis) and 4 species of nitrite oxidizers (Nitrobacter winogradskyi, N. agilis, Nitrooccus mobilis and Nitrospina gracilis). For details on nitrifying bacteria and their mechanisms of chemo-autotrophy, consult Volume II (SCHLEGEL, 1975, pp. 12–14, pp. 22–25; see also Chapter 3 of the present volume).

Microbial water treatment has received much attention from investigators concerned with the removal of organic substances from municipal waste water. Since biological denitrification is one of the few economic methods available for removing low concentrations of nitrogenous compounds from large volumes of water (e.g. JOHNSON and SCHROEPFER, 1964; WUHRMANN, 1964a, b; BARTH and co-authors, 1968), bacterial denitrification has become a focal point of water research studies (e.g. NASON and TAKAHASHI, 1958; NASON, 1962; NICHOLAS, 1963; PASVEER, 1965a, b; DU TOIT and co-authors, 1970; DAVIES and co-authors, 1971; DAVIES and TOERKIN, 1971). For proper microbial water treatment, both in culture systems and sewage plants, it is essential that we obtain more information on the biochemistry of bacterial denitrification.
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

Microbial filtration in mechanical filters

In a properly designed culture system, microbial filtration facilitates fast and efficient decomposition of potentially detrimental nitrogenous compounds. If adequately dimensioned and used continuously, mechanical filters—especially sand-gravel filters—tend to support a microflora abundant and diverse enough to process all major organic pollutants of the system. Permanent support of multispecific micro-organism populations requires balancing of system-components, sufficient oxygen and filter management.

A new cultivation system requires time for the sand-gravel filter to acquire the necessary types and numbers of bacteria responsible for breakdown of waste products. Before introducing the animals, the water must be circulated for several weeks, and appropriate amounts of ammonia, nitrate, nitrite and phosphate continuously added. Microbial decomposition requires certain minimal quantities of nitrogen and phosphorus for bioconversion and energy liberation; other elements required, such as calcium and potassium, are usually present in sufficient quantities.

SAEKI (1968) studied the dynamics of microbial filtration in a closed culture system consisting of filter bed, sediment collector, fish tank, as well as circulation and aeration equipment. The ammonia level of a well-aerated closed system can serve as a convenient criterion for assessing the quality of the culture water. SAEKI presents the following equation:

\[
\frac{dF}{dt} = \frac{dX_1}{dt} + \frac{dX_2}{dt}
\]

where \( F \) = nitrogen compounds secreted by experimental fish, \( X_1 \) = ammonia oxidized by bacteria and \( X_2 \) = nitrogen compounds assimilated. From fish culture experiments, SAEKI found \( \frac{dF}{dt} \) to be 50 mg day\(^{-1}\) 100 g\(^{-1}\) of fish. Bacterial oxidation removes about 25% of the ammonia; a very small fraction evaporates at the water surface. The velocity of ammonia oxidation is proportional to the ratio exchangeable ammonia of the sand to total ammonia. \( \frac{dX_1}{dt} \) is 0.14 mg day\(^{-1}\) 10 g\(^{-1}\) of sand, and \( \frac{dX_2}{dt} \) is 0.07 mg day\(^{-1}\) (grain size: 2 to 5 mm; calcite or weathered granite). 300 g filter sand should be used for 10 g of fish cultured. Long-term cultivation requires control of alkalinity and pH.

Unfortunately, little is known about quantitative aspects of nutritional requirements of marine filter-bed micro-organisms. In municipal waste-water treatment, microbial requirements can be assessed from: (i) differences between the nutrients present in influent and effluent—providing the amount of nutrients present in the effluent represents the minimum level; (ii) the weight of nutrients required per 100 kg of organics removed from the system (HELMERS and co-authors, 1951). Experiments with different organic wastes gave a maximum nitrogen requirement of 5 to 6 kg N for each 100 kg of organic matter removed and a minimum requirement of 3 to 4 kg N 100 kg\(^{-1}\) organic matter removed; the respective values for phosphorus are 1.0 kg P 100 kg\(^{-1}\), and 0.6 kg P 100 kg\(^{-1}\). A ratio organic matter oxidized:N:P of 100:5:1 in waste material will usually ensure adequate microbial nutrition (ECKENFELDER and O’CONNOR, 1961). The important variables to be considered for detailed estimation are BOD (p. 108), availability of nutrients, temperature, solids and time. ECKENFELDER and O’CONNOR have presented a detailed example for calculating nutritional requirements of the micro-organisms in a sludge plant.
with defined waste-processing rates. Nitrogen may be fed to a system as gas (anhydrous ammonia), liquid or salt; phosphorus, as acid or salt. Total denitrification requires addition of an extraneous carbon source (e.g. Finsen and Sampaion, 1959; St. Amant and McCarty, 1969; Balakrishnan and Eckenfelder, 1970; Seidel and Crites, 1970; Du Toit and Davies, 1973).

Table 2-24
Inorganic nitrogenous compounds in the sea water of the Ueno Aquarium (Ueno, Japan) during the first 82 days after its opening on April 12, 1952 (After Saeki, 1963; modified; reproduced by permission of the author)

<table>
<thead>
<tr>
<th>Time after opening (days)</th>
<th>Ammonia-N (ppm)</th>
<th>Nitrite-N (ppm)</th>
<th>Nitrate-N (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.38</td>
<td>1.80</td>
<td>7.9</td>
</tr>
<tr>
<td>45</td>
<td>0.10</td>
<td>1.00</td>
<td>22</td>
</tr>
<tr>
<td>52</td>
<td>—</td>
<td>0.625</td>
<td>26</td>
</tr>
<tr>
<td>68</td>
<td>0.10</td>
<td>0.240</td>
<td>33</td>
</tr>
<tr>
<td>82</td>
<td>0.11</td>
<td>0.083</td>
<td>69</td>
</tr>
</tbody>
</table>

The latent period of a filter bed for developing sufficient purification capacity has been investigated by Saeki (1963) in the closed sea-water system of the public aquarium at Ueno (Japan). Table 2-24 shows the amounts of major waste products found in the culture water within the first 82 days following the opening of the Ueno Aquarium. The high ammonia concentration, 0.38 ppm, 40 days after the opening, decreased within the next 40 days to about 0.1 ppm. In the very first days, nitrite could not be detected; it increased to a maximum of 1.80 ppm after 40 days, and then decreased again to 0.08 ppm on the 82nd day. Nitrate attained 7.9 ppm on the 40th day and continued to rise throughout the test period. These measurements suggest that the full purification capacity of the filter bed was not attained until about 2 months after the opening. Another example of microbial decomposition of nitrogenous waste products determined in a culture system, is illustrated in Fig. 2-67. Details of the sequence shown depend primarily on: (i) animal load (or controlled addition of ammonia, nitrate and nitrite); (ii) feeding level, intensity of defecation and the quantity of food not consumed; (iii) temperature; and (iv) amount of oxygen available.

In large-scale organic waste management, three steps—pretreatment, biological oxidation, and sludge treatment plus disposal—are distinguished (e.g. Eckenfelder and O'Connor, 1961). Pretreatment includes screening, grit removal and sedimentation or flotation. Biological oxidation is accomplished by microorganisms in fixed-bed units (trickling filters; see below) or in fluid-bed systems (activated sludge, p. 127).

Biological filters for municipal waste-water treatment may be either standard or high-rate trickling filters. Standard filters operate at a low hydraulic and organic
loading, and produce a stabilized effluent. High-rate trickling filters have high hydraulic and organic loading. A trickling filter consists of a circular structure containing a suitably deep bed of crushed stone, and receives wastes sprayed by rotary distributors or fixed nozzles. The liquid wastes trickle downward through the stone bed to the underdrains (clay blocks with openings for drainage and ventilation). As organic wastes are sprayed continuously, a gelatinous film of microorganisms develops on the stone surfaces, adsorbing and oxidizing organic components. The use of crushed oyster shells over crushed rock in the construction of trickling filters for salmon propagation has been described by Burrows and Combs (1968). Water purification by means of trickling filters has received further attention from Balakrishnan and Eckenerder (1969b), Middlebrooks and Coogan (1969), Rincke and Neumann (1969), Rincke and Born (1970), Born (1970, 1971) and others. For trout farming, Anderson (1974) constructed a biological trickling filter capable of removing ammonia from fish raceway effluent at a maximum rate of $6.4 \times 10^{-5}$ pounds of NH$_3$—N ft$^2$ of media surface.

The similarities and differences between trickling filters and activated sludge (see next section) have been discussed by Kincannon and Sherrard (1974). Com-
pared on an equitable basis (i.e. high-rate or low-rate process), operational characteristics and effluent quality are similar. Hence, process selection should be based on treatment objectives and economic considerations related to the geographical location of the proposed treatment process.

**Microbial filtration in activated sludge**

Large-scale water treatment tends to produce considerable amounts of precipitated particulate matter—the sludge. Populated with micro-organisms, the sludge becomes 'activated' and may attain high capacities for eliminating unwanted organics, especially nitrogenous compounds. Water treatment with activated sludge involves continuous circulation of fluid containing flocculated biological growth, under conditions which provide maximum contact with organic waste products in the presence of oxygen, i.e. air bubbles (Fig. 2-68). In municipal water treatment, excess sludge is concentrated by gravity thickening, dewatering, flotation, or digestion before removal (landfill; fertilizer) or disposal (incineration).

Research on, and use of activated-sludge plants was originally stimulated largely by the need for effective large-scale nitrogenous waste elimination in sanitary sewage processing. However, activated sludge is also of considerable importance in large-scale cultivation projects, especially in aquaculture. The activated-sludge process could play an important role in avoiding or reducing environmental pollution from large aquaculture farms.

Discussing the current literature on activated-sludge plants, **Drews and Greeff** (1973) conclude that the removal of nitrogenous compounds from liquid wastes by microbiological filtration (nitrification-denitrification) is now regarded as a technique requiring only proper designing and dimensioning. Most tests indicate removal of carbonaceous matter, complete nitrification under optimum aerobic conditions, and denitrification under anaerobic conditions, with controlled supplementation of a carbon source (hydrogen donor) for the denitrifying bacteria. Under these conditions, the three-sludge system (**Barth, 1971**; **Mulbarger, 1971**) gives consistently good results. However, the complex three-sludge system is expensive and requires addition of exogenous H-donors (e.g. methanol; see also **Balakrishnan and Eckenfelder, 1970**; **Davies, 1973**; **Du Toit and Davies, 1973**). Attempts to develop denitrification sludge plants which can be operated with endogenous H-donors (avoiding the need to supply additional carbonaceous matter) have thus far failed. Comparing maximum nitrogen-elimination capacities of 'orbital' (endless channel) activated-sludge plants, **Drews and Greeff** (1973) report that maximum efficiencies require very careful balancing of sludge concentration, organic load and oxygen input, so as to produce a rapid alternation of aerobic versus anaerobic conditions between aeration points in each of the orbital channels. After such treatment, the amounts of ammonia and nitrate in the effluent are minimal. Automated control of oxygen input and of sludge concentration is necessary for constant maximum nitrogen removal. Important studies on bacterial growth kinetics in activated sludge have been conducted by **Jones (1973)**. The activated-sludge process has been shown to be relatively resistant to drugs used to control fish diseases (**Knösch, 1974a**).
Thus far, most contributions devoted to studying the activated-sludge process are restricted to freshwater conditions, e.g. Levine and SoppeLand (1926), Baly (1931), Beedham (1931), Lume (1933), Butterfield (1935), Woolridge and Standfast (1936), Chuo (1937), Bardeley (1938), HaseHine (1938), Ruchhoft and co-authors (1940), Ruchhoft and Kachmar (1941), Keefer and Meisel (1951), Wuhrmann (1957, 1964a, b), Johnson and Schroepfer (1964), Pasveer (1965a, b), Bringmann and Kühn (1967), Barth and co-authors (1968), Sanzin (1968), MechalaS and co-authors (1970), Schuster (1970a, b), Barth (1971), Matsché (1971), Mulbarger (1971), Drews and co-authors (1972) and Pretorius (1973). Essential activated-sludge parameters have been assessed, for example, by Takamatsu and Naito (1967), Huber (1968), Balakrishnan and Eckenfelder (1969a, c), Naito and co-authors (1969), Scherb and Braun (1970) and Knösché (1974b). SherRard and co-authors (1974) developed equations to describe continuous flow dynamics in a completely mixed aerobic activated-sludge process.
They employed the yield of microbial cells as criterion. A comparison between the efficiencies of the activated sludge process and the use of trickling filters has been made by KINCANNON and SHEERRARD (1974).

What can the activated-sludge process do for reducing and balancing the organic load in recirculating sea-water systems? We do not yet know. A detailed assessment of the importance of activated sludge for marine research cultivation and commercial cultivation cannot yet be made with sufficient certainty. As has been pointed out, most activated-sludge studies have been conducted under freshwater conditions, and have involved heavily polluted waters with very high organic loads. We need to know more about the capability of the activated-sludge process to remove organic wastes from culture waters under salinity conditions and organic loads actually prevailing in culture systems of marine organisms. While activated sludge has been used with much success for cultivating freshwater fishes, such as the carp Carassius carassius at very high population densities (e.g. MESKE, 1969a, b), and while its usefulness in marine aquaculture farms seems probable, definite data on the biochemistry and efficiency of the process under marine conditions have still to be presented.

The effect of salinity on the activated-sludge process has been studied by LAWTON and EGGERT (1967), STEWART and co-authors (1962), LUDSACK and NORAN (1965), KINCANNON and GAUDY (1966), and BURNETT (1974). In most cases, however, shock effects rather than long-term effects have been studied. Responses to salinity of fully adapted activated sludge remain to be investigated.

Purification of effluents from aquaculture farms is rapidly becoming a problem of major concern. In limnic, brackish and marine waters of various tropical and subtropical coasts, gigantic commercial-cultivation projects are under consideration or have already been started. The effluents of these large aquaculture farms will pose a serious threat to local shallow-water communities unless adequate purification systems are constructed. Ideally, such purification systems should be designed as a link in a water-recycling chain, re-using organic wastes and inorganic nutrients for producing phytoplankton, part of which is then fed, via zooplankton or directly, to the commercial animals cultured. Presumably, the activated-sludge process can play an important role in avoiding or reducing environmental pollution from large mariculture plants.

**Algal Water Treatment**

Simulation of the effects exerted on living systems in the sea by autotrophic plants has been attempted by employing algal water treatment units or 'algal filters'. Algae remove from (and release into) the ambient water, a variety of organic and inorganic substances which may affect the quality of the culture water. Unfortunately, our knowledge on the functioning and usefulness of algal filters is still very limited. More information on exchange processes between alga and water may add important, new perspectives. It remains questionable, however, whether algal water-treatment units may become principal features of mariculture farms. Their efficiency and speed of nitrogenous-waste removal is lower than that of microbial water-treatment units, and their maintenance is more difficult.

In contrast to micro-organisms, algae are largely absent from mechanical filters.
A major reason for this is the lack of suitable light conditions. In some instances, the surfaces of mechanical filters have been exposed to light, so that photo-autotrophs could establish thriving populations. However, high irradiance tends to reduce not only the number of bacteria, but also their metabolic rates and hence, their capacity for oxidation (Volume I: GUNKEL, 1970, pp. 107-119). At the same time, extensive alga development reduces the capacity of mechanical filters, sometimes up to a point where the filter becomes clogged. Consequently, algal filters must be separated from mechanico-biological water treatment units (e.g. sand-gravel filters with established bacterial populations). The cultivation of marine plants, some of which may be suitable for use in algal filters, has been reviewed in Chapters 4.1 and 4.2. Algal filters must be designed so as to allow adequate light penetration.

Where benthonic algae are used, the culture water is forced to meander along the sections of a tray. These offer large surface areas for settlement (Fig. 2-69). The amount of water processed can be increased by using several filters in parallel (Fig. 2-70). The efficiency of water treatment increases as a function of total filter-tray length. In the presence of large amounts of nutrients, the algae tend to grow luxuriously, especially in the initial tray sections and hence, may tend to retard the water flow. Wider spacing of the first sections and regular harvesting eliminate such shortcomings.
For unicellular plankton algae, trays, bottles or columns with bottom filters are used. Figure 2-71 illustrates the working principle of a unicellular algal filter. The bottom sand-gravel layer (if necessary topped by a silt layer) prevents the unicells from entering the animal compartments.

Whether using benthonic or planktonic forms, algal filters should preferably contain members of defined species, and be exposed to defined conditions of light, temperature and salinity. For optimum filtration, several algal filters—each containing members of a different species—may have to be blended together. Since the filters must be replaced from time to time (counteraction of contamination with foreign species), additional filter sets must be kept ready for use. These are seeded long before they are required.

Algal water-treatment units tend to modify the gaseous content of the culture water, and may have to be operated at temperatures different from those in the animal compartments. The water processed must, therefore, be adjusted in regard to gaseous contents and temperature before returning it to the animal compartments. This can be accomplished by passing the water through a heat exchanger and a splash tower. Typically, the algal filter receives its water supply from the mechanico-biological filter, where the turbidity is reduced, and organic compounds are broken down to algal nutrients.
Experiments on algal filters have been conducted by Kinne (unpublished) some 18 years ago. Crustaceans, such as Gammarus locusta, Neomysis vulgaris, Idotea baltica and Sphaeroma hookeri, as well as the fishes Fundulus heteroclitus, Cyprinodon macularius, Gobius flavescens and Pleuronectes platessa, produced higher percentages of well-pigmented, low-mortality eggs after installation of plant filters. The filters employed a variety of benthonic (e.g. Enteromorpha sp. and Ulva lactuca) and planktonic (e.g. Dunaliella tertiolecta and Monochrysis lutheri) forms.

Enteromorpha sp. filters have also been used by Shelbourne and co-authors (1963) to remove CO₂ and other metabolites from the culture water, to stabilize pH and to add oxygen. Chlorella species have been employed for similar purposes by Sorokin (1971). Species of Oscillatoria and Chlorococccum reduced the nitrate level in a closed sea-water system by 68% (Siddall, 1972).

Many phytoplankters use appreciable amounts of ammonia, nitrite and nitrate for growth, and hence, avail themselves for use in algal filters. Of 26 species examined in axenic culture by Antia, Berland, Bonin and Maestrini (unpublished), all utilized NH₄⁺; 25 (exception: Hemiselmis virens) used also NO₂⁻ and NO₃⁺ (Table 2-25). With the exceptions of Chlamydomonas psila, Cyclotella cryptica and Porphyridium cruentum, the tested species generally utilized urea as efficiently as, or even more so, than NH₄⁺. Antia and co-authors measured algal growth by optical densities at 600 nm in 1- to 3-day intervals over a minimum period of 28 days in a growth medium previously described (Antia and Chorney, 1968), except that the sea water used had a salinity of 38%. All data listed in the table were corrected for growth obtained in the control medium.

Hemens and Stander (1969) investigated the removal of ammonia from anaerobically pretreated raw sewage by means of a rotating algal disc unit. They report that bacteria quickly overgrow the algae attached to the disc, rendering the unit incapable of removing ammonia. Their disc unit consisted of 9 parallel PVC discs on a horizontal shaft, 40 cm in diameter and spaced 6.3 cm apart. To increase the surface area of the discs, and to provide holdfast opportunities for a microbial film, each disc is perforated with 1336 holes, 0.6 cm in diameter and randomly distributed (Pretorius, 1971). The discs were rotated at a constant speed of 6-75 rpm. Pretorius operated a disc unit at 20°C and found that different micro-organisms develop on different discs. The bacterial flora changed from rapidly growing Pseudomonas putida, Cyclorella cryptica and Porphyridium cruentum, the tested species generally utilized urea as efficiently as, or even more so, than NH₄⁺. Antia and co-authors measured algal growth by optical densities at 600 nm in 1- to 3-day intervals over a minimum period of 28 days in a growth medium previously described (Antia and Chorney, 1968), except that the sea water used had a salinity of 38%. All data listed in the table were corrected for growth obtained in the control medium.

In an effort to remove ammonia and nitrate from waste waters, Goldman and co-authors (1974a, b) have developed a mixed plant-animal filter. This unicellular algae-oyster-seaweed system combines inorganic nitrogen removal with the commercial production of oysters. The prototype system consists of swimming-pool-sized containers for unicellular algae, oysters and seaweeds joined in series. The system was fed secondarily treated waste water, diluted 1:4 with sea water, for 11 weeks during the summer. According to Goldman and co-authors, the uni-
Table 2-25

Use of nitrogenous substances as sole N-source for growth of marine phytoplankters at a fixed N-concentration of 500 µg at l⁻¹. ++++ maximum growth; +++ sub-maximum growth; ++ small, but measurable growth; — no growth (After ANTIA, BERLAND, BONIN and MARSTRINI, unpublished)

<table>
<thead>
<tr>
<th>Algae</th>
<th>NH₄⁺</th>
<th>NO₂⁻</th>
<th>NO₃⁻</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chlorophyta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlamydomonas palla</td>
<td>M</td>
<td>++++</td>
<td>++++</td>
<td>—</td>
</tr>
<tr>
<td>Prasinocladius marinus</td>
<td>M</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Tetraselmis maculata</td>
<td>V</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Tetraselmis striata</td>
<td>M</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>V</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Nanochloris oculata</td>
<td>V</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Chrysophyta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emiliania huxleyi</td>
<td>V</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Isochrysis galbana</td>
<td>V</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Monochrysis bithiri</td>
<td>V</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td><strong>Xanthophyta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monellia salina</td>
<td>M</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>Heterocapsa sp.</td>
<td>M</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td><strong>Bacillariophyta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>M</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Nitzschia acicularis</em></td>
<td>M</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Cylindrotheca closterium</td>
<td>V</td>
<td>++++</td>
<td>++++</td>
<td>—</td>
</tr>
<tr>
<td><strong>Cryptophyta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monellia biskanleri</td>
<td>M</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Amphora hyalina</td>
<td>M</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td><strong>Cryptophyta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chroomonas salina</td>
<td>V</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Rhodomonas lens</td>
<td>V</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Hemiselmis virescens</td>
<td>V</td>
<td>+++</td>
<td>—</td>
<td>++++</td>
</tr>
<tr>
<td><strong>Pyroptera</strong> (dinoflagellates)</td>
<td>V</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Amphidinum cartieri</td>
<td>V</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Rhodopina</td>
<td>V</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Porphyridium sp.</td>
<td>M</td>
<td>++++</td>
<td>++++</td>
<td>—</td>
</tr>
<tr>
<td><strong>Cyanophyta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anacystis marina</td>
<td>V</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Agmenellum quadruplicatum</td>
<td>V</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Synechococcus strain 7335</td>
<td>S</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
</tr>
</tbody>
</table>

* M: Culture collection of Station Marine d’Endoume, Marseilles, France; V: Collection of Environment Canada, Fisheries & Marine Services, Vancouver Laboratory; S: Prof. Stanier’s collection at Institut Pasteur, Paris, France.

† Further tests revealed that Porphyridium sp. requires urea concentrations of 5 to 25 mg at N l⁻¹ for growth, i.e., much higher levels than the fixed N-concentration offered. Chlamydomonas palla and Cyclotella cryptica were totally unable to use urea, irrespective of its concentration.

Cellular algae removed 95% of the influent inorganic nitrogen; the oysters, in turn, removed 85% of the algae, but released as soluble ammonia, 16 to 18% of the nitrogen originally bound in the algal cells. All of the nitrogen released by the oysters was
finally removed by the seaweeds, so that the total inorganic nitrogen-removal efficiency was 95%. However, at the same time, only 45 to 60% of the phosphorus was removed. Such reconstitution of essential food-web-links may offer, in the long run, a most useful and efficient way of eliminating, or better, of recycling nitrogenous waste products.

(d) Physico-chemical Water Treatment

The objective of physico-chemical water treatment is the removal of excess dissolved organic substances from the circulating culture water. Physico-chemical water treatment also aids in removing inorganic forms of nitrogen and phosphorus. It counteracts eutrophication and reduces the oxygen consumption of filter-bed micro-organisms. Physico-chemical water treatment resorts to different techniques, but involves processes comparable, and aims similar, to mechanical or biological water treatment.

Physico-chemical water treatment is of particular importance in cases in which biological water treatment is insufficient or impractical. Where accumulation of excreta and food tax the capacity of microbial or algal water treatment, or where such treatment is too expensive, undesirable substances must be removed by physico-chemical methods. In large-scale culture projects (aquaculture) and in municipal waste-water processing, physico-chemical water treatment frequently becomes a major tool of water-quality management.

The methods involved in physico-chemical water treatment comprise activated carbon (charcoal) adsorption, foam separation, aeration, oxygenation, ozonation, ultra-violet irradiation, ion exchange and some other techniques used primarily in municipal waste-water treatment.

Activated Carbon (Charcoal) Adsorption

Activated carbon (charcoal) has been used in the cultivation of aquatic animals for many decades. It is a porous substance with a high adsorptive capacity. Adsorption occurs at all external and internal surfaces available to chemical attraction of organic molecules. One kg of high-grade powdered charcoal has an estimated total surface area of up to 1 million m$^2$; 1 kg of granular charcoal (mostly used in cultivation), of 10,000 to 100,000 m$^2$. Activated carbon is manufactured from cellulose-base materials such as coal, wood, nut shells (notably coconut and pecan) or from animal bones. Comparative studies of the effectiveness of the different types of charcoal have not yet been conducted. New carbon must be thoroughly washed in clean water to remove the dust. Regeneration of the adsorptive properties of used (saturated) carbon is effected by application of heat and steam pressure.

In culture systems of 500 l or less, corner-, inside- or outside-filters packed with granular activated carbon are used. Most outside filters consist of: (i) a top layer of filter wool (glass-wool or Perlon) which retains much of the particulate matter and can easily be replaced; (ii) a thick layer of activated carbon; (iii) a gravel layer; and (iv) a perforated PVC plate facilitating water collection at the bottom (Fig. 2-72). For larger culture systems (e.g. 500 to 5000 l), SPOTTE (1970) recommends the use of a carbon contactor. It can be constructed from a length of PVC pipe: each end of
the contactor should be threaded and fitted with caps for easy maintenance; the effluent end should have a perforated plate (or a plug of glass-wool) to prevent carbon from being sucked into the airlift. The contactor can be placed horizontally underneath the culture tank (Fig. 2-73), and may be shut down (while the water is recirculated only through the mechanico-biological filter), e.g. for carbon recharging.

![Diagram](image)

**Fig. 2-72:** Outside activated-carbon filter for small culture systems. (Original.)

For systems of 5000 to 50,000 l, suitable carbon contactors can be made from 200 l drums with removable lids (SPOTTE, 1970): the insides of the drums are first painted with two coats of a durable epoxy paint. Two holes are then drilled (one at the top, the other at the bottom), threaded PVC flanges sealed against each hole, and PVC flexible hose attachments screwed into the flanges from the outside. A subgravel plate suspends the carbon above the drum bottom. The influent into the contactor
comes from a 'tee' in the biological water-treatment return line. Each drum is three-quarters filled with washed granular carbon. One such drum should be used per 4000 l culture water.

Systems larger than 50,000 l require carbon contactors equipped with mechanical pumps. According to Spotte (1970), most sand pressure filters can be converted to carbon contactors; they are simply filled with granular carbon instead of with sand and gravel. Parkhurst and co-authors (1967) have described and tested the activated-carbon contactor illustrated in Fig. 2.74. It operates at 12.6 l sec\(^{-1}\) or at a hydraulic application rate of 285 l min\(^{-1}\) m\(^{-2}\). For removing suspended matter

![Diagram of Carbon Contactor](image)

Fig. 2.73: Carbon contactor for culture systems of 500 to 5000 l. Solid arrows: filtration through mechanico-biological filter and carbon contactor (valves 1 and 3 open, valve 2 closed); broken arrows: filtration only through mechanico-biological filter (valve 2 open, valves 1 and 3 closed). (After Spotte, 1970; modified; reproduced by permission of Wiley, New York.)

filtered from the secondary effluent, there is provision for 35% expansion of the carbon bed during backwash. A surface wash was added subsequently to the initial design to assist in breaking up surface scum. Secondary effluent enters through an annular distribution ring at the top of the column; the water flows downward through the packed carbon bed, passes through the support screen, and leaves through the effluent discharge; it then proceeds to the top of the next contactor in a series (Fig. 2.75). Backwashing is performed in the reverse direction. The lead contactor of a series is backwashed for 30 to 45 mins once a day. The total system-design pressure is 3.5 kg cm\(^{-2}\), and the head loss after backwashing amounts to between 0.4 and 0.7 kg cm\(^{-2}\). A schematic flow diagram for the pilot plant operated in Pomona (California, USA) is presented in Fig. 2.75. Piping is so arranged that the lead contactor may be any of Contactors 2 to 5, depending on the operation cycle. The filter material used is special-grade granular carbon with an initial iodine
number* approaching 1100 and an apparent density of about 0.47 g ml⁻¹. The particle size is somewhat finer than in the usual commercially available granular activated carbon.

For more than 14 months, Parkinson and co-authors (1967) have applied secondary effluent directly to the pilot plant in Pomona and have obtained the following results: (i) The series of carbon contactors can remove successfully the 10 mg l⁻¹ of suspended solids found in activated-sludge-plant effluents (Table 2-26). (ii) Carbon regeneration can be accomplished very economically. (iii) Removal of dissolved contaminants (expressed as chemical oxygen demand, COD) amounts to about

---

*Iodine number is a relative measure of the total surface area of a unit volume of activated carbon. It decreases steadily with each regeneration step.*
0.5 kg for each kg of carbon. (iv) Micro-organisms establish themselves within the carbon beds, and contribute significantly to the total diminution of organic matter passing through. (v) Addition of organic nutrients to the contactor's feed water increases the natural denitriﬁcation efficiency of the carbon bed. The first contactor may be operated solely for the denitrification of the waste water and for the 10 to

Fig. 2.75: Carbon adsorption pilot plant. Schematic flow diagram. P: pump. (After PARKHURST and co-authors, 1967; modified; reproduced by permission of Water Pollution Control Federation.)

20% removal of dissolved COD that can be accomplished biologically. Regeneration of the total carbon charge of one of the contactors requires 3 days in the furnace at temperatures between 898° and 926° C on the bottom hearths, and between 260° and 315° C on the top hearth range (resulting in volatilization of odorous organic residues). The furnace must be provided, therefore, with an afterburner to prevent air pollution.

The main factors controlling the efficiency of pollutant adsorption to activated carbon are: size of granules, filtration period, filtration speed, temperature, pH, and
the concentration of pollutants in the water to be filtered. Since the surface area available for adsorption increases with decreasing granule size, smaller carbon particles have a higher adsorption capacity than larger ones. However, in culture systems, it is difficult to handle very fine carbon particles and to prevent them from entering the culture enclosure. While the amount of organics adsorbed increases with time, adsorption rate attains maximum values at the beginning of the filtration period (virgin or regenerated carbon); it decreases with carbon 'age' (degree of saturation).

Table 2-26

Removal of contaminants from waste water by an activated carbon plant (Pomona, California, USA). Average water-quality characteristics of main carbon column (June, 1965 to August, 1966). COD: chemical oxygen demand (After PARKHURST and co-authors, 1967; modified; reproduced by permission of Water Pollution Control Federation)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Influent</th>
<th>Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total COD (mg l⁻¹)</td>
<td>47</td>
<td>9.5</td>
</tr>
<tr>
<td>Dissolved COD (mg l⁻¹)</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>Total organic carbon (mg l⁻¹)</td>
<td>13</td>
<td>2.5</td>
</tr>
<tr>
<td>Suspended solids (mg l⁻¹)</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Turbidity (Jackson Turbidity Units)</td>
<td>10.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Nitrate-N (mg l⁻¹)</td>
<td>6.7</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Uptake of pollutants from dilute solution proceeds slowly for compounds with high molecular weight. The rate of adsorption is a linear function of the square root of concentration: a greater fraction of solute is adsorbed per unit time the more dilute the solution (MORRIS and WEBER, 1964). Uptake rate is affected by molecule configuration: highly branched molecules are removed more slowly than those of identical molecular weight with configurations that permit coiling or attainment of compactness. Apparently, adsorption rate is controlled by the rate of diffusion of solute within the micropores of the carbon. The relationship between pollutant concentrations and adsorption efficiencies of activated carbon requires further investigation.

An important parameter for the adsorption of dissolved material is the surface electrical charge at the carbon-water interphase. This may originate from dissociation of chemical groups fixed to the surface of solids or from adsorption of ions from the ambient solution. NEHOF and LOEB (1972) investigated the surface charge of sea water (by microelectrophoresis) from estuarine and coastal sources. Seston, consisting mostly of bacteria, small algae and detritus, exhibited considerable ranges of mobilities; all were negatively charged. A method for rapid determination of the amount of organic substances adsorbed on activated carbon has been described by
HEIL and SOESTHEIMER (1972). The method is based on catalytic H$_2$O$_2$ decomposition by free active centres on the carbon; occupied adsorption sites are catalytically non-effective. The data obtained yield additional information on type of binding between adsorbed substance and adsorbent, as well as on the specific space requirement of adsorbed molecules. Preliminary experiments indicate that slow chemical or microbiological changes at the adsorption sites can also be detected by the H$_2$O$_2$-decomposition method.

Continuous accumulation of organics at the filtrant surface supports dense microbial populations (e.g. WEINBERGER and co-authors, 1966; WALLIS and co-authors, 1974). The micro-organisms participate significantly in the water-treatment process, and turn the activated-charcoal filter into a system combining mechanical, biological and physico-chemical methods.

How often should the carbon filter be changed? Difficulties in determining load and capacity of filtration and in predicting the time course of carbon saturation force the cultivator to resort to monitoring effluent characteristics. SPOTTE (1970, p. 48) writes:

‘The only sure way of knowing when a carbon bed is saturated is to monitor the organics in the contactor effluent. When they start increasing, it is time to change the carbon.’

**Foam Separation**

Foam separation, also known as foam fractionation, air stripping or protein foaming, is an efficient and practical method for removing excess dissolved organic material from culture systems. At the water-air-bubble interphase, surface-active dissolved organic molecules re-orient and form a thin ‘film’ or ‘skin’. When the air bubbles burst, the collapsed skins are left behind. They accumulate in the foam collector from where they can be easily removed from the culture system. Foam separation secures proper gas exchange and assists in stabilizing the pH of the culture water by removing weakly acidic substances from solution.

A basic physical process, the adsorption of dissolved, surface-active organic substances to the water-air interphase has been investigated by numerous authors. GARRETT (1967), for example, has shown that alcohols and fatty acids of high molecular weight compete for adsorption sites with more soluble substances; they force these out of surface positions and thus, in a stream of air bubbles, cause foaming. BAYLOR and co-authors (1962) and SUTCLIFFE and co-authors (1963) focused attention on the significance of this process in biological oceanography (see also HARVEY, 1941; BADER and co-authors, 1960; BAYLOR and SUTCLIFFE, 1963; RILEY, 1963; RILEY and co-authors, 1964; WALLACE and WILSON, 1969; PARKER and BARSON, 1970; NEIHOF and LOEB, 1972). Under in situ conditions, the collapsed skins slowly sink through the water column and provide seeds for the adsorption and aggregation of additional organics. Newly formed skins constitute a source of energy for bacteria, protozoans and multicellular filter feeders, and contribute to detritus formation. Experiments conducted by BATOOSINGH and co-authors (1969) have confirmed that small particles (0.22 to 1.2 µm) serve as nuclei for the formation of larger particles. However, in contrast to BARBER (1966), who was unable to produce particles unless
bacteria were present, BATOOSINGH and co-authors found no evidence that bacteria would be more important as seeds than non-living particles of comparable size.

The efficiency of foam separation depends primarily upon the extent and time course of the contact between water and air. It increases with the size of the contact area (total electrostatic potential between charged organics and bubble surfaces) and with contact time; in addition, it increases as a function of temperature. Maximum rates of particle formation are obtained in continuous-yield experiments, in which the particles are removed from suspension as they are produced. This fact is of importance, both in natural habitats and in culture systems. Foam separators are capable of reducing the dissolved protein concentration of the culture water down to protein levels prevailing in deep oceanic waters. According to ABE (1953), the mean 'life span' of air bubbles in sea-water foam is a function of bubble diameter. Of the sizes tested, a 0.1-mm diameter bubble has the shortest life span (ca 5.2 secs), that of 1.9-mm diameter the longest (ca 8.6 secs).

During foam separation, organic pollutants become concentrated in the foam (e.g. MIYAKE and ABE, 1948), thus facilitating their removal from the culture water. Although only surface-active substances adsorb on the water-air interphase, non-surface-active organic compounds may combine with the accumulating surface-active matter (RUBIN and co-authors, 1963). SHORT and OLSON (1970) employed foam separation for improving culture-water quality in a fish hatchery. By-products of the hatchery process were largely eliminated with the foam; ammonia-N and total nitrogen remained negligible.

In a sewage effluent containing 118 ppm COD, RUBIN and co-authors (1963) found that foam separation removed up to 40% of the initial concentration of organics. KUHN (1956) studied the feasibility of foam separation (in packed waste-water treatment towers) to remove ammonia. At pH 11 (most ammonia present as NH₃), a 2-m, 20-cm diameter column, packed with 1.3-cm Raschig rings and loaded with 1.5 to 1.6 m³ min⁻¹ of air and 0.46 l min⁻¹ of effluent, removed about 92% of the ammonia-N. CULP and SLECHTA (1966) tested a waste-water-treatment pilot plant and found that foam separation removed approximately 98% of the ammonia-N. The application of foam fractionation to sanitary waste-water treatment has been reviewed by JENKINS (1966).

In collapsed foam (foamate) from pure phytoplankton cultures and filtrates of natural sea water, evidence has been obtained which indicates the presence of proteins and/or humic acids, fatty acids, polysaccharides, as well as possible phospholipids and steroids (WALLACE and WILSON, 1969). In artificial sea water, WALLACE and WILSON recovered practically all bovine serum albumin from initial concentrations as low as 5 μg l⁻¹. They consider, in detail, the efficiency of the foam-separation process, and discuss essential dimensions of foam-tower operation (column dimensions, gas flow rate, bubble size, liquid turbulence, stability and drainage of foam).

Most foam separators can be easily constructed. The simplest design consists of a vertical water pipe with an airlift. Two foam separators, developed for home-fish aquaria by SANDER (1967), are illustrated in Figs 2-76 and 2-77. In the design shown in Fig. 2-76, air from the compressor enters the disperser (air stone in small systems; Raschig rings or perforated PVC pipes in large systems) and bubbles upward in the mixing column. Organic particles separate from the culture water in the separator compartment. As the foam builds up, it is forced through a connecting tube into the
foam collector, which is periodically removed and cleaned. In the counter-current foam separator (Fig. 2-77), water drawn by the airlift (left) flows against the main air-bubble direction. This increases the contact time between water and air.

A foam tower used in a culture system for planktonic copepods (Chapter 5.1)

![Diagram of foam separator with separator compartment and foam collector.](image)

Fig. 2.78: Foam separator with separator compartment and foam collector. (After Sander, 1967; modified; reproduced by permission of the author.)

has been described by Zilliox (1969a, b). It consists of a glass tube (123 cm high, 8.8 cm diameter). Air is supplied at 400 l hr⁻¹ through a 40-mm diameter, fritted glass disc, which is located at the base of the tower. Culture effluent enters (at a rate of 250 ml min⁻¹) and leaves the tower at the base through 1-cm diameter glass tubes. Tube height is adjusted, so that the culture water enters about 2/3 the way up the liquid-filled section and drains at the bottom below the level of air input. An external manometer tube maintains the liquid level 12 cm below the top of the column. A counter current facilitates processing of the culture water. Liquid residence time in
the tower is 27 mins (much shorter periods suffice for effective separation). To improve foam separation, Zilloux had the foam removed manually each day, and the wall of the column above the liquid level wiped clean. Increased foam-tower efficiency has been achieved by Zilloux and Lackie (1970), modifying a tower design by Wallace and Wilson (1969). Air enters the tower (120 cm high, 7.7 cm diameter) at its base through a 60-mm-diameter, fritted glass disc at a rate of 400 l hr⁻¹. The tower top terminates in a side arm which is inclined 95° from the vertical.

Fig. 2.77: Foam separator. Countercurrent design. (After Sander, 1987; modified; reproduced by permission of the author.)

Foam collects in the side arm until a plug is formed and forced out by the rising air. The tower is constructed in two sections for easy cleaning and is joined by a bolted flange with a Teflon gasket. The inlet is located on the side (about 20 cm below the connecting flange), the outlet at the bottom just below the air-input level.

Zilloux and Lackie (1970) compared the efficiency of foaming in three assemblies: (i) the original foam tower (Zilloux, 1969a); (ii) the original foam tower with a glass-wool prefilter; (iii) the new tower with a glass-wool prefilter and improved foam removal. The results are presented in Table 2.27. Less protein occurs in the foam tower when a glass-wool prefilter is used; the prefilter limits the amount of cellular particulates entering the tower. The efficiency decreases successively in the three assemblies tested.
Table 2-27

Efficiencies of three different foam-separation assemblies installed in a continuous recirculating culture system for planktonic copepods (After Zmou and Lackie, 1970; modified; reproduced by permission of the Biologische Anstalt Helgoland)

<table>
<thead>
<tr>
<th>Foam separation assembly</th>
<th>Soluble protein (mg l(^{-1}))</th>
<th>Dissolved organic carbon (mg l(^{-1}))</th>
<th>Particles (5 to 100-(\mu)m diameter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before tower</td>
<td>After tower</td>
<td>Collapsed foam</td>
</tr>
<tr>
<td>Original foam tower without filter</td>
<td>18.7</td>
<td>26.2</td>
<td>1324</td>
</tr>
<tr>
<td>Original foam tower with glass-wool prefilter</td>
<td>11.2</td>
<td>11.2</td>
<td>498</td>
</tr>
<tr>
<td>New foam tower with glass-wool prefilter</td>
<td>1.7</td>
<td>2.6</td>
<td>782</td>
</tr>
</tbody>
</table>
The term 'aeration' designates effects on water due to interphase exchange with
air. In cultivation, the aim of aeration is the establishment of an equilibrium between
the concentration of gases contained in air and those dissolved in water. As is well
known, pure, natural air contains a mixture of nitrogen and oxygen (nearly 4
volumes to 1), 0.9% argon, ca 0.03% carbon dioxide, much smaller percentages of
helium, krypton, neon and xenon, and varying amounts of water vapour. Inter-
phase exchange with single gases is referred to as nitrogenation, oxygenation, car-
bon-dioxidation, ozonation, chlorination, etc., depending on the gas used. Gas
mixtures employed in aeration are named after the major gases involved.

Natural aeration is based on exchanges between atmosphere and water, facilitated
by wind, waves and water movement. Artificial aeration requires the provision of
maximum intimate contact between air and culture water by technical means. This
section is predominantly concerned with artificial aeration. The air (gases) used for
aeration should be free of contaminants, filtered, saturated with water vapour (to
reduce evaporation) and—if necessary—be sterile (p. 105).

Efficient interphase exchange between air and water requires maximum contact
between these two media. In principle, maximum contact is achieved by: (i) dividing
a volume of air and passing it through water; (ii) dividing a volume of water and
passing it through air; (iii) thoroughly mixing portions of air and water. In all three
cases, rapid movement of the air-water interphase is essential for maximum
gas exchange. We are concerned here primarily with oxygen and carbon dioxide ex-
changes.

Bubbling air through water requires the application of increased air pressures. A
large variety of compressors and air dispersers ('diffusers') are available. Several
types of air dispersers are listed under Aerators. Water can be passed through air at
normal atmospheric pressures. The equipment used for such surface aeration in-
cludes drippers, sprinklers, sprayers, splashers, cascaders and wave makers (p.
228). Thorough mixing of water and air is accomplished in aspirators, air-shear units
and related equipment.

Gas requirements

The gas requirements of aquatic organisms vary considerably. For a given species,
they depend on developmental stage, physiological condition, environmental his-
tory and concomitantly effective environmental factors. The importance of dis-
solved gases for marine organisms has been documented in Volume I (Kalle, 1972a;
Rheinheimer, 1972; Vernberg, 1972; Vidaver, 1972). For the cultivator, oxygen,
carbon dioxide and nitrogen are of primary importance. The solubility of these
gases in sea water is a function of temperature, salinity and pressure (Volume I:
Kalle, 1972a). Gas solubility increases with decreasing temperature and salinity
(Fig. 2-78). At any given temperature, sea water contains roughly 20% less oxygen
than fresh water. Solubility and saturation of oxygen, carbon dioxide and nitrogen
in waters of different temperatures and salinities have received attention in a number
of papers; the most important ones are Fox (1907, 1909), Carpenter (1966), Green
and Carrit (1967), Murray and co-authors (1969), Murray and Riley (1969);
see also the compilations by Barnes (1959) and Strickland and Parsons (1968). Theoretical aspects of aquatic gas exchange have been discussed by Truesdale and Jameson (1956) and Rahn (1966).

Fig. 2.78: Nomogram for oxygen solubility in sea water at equilibrium with water-saturated air, at 1 atm total pressure and oxygen 0.2094 mole fraction excluding water vapour. (Based on data of Green, 1966; after Green and Carritt, 1967; reproduced by permission of Sears Foundation for Marine Research.)

Adequate exchange equilibria between air and water are essential for the successful cultivation of aerobic marine micro-organisms, plants and animals. While normal exchange processes at the water-surface level may suffice in some cases (e.g. in culture enclosures with a large surface-to-volume ratio, at low temperatures, or for organisms with low oxygen demands), in most culture systems, the maintenance of adequate gas exchange equilibria requires artificial aeration. The degree of aeration can be considered ecologically adequate only if it maintains the gaseous contents of the culture water at normal saturation levels, both in terms of component quantity and proportionality. The concentration levels of gases dissolved in the culture water should be determined regularly and, if necessary, re-adjusted at suitable intervals.

Advantages of aeration

Artificial aeration substantially increases the surface area of the culture medium and provides water movement. Both phenomena are essential for efficient inter-
phase exchange and for improving the water quality. In cultivation, aeration compensates for deviations in the concentration of vital gases. The main sources of such deviations are oxygen consumption by animals and micro-organisms (particularly those of the filter bed, p. 124), carbon-dioxide release by heterotrophs, and carbon-dioxide consumption by autotrophs. Oxygen release by autotrophs (sometimes incorrectly referred to as aeration) can temporarily lead to oversaturation of oxygen—a condition which may be dangerous to animals, unless sufficient aeration restores normal equilibrium conditions.

Aeration counterbalances equilibrium deformations due not only to organismic activities, but also to technical failure and to temperature gradients, and it aids in removing foreign volatile substances.

Defective pumps, for example, tend to draw air and force it into the water under elevated pressures. The resulting air supersaturation can be detrimental to the organisms cultivated. Animals exposed to air-supersaturated water may develop the gas-bubble disease. After entering the animal’s body, supersaturated gas transforms into minute air bubbles which may block blood vessels and harm vital organs. In fishes, such bubbles often appear under the epidermis, especially on the fins. They result in exophthalmia, loss of equilibrium and, finally, death. Gas-bubble disease and its causes were first described in detail by Marsh and Gorham (1905). A more recent account has been presented by Harvey and Smith (1961). Nihrleit (in: Atz, 1964a) suggested that denitrifying bacteria may be a source of the nitrogen that can cause the gas-bubble disease. Temperature changes can significantly alter the gas contents of the culture water. Lower temperatures in the header tank than in the culture tank tend to cause air supersaturations comparable to those due to defective pumps. In both cases, aeration is the best and most efficient means for restoring normal gas levels.

Disadvantages of aeration

Where air is led directly into the culture enclosure, it may damage delicate forms—due to mechanical stress, physical injury (pushing against solid objects) or direct air contact (adherence to water-air surfaces caused by surface-tension phenomena). Such damage can be avoided, or significantly reduced, by dividing the culture system into a special aeration compartment and a culture compartment.

Pretreatment of air

Natural air tends to contain contaminants such as air pollutants, dust and micro-organisms. It may have to be pretreated before use in aeration. Air pretreatment includes charcoal-glasswool filtration, washing, water-vapour saturation and disinfection or sterilization (p. 101).

In animal cultures, it is often advantageous to reduce the carbon dioxide content of the incoming air (Fig. 2-79). Aeration with carbon-dioxide-poor air facilitates fast removal of respiratory CO₂ and augments pH stability.

Air with significantly reduced CO₂ content can easily be prepared by bubbling it through water containing NaOH (30 to 50 g l⁻¹); since 1 m³ of air consumes ca 1.1 g NaOH, the latter must be periodically replaced. The NaOH concentration should not drop below about 25 g l⁻¹.
Dynamics and theory of aeration

Most of the studies on dynamics and theory of aeration refer to municipal wastewater treatment. Aeration in microbial cultures has been reviewed by Brown (1970; see also Chapter 3). In algal cultures, primary requirements for aeration are to provide carbon dioxide and to produce water movement; pertinent information is scarce (Chapter 4). Droop (1969) has formulated the equilibrium relations between partial pressure of carbon dioxide in the air stream, bicarbonate (carbonate) concentration and the pH of the medium. The relations are approximately:

\[
\log [\text{HCO}_3^-] - \log P_{\text{CO}_2} = \text{pH} - 7.6 \tag{1}
\]
\[
\log [\text{CO}_3^-] - \log P_{\text{CO}_2} = 2\text{pH} - 16.6 \tag{2}
\]

The log \(P_{\text{CO}_2}\) of normal air, containing approximately 0.03% carbon dioxide, is \(-3.5\). Equations (1) and (2) are useful for setting up carbon dioxide generators and for stabilizing the pH of algal media bubbled with a mixture of air and carbon dioxide. According to Droop (1969), enrichments with more than 0.5% carbon dioxide are impractical with chemical generators, and cylinder gas is often more convenient. The problem of \(\text{CO}_2\) and pH control in marine culture systems containing algae has been discussed by Spencer (1966).

Fig. 2-79: Pretreatment of air: reduction in \(\text{CO}_2\) content (1), glasswool-charcoal-filtration (2), and water-vapour saturation (3). (After Kinne, 1960; modified; reproduced by permission of University of Chicago Press.)

In animal cultures, aeration has received attention, primarily with respect to respiratory requirements and the different types of aerators used (p. 183). Some essentials on the dynamics and theory of aeration are considered below.

Langlier (1932), Baylis (1935) and Haney (1954) have reviewed the early literature on theoretical aspects of aeration. Haney discusses fundamentals, such as equilibrium, solubility of gases, diffusion and interphase dynamics; his considerations on gas transfer rate have remained relevant, as have his aeration equations. Experiments conducted on air bubbles moving in liquids (Haberman and Morton,
PHYSICO-CHEMICAL WATER TREATMENT 149

1956) demonstrate that a complete description of air-bubble motion is not possible. However, for any liquid, bubble shapes and air-liquid exchange processes are a function of bubble volume (e.g. Miyagi, 1925; Oyama and Iwase, 1939; Shabalin and co-authors, 1939; Redding, 1942; Davies and Taylor, 1950; Peebles and Garber, 1953; Ladyzhenskii, 1954; Siemes, 1954; Downing and Trueal, 1953, 1956; Glibrecht and Harder, 1955). Glibrecht and Harder have measured relationships between air-bubble size, water-column height, water volume, and oxygen and carbon dioxide levels. According to these authors, bubbles of 1 mm diameter and smaller provide maximum gas exchange between water and air. For best results, the air bubbles should travel the longest possible distance through the water column.

For oxygen transfer from dispersed ('diffused') air to the ambient water, the important variables are: (i) type of air disperser; (ii) air-bubble diameter; (iii) depth of disperser submergence; (iv) air-flow rate; (v) water velocity; (vi) air-bubble velocity; and (vii) culture-tank dimensions. Bewtra and Nicholas (1964) present these and other variables mathematically and discuss their relative importance in waste-water treatment. Papers on oxygen transfer from air bubbles into the surrounding water have been presented by Ishii (1932), Eckfenelder (1959), Ippen and Carver (1954), King (1955), Carver (1956) and Eckfenelder and O'Connor (1961).

Eckfenelder and O'Connor (1961) have related velocity and shape of air bubbles in water to a modified Reynolds number, Re (Haberman and Morton, 1966). At Re less than 300, bubbles tend to be spherical and rise in rectilinear or helical patterns. Over a Re range of 300 to 4000, bubbles assume an ellipsoidal shape, and rise with a rectilinear, rocking motion. At Re greater than 4000, bubbles form spherical caps. Rising velocity of bubbles increases at high air-flow rates due to the proximity of other bubbles and resulting disturbances of bubble wakes. Eckfenelder and O'Connor found the size of air bubbles released by air dispersers to be related to both orifice diameter and air-flow rate; at low rates, bubble volume is proportional to orifice diameter and surface tension, and inversely proportional to water density. The size of the bubbles depends on the buoyant force, which separates the bubble from the orifice, and the shearing force necessary to break the surface tension across the orifice.

For evaluating the performance of commercial aeration equipment, Eckfenelder and O'Connor (1961) have considered the aeration process in terms of the overall mass transfer coefficient $K_{L}a$. This coefficient is a function of interphase area, water volume and other physico-chemical variables of the system. The number of bubbles generated per minute is:

$$\frac{\text{gas flow at orifice}}{\text{volume per bubble at orifice}} = \frac{G_{s}}{(\pi/6)d_{B}^{3}}$$

where $G_{s} = \text{air flow}$, $d_{B} = \text{mean bubble diameter}$. Total air-bubble surface area per minute amounts to:

$$A = \frac{G_{s}}{(\pi/6)d_{B}^{3}} \cdot \pi d_{B}^{2} = \frac{6G_{s}}{d_{B}}$$
Contact time between water and air bubble is:

\[
\frac{\text{tank depth}}{\text{bubble velocity}} = \frac{H}{v_B}
\]  

(5)

Therefore, total air-bubble surface area at any time is:

\[
\frac{6G_s H}{d_B v_B V}
\]

and the ratio of interphase area to water volume is:

\[
\frac{A}{V} = \frac{6G_s H}{d_B v_B V}
\]  

(6)

Since oxygen also is transferred from the turbulent water surface, Equation (6) becomes:

\[
\frac{6G_s H}{d_B v_B V} + F' \left( \frac{1}{H} \right)
\]  

(7)

For any aeration depth, oxygen transfer can be correlated according to the dimensionless Sherwood, Reynolds and Schmidt numbers (ECKENFELDER, 1959):

\[
\frac{K_L d_B}{D_L} = F' \left( \frac{d_B v_B}{v} \right) \left( \frac{v}{D_L} \right)^{1/2}
\]  

(8)

where \(v_B\) = bubble velocity; \(v\) = kinematic viscosity. For aeration depths greater than 1 m, Equation (8) can be generalized for all depths by applying an exponential depth correlation:

\[
\frac{K_L d_B}{D_L} H^{1/3} = F' \left( \frac{d_B v_B}{v} \right) \left( \frac{v}{D_L} \right)^{1/2}
\]  

(9)

Combining Equations (6) and (9), and neglecting the oxygen transfer from the surface, ECKENFELDER and O'CONNOR (1961) have shown \(K_L a\) to be:

\[
K_L a = \frac{F'' H^{2/3} G_s}{V d_B}
\]  

(10)

Equation (10) is applicable to aeration depths in excess of 1 m. Over the range of airflow rates normally encountered in waste-treatment aeration practice, the mean diameter of the bubbles produced is an exponential function of the gas rate:

\[
d_B \sim G_s^n
\]  

(11)

where \(d_B\) = mean bubble diameter; \(G_s\) = airflow; \(n\) = exponent. Equation (11) may be combined with Equation (10) for any operating temperature:

\[
K_L a \cdot V = F'' G_s^{(1-n)} H^{(1-\phi)}
\]  

(12)

Equation (12) can be used to characterize the performance of air-disperser devices.
Maximum possible efficiencies of oxygen transfer from dispersed air have been considered by Pasveer (1966). He stresses the importance of rapid renewal of the interphase water–air, in addition to producing large interphase areas. Efficiency improvements could be achieved in two ways: (i) increase of contact time between air bubble and water by means of ‘horizontal flow’ systems; (ii) decrease in air-bubble size. Because of the practical difficulties involved in producing very small air bubbles, Pasveer favours the first possibility. He discusses parameters of oxygen solution and of oxygen supply. We present here some of his considerations.

If air is brought into contact with oxygen-free water, the monomolecular interphase film becomes saturated with oxygen within a one ten-millionth part of a second. In stagnant water, the oxygen penetrates further into the water by molecular diffusion. This process is very slow. The amount of oxygen diffused in stagnant water is directly proportional to the square root of the time of existence of the interphase layer:

\[
Q = 2A(c_s - c_L) \sqrt{\frac{k \cdot t}{\pi}}
\]

where \(Q\) = quantity of oxygen which diffuses through the interphase layer \(A\) in time \(t\); \(c_L\) = oxygen concentration in the water at the beginning of the diffusion; \(c_s\) = saturation concentration of oxygen in water; \(k\) = constant of diffusivity.

The speed of oxygen diffusion in stagnant water decreases with time (Fig. 2-80).

![Graph showing rate of oxygen diffusion in stagnant oxygen-free water](image-url)
It is inversely proportional to the square root of the time of existence of the interphase layer.

\[
\frac{dQ}{dt} = \frac{A}{\sqrt{t}} \frac{c_L - c_s}{kn} \sqrt{1/\eta}
\]  

(14)

Hence, quick renewal of the interphase layer is essential for maximum efficiency of oxygen transfer.

In air bubbles rising through the culture water, the size of the air-water interphase area (as a function of total air volume) increases with decreasing bubble dimensions (Table 2-28). Air bubbles smaller than 1.2 mm ascend more slowly to the surface (Fig. 2-81); hence, contact time increases and gas exchange is enhanced. The amount of oxygen transferred (per unit air volume) from air bubbles to the ambient water increases with ascent height (water-column depth) and decreasing bubble

![Fig. 2-81: Velocity of bubble ascent as a function of bubble size. (After Pásveer, 1966; reproduced by permission of Pergamon Press Ltd.)](image)

<table>
<thead>
<tr>
<th>Interphase air-water ml⁻¹ air (cm²)</th>
<th>Velocity of ascent cm sec⁻¹</th>
<th>Long axis cm</th>
<th>Bubble number ml⁻¹ air</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>5.8</td>
<td>0.04</td>
<td>30,000</td>
</tr>
<tr>
<td>75</td>
<td>14.6</td>
<td>0.08</td>
<td>4,000</td>
</tr>
<tr>
<td>51.6</td>
<td>23.5</td>
<td>0.12</td>
<td>1,200</td>
</tr>
<tr>
<td>31.8</td>
<td>28.8</td>
<td>0.20</td>
<td>300</td>
</tr>
<tr>
<td>23.4</td>
<td>25.4</td>
<td>0.30</td>
<td>100</td>
</tr>
<tr>
<td>13.8</td>
<td>25</td>
<td>0.60</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2-28

Size of air-water interphase area and velocity of bubble ascent as a function of size and approximate number of air bubbles (After Pásveer, 1966; modified; reproduced by permission of Pergamon Press Ltd)
diameter (Fig. 2-82). When air bubbles were introduced into horizontally flowing water (Fig. 2-83), PASVEER (1966) obtained 2 to 2.5 times the oxygen transfer characteristic of 'vertical' systems (see also PASVEER and SWEERIS, 1965).

The supply of oxygen to a culture system can be considerably increased by bubbling oxygen instead of air through the culture water. Oxygenation is a very helpful rescue measure at times of acute oxygen depletion (e.g. after accidental overnight aeration failure or in other stress situations). Permanent oxygenation is, in general, not advisable. Bottled oxygen is expensive. A more efficient and less costly way of providing additional oxygen is careful ozonation.

**Oxygenation**

The supply of oxygen to a culture system can be considerably increased by bubbling oxygen instead of air through the culture water. Oxygenation is a very helpful rescue measure at times of acute oxygen depletion (e.g. after accidental overnight aeration failure or in other stress situations). Permanent oxygenation is, in general, not advisable. Bottled oxygen is expensive. A more efficient and less costly way of providing additional oxygen is careful ozonation.
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

Chemical preparations (tablets containing urea hydrogen peroxide, sodium perborate, potassium permanganate) have been developed for fast oxygenation of critically polluted culture water, and for providing an oxygen reserve during transportation of aquatic animals. Urea hydrogen peroxide quickly dissolves in water forming urea and hydrogen peroxide; the latter forms H₂O and O₂. KÜHL and MANN (1963) tested a number of preparations (see also PESCHEK, 1960). They found that oxygen production can be very intensive, and recorded dangerous supersaturations (200 to 300%). As long as it remains difficult to administer proper doses, and to control possible side effects, these oxygen providers can be applied only with considerable reservation.

Ozonation

The high oxidative capacities of ozone gas may be both beneficial and dangerous. Beneficial effects are: (i) culture-water disinfection and sterilization (p. 101); i.e. partial or complete inactivation of pathogenic and other unwanted microorganisms and viruses; (ii) reduction in the amount of life-endangering oxidizable substances; (iii) augmentation of the redox potential. Dangerous effects of ozonation are: (i) high toxicity to man, cultured organisms (e.g. cytological and cytogenic damage, including chromosome breakage and inhibition of mitotic activities, especially in early ontogenetic stages) and potential damage to equipment; (ii) possible depletion of life-supporting substances including trace elements.

Proper dosage of ozonation remains a problem. There are two major reasons for this: (i) The high speeds of ozone reaction and decomposition, as well as the presence of other free radicals (e.g. Cl⁻), interfere with direct ozone determinations. (ii) The safety limit below which detrimental effects can be excluded is still a matter of dispute. It remains to be investigated whether certain conditions (e.g. high organic load or presence of certain organic compounds) favour the formation of stable, toxic ozonides (e.g. HOLLUTA, 1963; HEIDT and LANDI, 1964; LOAN and co-authors, 1965; DIAPER and co-authors, 1968; CORTESI and PIVETT, 1972). The rate of ozone decomposition depends mainly on organic load and pH (SULZER, 1958). Since hydroxyl ions accelerate decomposition (WEISS, 1935; STUMM, 1956), breakdown is usually more rapid in sea water (high pH values) than in fresh water. Strongly alkaline solutions tend to stabilize ozone (HEIDT and LANDI, 1964). The solubility of ozone in water depends on temperature, salinity, pH and turbidity (HOATHER, 1948; STUMM, 1954, 1956; SHECHTER, 1973). Interrupted (temporal) ozone treatment and low dosages are recommended in all cases where potential ozone damage cannot be ruled out with certainty.

Ozonation lowers the level of organic substances dissolved in the culture water, oxidizes some potentially dangerous end-products of nitrogen metabolism, and produces very clear water. Nitrite is readily oxidized to nitrate, and humic acids are largely degraded after a 5-min period of appropriate ozone treatment; however, ozonation does not contribute significantly to the oxidation of ammonia.

Ozone was discovered in 1840 by SCHÖNBEIN. It represents a specific molecular state of oxygen: the ozone molecule (molecular weight: 48.00) consists of 3 oxygen atoms and has a very low dipole moment (0.49-0.58 debye). In the atmosphere, ozone is important as an ultra-violet shield. In contact with biological systems, ozone reacts with the carbon–carbon double bonds of unsaturated organic compounds to
PHYSCO-CHEMICAL WATER TREATMENT

form ozonides (addition of all three oxygen atoms at a double or triple bond). When these bonds break, aldehydes, ketones and acids form. Apparently, ozone reacts more readily with carbon–carbon double bonds than with carbon–nitrogen double bonds. It is not effective in oxidizing saturated compounds (Smith and Cristol, 1966). Examples of functional groups oxidized by ozone are \(-\text{SH}\), \(-\text{NO}_2\), \(-\text{NH}_2\), \(-\text{OH}\) and \(-\text{CHO}\). The organic chemistry of ozone has been dealt with by Bailey and co-authors (1959), Beachell and Nemhos (1959), Briner (1959), Mosher (1959) and others (see Ozone Chemistry, 1959); its biological effects have been investigated, for example, by Giese and Christensen (1954), Goldsmith and Nadel (1969), Klein (1969), Mudd and co-authors (1969), Drutt and Packman (1972) and Blogoslawski and co-authors (1973). The most recent bibliography on the biological effects of ozone and its technical application has been compiled by Rosenthal (1974).

Heavy ozonation kills a variety of organisms, due to protoplasmic oxidation (e.g. Bringmann, 1953, 1954; Dickerman and co-authors, 1954; Thorp, 1954, 1965; Fetner and Ingols, 1959; Ozone Chemistry, 1959); hence, ozonation must be used with care in order to avoid undesirable side effects. 0.1 mg l\(^{-1}\) of ozone kills 60,000 Escherichia coli ml\(^{-1}\) in 5 secs, while 0.1 mg l\(^{-1}\) of active chlorine requires 15,000 secs (4 hrs 10 mins) to yield the same result (Bringmann, 1953). Spores of soil bacteria (120 spores ml\(^{-1}\)) which survive 20 hrs of water-steam treatment and require a lethal dose of 10 mg l\(^{-1}\) chlorine effective for 35 mins, are killed by 0.36 mg l\(^{-1}\) ozone after 14 mins. Ozone also rapidly kills phyto- and zooplankton (lethal level about 0.3 mg l\(^{-1}\) in 1 to 3 mins), insect larvae and Tubifex sp. (0.5 mg l\(^{-1}\) in 10 mins), as well as isopods (0.8 mg l\(^{-1}\) in 10 mins). In waste water, 1.5 ppm of ozone brings about a decrease in CFUs from 70,000 cells ml\(^{-1}\) to practically zero within 5 mins (Dickerman and co-authors, 1954). In a Paris ozone plant, installed to disinfect filtered Marne River water, ozone is added at an average dose of 1.1 mg l\(^{-1}\) (0.6 to 1.6 mg l\(^{-1}\)); according to Guinvarc'h (1959), the treated water never contained living E. coli cells; the number of Clostridium perfringens (CFUs) was reduced by about 50%, but the reduction in organic matter was only slight. Even at the low temperature of 1°C, 0.4 to 0.5 mg ozone l\(^{-1}\) inactivated all E. coli present (Fetner and Ingols, 1959).

A comparison of the bactericidal activity of ozone and chlorine discloses different modes of action. While the surviving fraction of chlorine-treated bacteria decreases in geometric progression as a function of time or concentration, ozone produces an all-or-none response within a contact time of 1 min; there is no detectable effect until a certain critical concentration is attained, and then survival drops practically to zero. This response supports the theory of Bringmann (1954), that ozone acts as a general protoplasmic oxidant (Fetner and Ingols, 1959). In cultures of Mytilus galloprovincialis and Tapes decussatus, suppression of coliform bacteria was more efficient with ozone than with chlorine treatment (Fauvel, 1964); clams exposed to ozonation retained their original flavour, whereas after addition of chlorine and hyposulphite, they tasted sweet (‘adoucir’). Further papers on bactericidal activity of ozone have been published by Miller and co-authors (1959), Torricelli (1959), Franz and Gagnaux (1971) and others.

Maximum efficiency of the ozonation process requires intimate ‘contacting’ at the liquid–gas interphase (e.g. Jacobson, 1973; Nebel and co-authors, 1973a, b).
Several factors modify the oxidizing capacity of ozone, especially temperature, pH, salinity, and contact time. For details consult the compilation by Rosenthal (1974).

The potential effectiveness of ozone for inactivating viruses is largely unexplored (Carazzone and Vanini, 1969; Pavoni and co-authors, 1972; Majumdar and co-authors, 1973). In a series of experiments, Pavoni and co-authors were able to demonstrate virtually 100% inactivation of F2 virus after a contact time of 5 mins at a total ozone dosage of ca 15 ppm and a residual of 0.015 ppm. Coin (1964) found that a residual ozone concentration of 0.3 mg l\(^{-1}\) produced 99.99% or more inactivation in the poliomyelitis virus after 4 mins in distilled water. Coin emphasizes the importance of virus-particle density, type of water, contact time and concentration of free residual ozone. According to Perlman (1969), a residual ozone concentration of 0.7 mg l\(^{-1}\) reduces the poliovirus level to 0.01% after 4 mins. In triple distilled water, an initial ozone concentration of 1.27 mg l\(^{-1}\) and a residual concentration of 0.23 mg l\(^{-1}\) caused 99.99% inactivation of the poliovirus after 2.5 mins (Schaeffer-Noth, 1970). In biologically treated wastewater, the same amount of inactivation was obtained with an initial concentration of 1.38 mg l\(^{-1}\) and a residual of 0.2 mg l\(^{-1}\) after 2.5 mins. Majumdar and co-authors (1973) obtained the results listed in Table 2.29. In all cases, the regeneration potential of virus particles after ozone inactivation remains to be investigated.

Table 2.29
Poliovirus inactivation by ozonation under continuous flow conditions (After Majumdar and co-authors, 1973; reproduced by permission of Water Pollution Control Federation)

<table>
<thead>
<tr>
<th>Type of waste water</th>
<th>Ozone concentration (mg l(^{-1}))</th>
<th>Residence time (mins)</th>
<th>Survival (%)</th>
<th>Average survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary waste water</td>
<td>0.84</td>
<td>8.0</td>
<td>1.800</td>
<td>1.820</td>
</tr>
<tr>
<td></td>
<td>0.84</td>
<td>8.0</td>
<td>1.479</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>1.47</td>
<td>2.0</td>
<td>0.013</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>4.44</td>
<td>1.0</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>4.44</td>
<td>1.0</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>Secondary waste water</td>
<td>0.79</td>
<td>8.0</td>
<td>2.210</td>
<td>2.055</td>
</tr>
<tr>
<td></td>
<td>0.79</td>
<td>8.0</td>
<td>1.900</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>1.77</td>
<td>2.0</td>
<td>0.013</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>1.77</td>
<td>2.0</td>
<td>0.013</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>5.05</td>
<td>1.0</td>
<td>0.006</td>
<td>0.006</td>
</tr>
</tbody>
</table>

The high toxicity of ozone reported by Hubbs (1930) discouraged experimental ecologists and hatchery operators for some time from considering ozone for seawater sterilization and disease control. Later workers, however, have used ozone with considerable success, and some (e.g. Benoit and Matlin, 1966) have suggested re-examining Hubbs' results with the aim of determining whether they were truly due to ozone poisoning. Evaluating the pros and cons of ozonation,
Hückstedt (1969) recommends intermittent operation. He warns against continuous ozonation, not least because ozonation produces additional amounts of nitrate. Hückstedt found that an ozonator producing 200 mg ozone hr\(^{-1}\) yielded 7.3 mg nitrate hr\(^{-1}\); a smaller unit producing 25 mg ozone hr\(^{-1}\) yielded 1.3 to 1.8 mg nitrate hr\(^{-1}\). On the other hand, Bean (1959) was able to remove manganese from solution in waste waters by ozonation; his finding supports the notion that prolonged application of ozone may remove essential trace substances. Water replacement (10% every two weeks) has been recommended by Spotte (1970) to compensate for possible depletion of trace elements in ozonated marine culture systems. For further information regarding the potential depletion of trace elements in ozonated systems, the reader is referred to Rosenthal (1974).

As is the case with chlorine, ozone, if improperly used, can be dangerous to man and equipment. Hence, care must be taken to prevent excess ozone from entering the laboratory (Mittler and co-authors, 1959; Kroke, 1964; Goldizen, 1970). Even at concentrations at which ozone odour is barely perceptible, daily 8-hr exposures may cause detrimental effects to man.

In aquaculture research, ozone gas has been used for disinfection of make-up water (disease control), for oxidation of organic matter, for elimination of fouling organisms, and for inactivation of non-desirable phytoplankton by-products (e.g. Blogoslawski, in: MacLean and co-authors, 1973). At least 0.4 ppm ozone residual was required to disinfect the sea water. Ozone treatment (30 standard cubic feet hr\(^{-1}\) in a 15-l bucket for 3 mins by a 2% ozone-air mixture) at room temperature revealed the following effects on spawned, fertilized, meiotic and cleaving eggs of the commercial American oyster Crassostrea virginica (MacLean and co-authors, 1973): (i) unfertilized eggs showed no cytological changes; (ii) fertilization occurred less readily (decrease in polyspermy, increase in parthenogenesis) than in untreated sea water; (iii) the incidence of abnormal polar bodies increased; (iv) numerous cleaving eggs had abnormal nuclei (signs of metabolic difficulties or of degeneration; the nuclei were pycnotic, pale, diffuse or even fragmented). The results document considerable danger in the application of ozone, especially with regard to early ontogenetic stages. Decomposition of ozone in water produces the same positive radicals (OH and HO\(_2\)) which are generally considered the biologically active products of protoplasm irradiation. According to MacLean and co-authors, the cytological and cytogenic ozone effects observed are radiomimetic. Ozone has caused chromosome breakage in Vicia faba (Fetner, 1958) and in mammalian cell cultures (Fetner, 1962; Sachsenmaier and co-authors, 1965), and inhibited mitotic activity in Lemna perpusilla (Feder and Sullivan, 1969). While potential ozone damage remains a danger in cultivation, MacLean and co-authors believe that acceptable treatment steps could be developed and employed along with carbon filtration to remove or reduce the amount of life-endangering substances.

Ozonation can also be applied for pretreatment of culture water (p. 100). Blogoslawski and co-authors (1973), for example, suggest the use of ozone for detoxification of red-tide water prior to use in culture systems. A series of ozone doses (20, 40, 65, 110 ml min\(^{-1}\)) increasingly inactivated Gymnodinium breve toxin as tested by mouse (Mus musculus) and fish (Fundulus heteroclitus) injections. The highest dose of 110 ml min\(^{-1}\) rendered the material non-toxic to mice and significantly reduced the degree of toxicity to the fish. Inactivation of botulism toxin
(Clostridium botulinum) by ozone had been reported earlier by Miller and co-authors (1959). These findings are of importance to mariculturists who depend on natural raw sea water.

Ozone should never be bubbled freely into the culture enclosure. In culture systems, ozone is injected into a water-filled tube separated from the culture container. Ozonation can be applied together with, or as a substitute for, foam separation using air. Substitution of oxygen for air in the ozone-generator intake doubles the quantity of ozone produced (Spotte, 1970).

In sanitary waste-water treatment, ozone is used (Atkinson and Palin, 1973) to disinfect the water and render viruses ineffective, to remove colour, to eliminate bad taste, and to remove pesticides and other organic materials such as herbicides and synthetic detergents. In commercial water-treatment plants, ozone is produced from cold, dry air by a high-voltage silent electrical discharge. There are two basic types of ozonizers: the Otto plate ozonizer with flat electrodes and glass dielectrics, and the Welsbach type with cylindrical electrodes and dielectrics. According to Atkinson and Palin, the technically most difficult aspect of ozonation is the design of an efficient injection system. They list three possibilities: (i) an injector similar to the laboratory filter pump; (ii) the Kerag system where the gas is injected into the eye of a mechanical mixer at the base of the contact column; (iii) gas dispersers located in the column base. Numerous further papers on practical aspects of waste-water ozonation have been published, for example, by Marsh and Panula (1965), Arthur (1971), McNabney and Wynn (1971), Zenz (1971), McBride and Taylor (1973), Nebel and co-authors (1973a, b) and Rosen (1973).
An ozonator system for use in fish raising has been developed by Rosenthal and Sander (1974). The system (Fig. 2-84) combines dispergator ozonation and foam separation. Water from the culture enclosures passes through a pump, receives air, and is tangentially injected into a reaction tower. The fast-rotating (ca 2400 rpm) disc of a gas dispergator distributes small bubbles of ozone-containing air, thus providing for maximum water-air contact. The organic load of the culture water is considerably reduced, both by the ozonation process and by subsequent foam separation (p. 140). After pressurized-air aeration, the water re-enters the culture enclosures. Rosenthal and Sander directed part of their culture medium through the ozonator; the other part received biological water treatment (p. 122).

The 'Institut für Meereskunde' at Kiel (FRG) planned to recirculate the sea and brackish water in its public aquarium through an ozonator system consisting of ozone generator, foam- and disinfection towers, ozone absorber, and algal filter (Fig. 2-85). According to Sander (1971) and Schlesner (1973), sea water from the culture tanks (exhibition tanks) overflows through a 0.1-mm mesh size sieve (S₁) into a collector basin, from where it is pumped (P₁) through a foam tower operated with an air-ozone mixture. The foamate escapes through 3 sieves (S₂) into the foam collector on top of the column. A second pump (P₂) forces water into the disinfection tower (additional foam removal plus partial disinfection). The ozone absorber (O) was included to absorb excessive ozone; it releases the water into an illuminated algal filter (p. 129). From here, the water is pumped (P₃) back to the tanks. Due to the high water turnover rate, the activated charcoal in the ozone absorber tended to powderize and to pollute the tanks. Hence, the charcoal was removed, the ozone generation reduced from 30 g O₃ hr⁻¹ to 17 g O₃ hr⁻¹, and the ozone supply through the second injector (I₂) shut down. These alterations changed the disinfection tower to a second foam tower assisting in the release of excessive ozone. According to Trekel (personal communication), the Kiel aquarium is just as successful in
maintaining marine invertebrates and fishes as are other well-equipped public aquaria that employ conventional sand–gravel filters. However, the present degree of ozonation is insufficient for sterilization. In order to optimize the efficiency of the system, SCHLESNER (1973) recommends: (i) modifying the foam tower so as to achieve a continuous, uniform movement of the foam column; (ii) reactivating the disinfection tower and reintroducing the activated charcoal filter in such a way that it resists high turnover rates of the culture water.

Table 2-30

Methods for determining ozone concentrations in water (Compiled by ROSENTHAL, 1974, from the sources indicated)

<table>
<thead>
<tr>
<th>Method</th>
<th>Remarks</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manganese oxidation and colour reaction with orthotolidine</td>
<td>Mn$$^{++}$$—Mn$$^{+++}$$ (spectrophotometric determination at 440 m$$\mu$$, 5–20 $\mu$M l$$^{-1}$$)</td>
<td>ZEHRINGER and STUMM (1953) SULZER (1958)</td>
</tr>
<tr>
<td>Oxidation of leuco crystal violet</td>
<td>Redox indicator, colorimetric procedure at a wave length of 582 m$$\mu$$ in acidic solution (sub-ppm levels)</td>
<td>LAYTON and KINMAN (1970)</td>
</tr>
<tr>
<td>Spectrophotometry (visible region)</td>
<td>Typical absorption band at 280 m$$\mu$$ (&lt;0.4 $\mu$M l$$^{-1}$$)</td>
<td>ALDER and HILL (1960) KILPATRICK and co-authors (1956)</td>
</tr>
<tr>
<td>Reaction with potassium iodide and colorimetric determination of iodine</td>
<td>Two modified applications for low level (0.01–0.30 ppm) and high level determination (0.30–2.00 ppm)</td>
<td>SHECHTER (1973)</td>
</tr>
<tr>
<td>Reaction with potassium iodide and colorimetric determination of iodine</td>
<td>Stoichiometry</td>
<td>PARRY and HERN (1973)</td>
</tr>
<tr>
<td>Oxidative decoloration of indigo-sulphonate</td>
<td></td>
<td>DORTA-SCHAEPPI and TREADWELL (1949)</td>
</tr>
<tr>
<td>Reaction of ozone with potassium iodide</td>
<td>2Kl—I$_2$</td>
<td>WANNER (1971)</td>
</tr>
<tr>
<td>Reaction of ozone with potassium iodide</td>
<td>Colorimetric determination of the liberated iodine with starch</td>
<td>RAWSON (1953)</td>
</tr>
<tr>
<td>Automatic reading device</td>
<td></td>
<td>LITTMAN and BENOLIEL (1953)</td>
</tr>
</tbody>
</table>

Most methods for ozone determination (chemical, electro-chemical, optical) have been developed for measuring atmospheric ozone concentrations. Important methods of ozone determination in water are listed in Table 2-30. The classical method for determining residual ozone in water (ANONYMOUS, 1971) requires sample volumes of up to 1 l for low ozone concentrations of about 0.03 ppm, and thiosulphate titration of the iodine liberated by ozone has some limitations. For these reasons, SHECHTER (1973) developed a spectrophotometric method. At sample sizes of 5 to 10 ml, SHECHTER’s method involves oxidation of a buffered iodine solution and
CONCLUSIONS

spectrophotometric measurement of the triiodide ion liberated by ozone. Two procedures are used: one for low ozone concentrations (0.01-0.30 ppm); and another for high concentrations (0.30-2.0 ppm). At 0.20 ppm ozone, the standard deviation of the method is 0.9 to 3.4%. According to Rosenthal (personal communication), special problems exist in regard to determining ozone in sea water. Free radicals tend to reduce the specificity of the reaction. Hence, the methods listed in Table 2-30 may have to be amended or modified for application under sea-water conditions.

Ultra-violet Irradiation

Ultra-violet irradiation is frequently used for purification of sea water and other culture media. It oxidizes organic matter contained in the culture water and reduces the activity and reproductive capacity of micro-organisms. Ultra-violet exerts disinfecting, rather than sterilizing, effects. Ultra-violet irradiation comprises wavelengths below 400 nm (Volume I: Jerlov, 1970). For producing bacteriostatic and fungistatic effects, the most efficient wavelengths range from 240 to 280 nm; maximum efficiencies are attained near 254 nm.

The transmittance of ultra-violet irradiation in sea water varies considerably due to absorption by particles and yellow substance. In tropical ocean waters, transmittance ranges from 50 to 80% m⁻¹ of depth; in coastal areas, such as the Skager-rak, maximum transmittance does not exceed 10% m⁻¹ (Jerlov, 1968). Transmittance falls off with increasing concentration of dissolved substances (Hoather, 1955). In the surface waters of Liverpool Bay (UK), the distribution and magnitude of the ultra-violet (250–350 nm) absorption were, except during September, predominantly influenced by organic matter introduced from land sources, rather than by in situ biological activity (Foster and Morris, 1974). Penetration into most solids is virtually nil. However, some special glasses permit transmittance of bacteriostatic wavelengths, while ordinary glass and perspex are very opaque (e.g., Wood, 1961). Polished stainless steel, aluminium and surfaces coated with aluminium paint or chromium plating reflect a substantial portion of the radiation.

Part of the oxidizing capacity of ultra-violet discharge tubes, suspended directly over the culture container, results from ozone (p. 154) generation (e.g. Benoit and Matlin, 1966), particularly below 200 nm with a maximum near 185 nm. In addition to organic compounds, ultra-violet irradiation may also oxidize inorganic forms of nitrogen and phosphorus.

The disinfective potential of ultra-violet irradiation has been investigated primarily in fresh water—in most cases, with the main interest directed on drinking and swimming-pool waters, and in regard to heat-labile laboratory equipment. Fundamental data for ultra-violet disinfection of fresh waters have been provided by Luckiesh and Holladay (1944). Ultra-violet is equally effective against Gram-positive and Gram-negative bacteria, with lethal doses in the range 1 to 6 mW cm⁻². Bacterial spores require a 10 times higher, mould spores an up to 50 times higher dose. According to Sykes (1969), assessments of virus susceptibilities to ultra-violet irradiation vary considerably; the ratios reported of inactivating doses needed for viruses and bacteria range from 10:1 to 100:1, with extremes of 1:1 and 200:1. Buttolph and co-authors (1953) list the following irradiation requirements, in terms of mW cm⁻² to inactivate 63.2% of the micro-organisms present within 1 min: Alpha streptococci and Staphylococcus aureus, 1.3; Beta
haemolytic streptococci and *Escherichia coli*, 5-3; moulds, 10; mould spores, 2-8. Employing a 15-W lamp with an ultra-violet emission of 90 mW cm⁻², KAWABATA and HARA (1959) determined the tolerance of micro-organisms in aqueous suspension. They found the periods required to kill 99.9% of Gram-negative strains to be: *Proteus* sp., 42 secs; *Shigella* sp., 47; *Eberthella typhosa*, 49; *Escherichia coli*, 60. For Gram-positive forms, the periods were: Group A *Streptococcus*, 83 secs; *Staphylococcus aureus*, 103; *S. fecalis*, 165; *Bacillus subtilis* awamurana, 240; *B. subtilis* spores, 369. The yeast *Saccharomyces sake* required ca 217 secs, and *Willoanomalae*, 420 secs.

Using a discharge lamp similar to that employed by KAWABATA and HARA (1959), OKINAMI and co-authors (1952) investigated the feasibility of disinfecting seawater in cultures of oyster *Crassostrea virginica* with ultra-violet discharge units. Employing 'rather high' rates of recirculating water flow, a reduction of ca 90% in colony-forming coliform units was recorded after 1 passage. Utilizing six 15-W germicidal lamps, KAMIMURA and SUZUKI (1954) inactivated ca 90% of the bacteria present at a sea-water flow rate of 45 l min⁻¹ and an exposure period of 15 secs. KELLY (1961) states that ultra-violet irradiation is an effective means of destroying coliform micro-organisms in sea water. His disinfection unit (30-W ultra-violet lamps; 960 mW cm⁻²; 150 l min⁻¹ of sea water), developed for the treatment of sea water in laboratory experiments on oysters, accomplished more than 99.96% reduction of coliforms at 15 secs exposure.

WOOD (1961) and HERALD and co-authors (1962) have examined the usefulness of ultra-violet irradiation for controlling bacterial populations in closed sea-water systems. When the water was passed through an ultra-violet sterilizer at the rate of about 32 l min⁻¹, the number of suspended bacteria (CFU) was reduced to almost zero. However, water that appeared to be sterile after 48 hrs yielded bacterial colonies on test plates after about one week. Hence, the effect of ultra-violet irradiation was bacteriostatic rather than bactericidal. SHELBURNE (1964) modified Wood's design. He used disinfection boxes (each measuring 60 cm X 30 cm X 30 cm) made of resin-lined marine plywood. The box lid contains two 15-W low-pressure ultra-violet discharge tubes, backed by an aluminium reflector (Fig. 2-86). Filtered sea water enters the box at a low level and passes over a longitudinally arranged plywood weir. At a flow rate of 1000 l h⁻¹, the irradiated water turned out to be 'virtually sterile'. Within 6 hrs, the originally high CFU characteristic of inshore water dropped to a level closer to that of open-ocean water (Fig. 2-87). In an experiment, irradiation was continued overnight and sampling was resumed the following morning. By that time, the sea water in the reservoir was perfectly clear, and the filter coated with debris. The filter by-pass was then closed, and the entire water flow directed through the ultra-violet discharge unit. Figure 2-87 documents the degree of disinfection and the recontamination capacity of a dirty filter.

BURROWS and COMBS (1968) obtained significantly reduced incidences of infectious diseases in cultured salmon after ultra-violet treatment of the culture water. The efficiency of ultra-violet irradiation increased with exposure time and with decrease in turbidity. Filtration of raw river water (rapid sand filter) doubled the efficiency of an 18-W ultra-violet irradiation unit (divided into 3 series of 6 40-W lamps each) with a capacity of about 160 l min⁻¹.

In barnacle cultures, heavy bacterial growth developed at irregular intervals on
the water surface of the heating tank. To prevent this, Tighe-Ford (1967a, b) employed two 15-W, 46-cm ultra-violet lamps, housed in a Tufnol lid on top of the tank. These lamps emitted bacteriostatic radiant energy (254 nm) which kept the water surface free from microbial growth. Eagleson and Herald (1968) used ultra-violet irradiation for disinfecting marine aquaria.

The Fishery-Oceanography Center at La Jolla (California, USA) has installed in its experimental sea-water aquarium an ultra-violet unit (Aquafine Sterilizer Model PVD-24) capable of disinfecting about 1000 l of filtered sea water min⁻¹ by spinning
the water with helical baffles past 26 quartz-jacketed ultra-violet lamp units en-
cased in a PVC cabinet. In 4 years of operation, bacterial contamination and fouling
have been insignificant (LASKER and VLYMEN, 1969). The unit is cleaned once a
month to remove accumulated minerals from the exterior surfaces of the quartz
sleeves (flushing with a dilute solution of hydrochloric acid; MURPHY, 1969). At
the bottom of the ultra-violet unit, dielectric probes are mounted in a tray. In the
case of leakage, the tray fills with sea water, shorting out the probes and tripping a
relay which shuts off electricity to the ultra-violet lamps and sounds a bell alarm.

Ion-exchange

Ion-exchange resins are electrochemically charged. They remove ions from solu-
tion by exchanging them with other ions. Eventually, the resin becomes exhausted
and must be recharged. Depending upon the pollutants to be removed from solution,
different resin types (strongly or weakly acidic cations, strongly or weakly basic
anions) are employed (e.g. KUNN, 1963).

The usefulness of ion-exchange in cultivation is rather limited. In sea water, the
large number and high concentration of ions present interfere with the exchange
process. Even in freshwater cultures, ion-exchange resins can be used only with
reservation. Organic substances leach out of the resin and may contaminate the
culture water (Chapter 7). In closed systems, the exchange process may modify the
ionic composition of the culture water.

Ammonia removal from effluents of large aquaculture farms is a problem of in-
creasing concern. In fresh and brackish waters, ion exchange may help. In secondary
municipal waste effluents, MERCER and co-authors (1970) tested the efficiency of
the ion-exchange process for reducing the extremely high ammonia loads. More
than 99% of the ammonia was removed in the laboratory with 2 Zeolite® columns
in series; however, the purified secondary effluent still contained 10 to 19 mg amm-
onia-N l⁻¹. An average of 97% ammonia was removed by two 1900 l clinoptilolite
columns in a mobile demonstration plant, with the clarified secondary effluent
containing 16 mg ammonia-N l⁻¹. Flow rate was 244 l min⁻¹ m⁻² (see also AMES,
1967; MERCER and co-authors, 1967). Further studies on the removal of ammonia
and nitrate from municipal waste water by ion-exchange resins have been published
by NESSELSON (1954), MARTINEZ (1962), ELLASSEN and co-authors (1965) and CLESCERI
and SLECHTA (1966). Phosphate removal has received attention from MARTINEZ
(1962), ELLASSEN and co-authors (1965), RAND and NEMEROW (1965) and CLESCERI
(1968).

Other Techniques

Other techniques of physico-chemical water treatment have thus far failed to
reveal promise in regard to cultivation. In municipal waste-water treatment,
CLESCERI (1968) has applied lime, alum, aluminate, iron and activated alumina.
Flocculation and precipitation of settleable and suspended particulate matter,
combined with separate removal of colloidal dissolved substances by means of
reversible osmosis (plus activated carbon and ion-exchange), has been discussed by
MALZ (1971). Flocculation and coagulation processes have also been considered by
MALHOTRA and co-authors (1964), STUMM and HAHN (1967), STUMM and O’MELIA (1968) and BERNHARDT and WILHELM (1971).

For removing ammonia and phosphate compounds from domestic waste water, FOYN (1964) has developed an ‘electrolytic sewage-purification method’. Raw sewage, mixed with 10 to 15% of sea water, is conveyed to a cathode chamber, and sea water alone is placed in the anode chamber. After applying electrical current, the pH at the cathode increases; ammonia precipitates as a magnesium ammonium phosphate, and phosphorus as a calcio-phosphato compound (e.g. hydroxylapatite). At the cathode, magnesium hydroxide and hydrogen gas bubbles are formed, supporting the removal of insoluble ammonia and phosphate compounds by floatation. The resulting sludge is skimmed off and treated by applying the usual sludge disposal techniques. At the anode, chlorine develops; it oxidizes and sterilizes the effluent. The electrolytic process lasts about 30 mins (400 kWh per 4 million l sewage water). It removes about 75% of the ammonia-N. The applicability of the process to effluents of large and heavily loaded aquaculture systems is open to debate.

(c) Conclusions

Culture-water treatment is of fundamental importance in all closed-system operations. The recirculating water is subject to pollution from the organisms cultured, from substances added by the cultivator (e.g. food), or from the equipment and materials used. For countering such degradation of the culture water, a variety of procedures have been developed which may be grossly subdivided into pretreatment, reconditioning treatment and post-treatment. Pretreatment of the culture water includes cleaning, disinfection, aeration and adjustments in temperature, salinity, gaseous contents, etc., before adding the water to the culture system. Reconditioning treatment constitutes the basis of culture-water treatment. Post-treatment before culture-water release becomes necessary in large systems, such as aquaculture farms, in order to avoid environmental pollution.

Reconditioning treatment comprises mechanical, biological and physico-chemical procedures. These procedures share a number of characteristics and, sometimes, defy exact distinction. Mechanical water treatment removes excessive suspended particulate and colloidal substances by sedimentation, centrifugation or filtration. Of these three methods, filtration is the most important. It involves sand-gravel filters, rapid sand filters, diatomaceous-earth filters or disposable cartridge filters. The most commonly used filter is the sand-gravel filter. Biological water treatment aims at maintaining the life-endangering substances, produced by the organisms cultured, at acceptable levels. Micro-organisms accommodated in the filter bed remove excessive loads of organic matter from the recycling water. Algae remove from, and release into, the water a variety of substances and thus may assist in improving the quality of the culture water. While microbial water treatment is an essential component in water-quality management, algal water treatment has not yet passed the exploratory phase. The contribution that algal filters can make to the cultivation of multispecies systems remains to be fully investigated. Physico-chemical water treatment aids in removing excess dissolved organic substances, e.g. by activated carbon adsorption, foam separation, aeration, oxygenation, or ozonation. It is of importance in cases where biological water treatment is insufficient or impractical.
In the last decades, considerable information has accumulated on culture-water treatment. Many impulses for new procedures and techniques came from the related field of municipal waste-water treatment. However, the degree of communication and cooperation between ecologists and sanitary engineers and, in fact, between ecologists engaged in the cultivation of micro-organisms, plants or animals, is still insufficient.

The most important perspectives for future research may be summarized as follows: (i) We need more and better methods for recording, evaluating and controlling chemical properties of the water body managed. (ii) The possible contribution of life-supporting substances, including those released by aquatic plants, deserves more attention. (iii) Special culture-water treatment techniques must be worked out for multispecies cultures and microcosms.

(8) Capacity of Culture Systems for Supporting Aquatic Animals

(a) General Aspects

The total amount of living animals that can be supported by a given closed culture system is referred to as the 'carrying capacity' or 'animal load'. It is expressed in terms of the weight or volume ratio culture water to cultured animals. The carrying capacity depends on water turn-over rate, water treatment (e.g. type, size and efficiency of the filter), as well as on type and size of the culture enclosure. Studies which relate these parameters to the weight (volume) of the animals supported are quite limited in number. In fact, proper relative dimensioning of essential culture-system components has largely remained a virgin field. This very promising branch of cultivation invites the combined efforts and talents of both ecologists and engineers.

Carrying capacity is viewed from different perspectives in research cultivation and in commercial cultivation. In research cultivation, there can hardly be too much sea water. The experimental ecologist usually aims at establishing conditions in which the amount of culture water neither limits, nor interferes with, the natural responses of the animals cultivated. The sea-food farmer, on the other hand, wants as little water as possible; he must produce maximum amounts of sea food at minimum cost.

Most of the scant information available on the carrying capacity of culture systems has been produced with an eye on commercial cultivation. Ecologists have largely worked under undefined 'green-thumb' conditions—a situation which is no longer tolerable, if we want to comprehend and manage life processes in oceans and coastal waters.

A number of ecologists engaged in research cultivation have emphasized that the weight (volume) ratio sea water to animals should be as large as possible. HINTON (1958) says it is difficult to have too much sea water, and Atz (1964a) refers to a cardinal, though unproven, principle holding that the greater the water volume, the slower its rate of deterioration. STOWELL and CLANCY (1927) and WILSON (1952, 1960), on the other hand, have suggested that the useful life of a volume of sea water
may be lengthened by dividing it into equal parts, alternately ‘resting’ half of the water in a dark reservoir for 1 or 2 months. In the Amsterdam Aquarium (Netherlands), the culture tanks comprise only one sixth of the total water volume of the system (Sunier, 1951). Based on Saeki’s (1958) work and his own experiments conducted at the Cleveland Aquarium (USA), Kelley (1963) advises that the relation of water volume to the weight of animals supported should be about 3801: 0.45 kg. Of course, the carrying capacity of a given water volume depends upon its turnover rate (p. 118). According to Kelley, the water should circulate completely once every hour and pass through the filter at a rate of about 41 0.1 m\(^{-2}\) min\(^{-1}\). His filter material consisted of 2 to 6 mm grains of silica gravel (75%) and calcareous gravel (25%) with 0.03 m\(^3\) of filter material for each 0.45 kg of animals.

(h) Assessment of Carrying Capacity

The first detailed account on standards for design and planning of closed systems for the cultivation of invertebrates and fishes was presented by Saeki (1958). He argues that—provided the basic environmental and nutritional requirements are met and the animals are healthy—critical consideration must be given those substances that are metabolized in large quantities: oxygen, carbon dioxide, ammonia, nitrite, nitrate, phosphate and complex organics. Either excess or insufficiency of these substances soon causes damage or death.

Saeki (1958) used the ammonia content of the culture water as a criterion for assessing the standards for system design and planning (see also Saeki, 1963, 1965). He measured and calculated—at temperatures between 20° and 25° C—the rates of ammonia production by the animals cultured and those of bacterial nitrogen assimilation in the filter bed.* Saeki used various freshwater and marine invertebrates and fishes as test animals. Although there are some discrepancies in the figures, carp, goldfish, killifish, rainbow trout, brook trout, etc., excrete about 25 mg ammonia day\(^{-1}\) 100 g\(^{-1}\) wet body weight.

Bacterial ammonia oxidation was measured by Saeki (1958) in a percolator (Fig. 2-88) containing 60 g of washed sand (weathered granite sand; grain size 2 to 5 mm) from the bottom of the balanced public aquarium at Ueno (Japan), and about 1.5 l of well water containing ca 10 ppm of ammonia. Percolation at a speed of ca 150 cm\(^3\) min\(^{-1}\) was effected by an airlift. The percolator was kept at 22° C in darkness. The results obtained are illustrated in Fig. 2-89. After 4 days, the original ammonia contents had decreased by about half, and after 7 days the ammonia had practically disappeared. The velocity coefficient \(K\) in this experiment was \(3.7 \times 10^{-3}\); when 30 g of sand were used for 1 l of water, the coefficient was \(K_{30} = 2.5 \times 10^{-3}\). Further experiments with sands of different detritus content, and after different periods of sand use, gave an average value of \(K_{30} = 2.0 \times 10^{-3}\). This value is the same for sea

* Filter sand: calcite (limestone chip) or weathered granite sand of 2 to 5 mm grain size. Salt water: stored sea water. Fresh water: well water of pH 7.9; 40 mg l\(^{-1}\) Ca; alkalinity ~3 meq. l\(^{-1}\). Ammonia was determined with Nessler reagent; nitrite with G.R. reagent; nitrate by strychnine reduction; phosphate by Déniges–Atkins’ colorimetric method; alkalinity by titration.
water and fresh water. The processes of ammonia oxidation and nitrification may be represented by the following equations:

\[
\log \frac{A_A - x}{x} = A_A K_A (t - t_1)
\]

\[
\log \frac{A_N - y}{y} = K_N (t - t_1)
\]

\[- \frac{dx}{dt} = \frac{K_A}{M} (A_A - x)x\]

Fig. 2-88: Percolator for water-treatment studies. (After SAEKI, 1958; modified; reproduced by permission of the author.)

where \(A_A\) and \(A_N\) = initial maximum amounts of ammonia-N and nitrate-N, respectively; \(x\) and \(y\) = the amounts thereof at time \(t\); \(t_1\) and \(t_2\) = half-times of decrease or increase; \(K_A\) and \(K_N\) = reaction velocity constants.

If reaction velocity is constant, and if the amount of ammonia at a given time, as well as the initial amount of ammonia, is known, the rate of ammonia oxidation can be calculated. The equations, however, refer to circumstances under which ammonia gradually decreases. In actual cultivation, the 'initial amount of ammonia' may be represented by the amount of increased nitrate. Ordinarily, the culture-water nitrate content is 20 to 100 mg l\(^{-1}\). For the oxidation rate to reach equilibrium, a nitrate increase of about 15 mg l\(^{-1}\) is necessary. Since the amount of ammonia in a healthy fish culture is 0.1 to 0.2 mg l\(^{-1}\), \(x \approx 0.15\) mg l\(^{-1}\), the reaction velocity constant, as already noted, is \(2.0 \times 10^{-3}\), and the value of ammonia-N corresponding to the initial ammonia is 15 mg l\(^{-1}\). Then,

\[- \frac{dx}{dt} = \frac{2.0 \times 10^{-3}}{M} \times 0.15 \times (15 - 0.15)\]

\[\approx 19.6 \mu g hr^{-1} or 0.47 mg day^{-1}\]
This means that the bacteria proliferating in 30 g of sand oxidize ammonia at a rate of 0.47 mg day\(^{-1}\).

The rate of ammonia oxidation by the bacteria is proportional to the ratio \(r\) between the exchangeable ammonia in the filter bed and that of the system as a whole. SAEKI (1958) took bottom sand from the tropical fish tank of the Ueno Aquarium and sand from the filter tank during the closed-circulation culture of goldfish and eels. He then placed amounts varying between 20 and 330 g of each of these sands into the aforementioned percolation apparatus, through which he circulated well water in amounts from 0.8 to 1.5 l, with added ammonium sulphate, and for several days, measured the variations of inorganic nitrogen compounds therein. Finally, he estimated the exchangeable ammonia of the sand and calculated the corresponding rate of ammonia oxidation and the constant of proportionality \(C\). For weights of sand varying from 19 to 330 g, he found exchangeable ammonia values of 30 to 1390 \(\mu\)g; \(r\) values of 0.0037 to 0.166; rates of ammonia oxidation of 0.35 to 2.4 mg day\(^{-1}\); values of constant \(C\) of 14.5 to 54.4. Further considerations led SAEKI to assume a constant of proportionality of about 20.

With an ammonia content of 100 to 300 \(\mu\)g l\(^{-1}\) in the culture water, what will be the amount of exchangeable ammonia in the filter sand under favourable cultivation conditions? For eel cultures and for the tropical water tank and the sea-water aquarium at Ueno, the exchangeable ammonia per 10 g of sand ranges from 1.9 to 3.8 \(\mu\)g (in a non-circulating system the exchangeable ammonia is 11.8 to 12.2 \(\mu\)g);
the dissolved ammonia ranges from 200 to 280 \( \mu g \) l\(^{-1}\). From these and related results one may reckon with 2-0 \( \mu g \) of exchangeable ammonia per each 10 g\(^{-1}\) of sand.

According to these values, the rate of filter-sand ammonia oxidation is:

\[
\frac{dx_1}{dt} = Cr
\]

where \( r = x/(x + x_i) \); \( x_i \) = weight of dissolved ammonia; \( x \) = weight of exchangeable ammonia in filter sand (= 0.002 mg per 10 g of sand); \( C \) = constant of proportionality (= 20).

If \( x_1 \) is taken to be 0.1 to 0.3 mg l\(^{-1}\), the value of \( x \) does not vary greatly; one can safely take 0.3 mg l\(^{-1}\) as the value of \( x_1 \).

\[
x_1 = 0.3 \text{ mg l}^{-1}
\]

\[
\frac{dx_1}{dt} = 0.13 \text{ mg day}^{-1}
\]

Since this rate is 0.16 mg day\(^{-1}\) per 10 g of sand when employing the percolation method (p. 167), the mean of the two values obtained will be used for computation:

\[
\frac{dx_1}{dt} = 0.14 \text{ mg of ammonia day}^{-1} \text{ per 10 g of sand}
\]

The amount of nitrogen compounds assimilated by the filter-sand bacteria ranges between 45 and 100\% of the amount oxidized. The rate of assimilation is proportional to the increase of bacteria, while the oxidation rate is proportional to the weight of the bacteria. Hence, oxidation may proceed even when there is no bacterial growth. For purposes of calculation, SAEKI (1958) takes the assimilation rate to be 50\% of the oxidation rate:

\[
\frac{dx_2}{dt} = 0.5 \frac{dx_1}{dt}
\]

The amount of nitrogen compounds processed by the filter-bed bacteria must equal the amount of nitrogen compounds excreted by the animals cultivated. Unless this prerequisite is met, cultivation is not possible. In order to satisfy the basic requirements for short-term cultivation with a sufficient margin of safety, the weight of filter sand should be about 30 times that of the animals cultured.

In order to meet the requirements for long-term cultivation, additional considerations are necessary. In a closed-circulation system, the culture water tends to exhibit three major deviations from healthy, natural waters: (i) higher amounts of nitrates (100 to 1000 times higher); (ii) higher amounts of phosphates (about 100 times); (iii) lower alkalinity and pH. The excreta of the animals cultivated are made up mostly of the elements H, O, C, N, P, Ca, Na, Cl and Mg. Among these, accumulations of N and P are most important. The changes in water quality due to long-term cultivation involve, first of all, a decrease in excess base due to gradual increase in nitrate and accompanying oxidation processes. The increase in nitrate is limited, due to bacterial denitrification. In the Ueno Aquarium, for example, SAEKI (1958) found the limiting level to be around 100 mg l\(^{-1}\). Increases in phosphate contents, in conjunction with the Ca and Mg in the culture water, lead to precipitation, which occasionally may cause increased turbidity. Because of precipitation, the level of dissolved phosphates
does not exceed a certain amount (≤ 6 mg l\(^{-1}\)). Since portions of Ca and Mg are removed from the culture water, this mechanism reduces the alkalinity and may cause acidification. Examination of the deposits in the filter tank and circulating pipes of the Ueno Aquarium revealed that 49% and 0.7% respectively of the dry substance was calcium phosphate. Moreover, the surface of the filter sand, which had been used for some time was covered with a substance containing phosphate of lime or magnesium. When SAEKI added lime or magnesia to water— with a content of 2.56 mg l\(^{-1}\) of phosphate—that had been used in eel cultivation, the phosphate content (determined in the supernatant layer) decreased considerably (0 to 0.4 mg l\(^{-1}\)) within 24 hrs (precipitation with Ca and Mg). The results of this experiment are listed in Table 2-31.

### Table 2-31

Dephosphorization of eel-culture water by magnesia or lime treatment. To each 100 cm\(^3\) of original culture water, 0.2 g of MgO, CaO or MgCO\(_3\) were added. After 24 hrs, pH, alkalinity and phosphate-P of the water were determined. 22° C (After SAEKI, 1958; modified; reproduced by permission of the author)

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>pH</th>
<th>Alkalinity (mM)</th>
<th>Phosphate-P (mg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original culture water</td>
<td>8.2</td>
<td>2.56</td>
<td>2.64</td>
</tr>
<tr>
<td>After MgO treatment</td>
<td>9.2</td>
<td>2.44</td>
<td>0.024</td>
</tr>
<tr>
<td>After CaO treatment</td>
<td>&gt;9.2</td>
<td>3.94</td>
<td>0.000</td>
</tr>
<tr>
<td>After MgCO(_3) treatment</td>
<td>7.7</td>
<td>4.50</td>
<td>0.450</td>
</tr>
</tbody>
</table>

At pH 7.7, magnesium and phosphate ion concentration is extremely low (ca 2 × 10\(^{-8}\) mole). Under these circumstances, H ions decrease in number, solubility of CO\(_2\) increases, alkalinity decreases, and buffer capacity becomes reduced. The result is harm to the animals cultivated and reduction of bacterial ammonia oxidation.

Long-term cultivation leads to increasing acidification of the culture water (Tables 2-32, 2-33). The acidifying velocity approximates 2.6 equivalents (amount of nitrate increase = 2.1 equivalents + amount of phosphate increase = 0.5) per ton of animals cultivated per day (SAEKI, 1963). Acidification can be counteracted by addition of lime or sodium bicarbonate. In view of the fact that one cause of acidification is precipitation of calcium and magnesium, lime is preferred. When large amounts of lime are added, a portion does not dissolve immediately; the resulting lime deposit counteracts acidification for some time.

Capacity and efficiency of filtration (sand–gravel filter) in a small closed system have also been studied by HIRAYAMA (1966a, b, 1966a). He used oxygen consumption of the filter-bed micro-organisms (OCF) as criterion for assessing the filter’s capacity to avoid water pollution due to excreta and waste food (HIRAYAMA, 1965a). Analyzing the dynamic balance between rates of pollution and purification, HIRAYAMA (1966b) employed the closed system illustrated in Fig. 2-90, and used the
sea bream *Chrysophrys major* as test fish. The rate of pollution $X$ (mg O$_2$ consumed min$^{-1}$) assumes the following relation to the body weight $B$ (g) of the fish and the amount of food $F$ (g day$^{-1}$) offered:

$$X = \sum_{j=1}^{g} \left( B_j^{0.544} \times 10^{-2} \right) + 0.51F$$  \hspace{1cm} (1)

### Table 2-32

Acidification of culture water and depression of microbial nitrification with repeated percolation. A: nitrification with weathered granite sand; B: with calcite sand (limestone chip). 1.5 l of well water and 15 g of sand (2 to 5 mm grain size) were used in each experiment (After Sæki, 1958; modified; reproduced by permission of the author).

<table>
<thead>
<tr>
<th>Repeat cycle</th>
<th>Ammonia content (mg l$^{-1}$)</th>
<th>Maximum rate of ammonia oxidation (mg l$^{-1}$)</th>
<th>Alkalinity (mN)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at beginning</td>
<td>at end</td>
<td>at beginning</td>
<td>at end</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>6.6</td>
<td>0.1</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.9</td>
<td>0.05</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(7)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>4*</td>
<td>10.2</td>
<td>10.1</td>
<td>0.0</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>7.4</td>
<td>0.1</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.6</td>
<td>0.05</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(7)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>4*</td>
<td>8.8</td>
<td>0.05</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Calcium content (mg l$^{-1}$)

<table>
<thead>
<tr>
<th></th>
<th>at beginning</th>
<th>at end</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>40.5</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>58.0</td>
</tr>
</tbody>
</table>

where $g$ represents the number of fish. The water quality requirements of the fish can be expressed by the equation:

$$\sum_{j=1}^{g} \left( B_j^{0.544} \times 10^{-2} \right) + 0.051F \leq \sum_{i=1}^{p} \frac{10W_i}{V_i + 0.95 \times 10^3}$$  \hspace{1cm} (2)

where $p = \text{number of filters}$, $V = \text{filtering velocity (cm min}^{-1})$, $W = \text{surface area of the filter bed (m}^2)$, $G = \text{grain-size coefficient}$, and $D = \text{sand depth (cm)}$.

Grain size is determined by:

$$\frac{1}{R_1}x_1 + \frac{1}{R_2}x_2 + \frac{1}{R_3}x_3 + \cdots + \frac{1}{R_n}x_n$$  \hspace{1cm} (3)

where $R = \text{main grain size (mm) of each fraction of graded sand}$, $x = \text{percentage weight of each fraction}$.
### Table 2-33

Acid-alkali balance and change of alkalinity in repeated percolation experiments. Experiments A and B as in Table 2-32 (After SAEKI, 1958; modified; reproduced by permission of the author.)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Extinction of ammonia (mg [meq.] l⁻¹)</th>
<th>Formation of nitrate (mg [meq.] l⁻¹)</th>
<th>Acidification (meq. l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22.4</td>
<td>1.60</td>
<td>2.72</td>
</tr>
<tr>
<td>B</td>
<td>32.6</td>
<td>2.33</td>
<td>3.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Decrease of alkalinity (meq. l⁻¹)</th>
<th>Solvation of Ca (meq. l⁻¹)</th>
<th>Alkalization (meq. l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.23</td>
<td>0.19</td>
<td>2.42</td>
</tr>
<tr>
<td>B</td>
<td>2.79</td>
<td>1.38</td>
<td>4.17</td>
</tr>
</tbody>
</table>

---

![Diagram](image)

**Fig. 2-90:** Closed system for studying relationships between culture-water pollution, due to excreta plus food, and filtration. Test fish: *Chrysochrys* major. Total water volume: 300 l; filtration rate: 3.5 cm² min⁻¹; 20° C, 17–20% S; pH 7.3. (After HIRAYAMA, 1966b; modified; reproduced by permission of the author.)
The capacity of the filter—as assessed by OCF—must be larger than or equal to the rate of pollution by the fish. Equation (2) further reveals that the filter capacity decreases as a function of the weight of the individual fish: A filter which supports one 100-g fish may not be capable of supporting 10 fish of 10 g each. This finding is not surprising, since small individuals tend to have a higher turnover of energy and matter than larger ones. The relations obtained by Hirayama (1966b) between fish-body weight, OCF and nitrite-N and ammonia-N are illustrated in Fig. 2-91. OCF increases with fish-body weight, as do the concentrations of nitrite-N and ammonia-N. The test fish, kept in the closed system shown in Fig. 2-90, were not
fed during the experiment. Calculations by Hirayama reveal that the OCF increases with fish-body weight as follows:

$$\log A = 0.544 \log B - 1.739$$  (4)

where $A = \text{OCF (mg min}^{-1})$ and $B$ body wet weight (g).

OCF values obtained after polluting the culture water (by adding different amounts of food not eaten by the fish) are illustrated in Fig. 2-92. The difference between a situation in which the food was not eaten and one in which it was swallowed shortly after feeding is shown in Fig. 2-93. Mean OCF values are much lower when the food is consumed.

![Fig. 2-93: Changes in OCF as a function of daily food ration. Filled circles: food not eaten; open circles: food consumed shortly after feeding. Test fish: Chrysophrys major. The system used is illustrated in Fig. 2-90. (After Hirayama, 1966b; modified; reproduced by permission of the author.)](image)

In his 1966 papers, Hirayama investigated nitrate accumulation in the water of closed systems containing Octopus vulgaris. The values found on filter capacity (Hirayama, 1965a, b, 1966a, b) correlate well with criteria established by Goldizen (1970) on cultivation requirements of the stenoplastic marine $O$. bimaculatus: (i) 500 l of culture water per kg animal; (ii) 0.1 m$^3$ filter bed consisting of 2 to 5-mm grains of a magnesium-bearing calcium carbonate filtrant; (iii) a turnover rate of 80 l per m$^2$ filter-bed surface min$^{-1}$; (iv) replacement of 25% of the culture water per month. Systems of similar design, according to Goldizen, will maintain 5 kg of sea urchins Strongylocentrotus purpuratus, or 7 kg of American lobster Homarus americanus. However, in rearing and breeding experiments, the animal load should remain below 1 kg. The criteria listed above have been successfully applied to systems ranging in capacity from 80 to 300,000 l and containing animals as diverse as the sponge Microciona prolifera and the dolphin Tursiops truncatus.
Acidification as a function of food and animal load has been studied by Hirayama (1970) in a 50-l experimental aquarium (Fig. 2-94). The sea water used had a specific gravity of about 1.023 (15°C), a temperature of 23°C to 25°C and a recirculation rate of 2 l min⁻¹. The sand filter (diameter at surface: 21 cm, at bottom: 19 cm; depth: 16.5 cm) contained sand of 2.1-, 3.9- or 8.3-mm grain diameter. The aquarium was stocked with the black sea bream Mylio macrophalus, fed with shrimp. Hirayama reports that the acidifying velocity of the culture water is not affected by the different filter-sand grain sizes. The relation between acidification (V eq. day⁻¹) and feeding rate (F g day⁻¹) can be expressed by the equation

\[ V = 0.92 F \times 10^{-1} \]

Fig. 2-94: Experimental aquarium used for acidification tests. Airlift-operated recirculation through sand filter. (After Hirayama, 1970; modified; reproduced by permission of the author.)

Compared with the effect of feeding rate, variations in fish-body weight remain insignificant. CaCO₃ (coral, mollusc-shell pieces, sand) can maintain the alkalinity above 1.0 meq. 1⁻¹ and the pH above 7.5; this positive effect is due to calcium dissolution. In the absence of calcium, normal alkalinity values of about 2.0 may drop to nearly 0 meq. 1⁻¹ within 20 days in sea breams cultivated in 100 l of sea water and fed 10 g of shrimp per day. In each experiment, alkalinity decreased quite linearly with time until it reached 0.2 meq. 1⁻¹. According to Hirayama, the low alkalinity values encountered cause practically no harmful effects to the fish cultured over the experimental period (up to about 50 days). However, long-term effects remain to be examined. The nitrifying capacity of the sand filter was sufficient to keep ammonia-N at a low level, even at pH values below 7.

The total carrying capacity of a culture system depends upon the physiological condition of the animals cultivated, their feeding habits and the food sources. However, some generalizations seem in order: The carrying capacity tends to (i) be higher per unit weight of animal in larger than in smaller individuals of the same species; (ii) decrease with increasing metabolic activity of the animals; (iii) be lower if the animals are to breed, and to increase if the cultivation goal shifts from rearing
to maintenance; (iv) be higher during short-term than during long-term cultivation; (v) decrease with increasing pollution due to feeding procedures.

For commercial cultivation (aquaculture), it is necessary to determine the carrying capacity consistent with the cultivation goal (maintenance, rearing or breeding, p. 2) in the light of expenses for construction and maintenance of the system. The ratio, animal load to carrying capacity, may be referred to as the percentage of saturation of the carrying capacity. Values under 100% indicate use of the system below its capacity; percentages above 100% indicate overloading. When fattening is the objective of aquaculture, cultivation may begin at 30 to 70% saturation, depending upon the growth rate of the animal and the intended time span for use of the system. If the purpose is merely to maintain the animals for limited periods

Table 2-34

*Ictalurus punctatus*. Solid and soluble wastes produced by two adult fish, based on weekly measurements. 950-l tank; 24°C; feed contents: 40 g pulverized liver, 80 g fish pellets; daily ration: 2% of body weight. In the closed system, 1 tank volume was displaced every 24 hrs (After Murphy and Lipper, 1970; not copyrighted)

<table>
<thead>
<tr>
<th>Volume of solids (ml week⁻¹)</th>
<th>Solid waste BOD (g week⁻¹)</th>
<th>Soluble waste BOD (g week⁻¹)</th>
<th>Total wastes BOD (g week⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2420</td>
<td>47.02</td>
<td>82.82</td>
<td>129.94</td>
</tr>
<tr>
<td>2540</td>
<td>62.46</td>
<td>78.34</td>
<td>140.80</td>
</tr>
<tr>
<td>2350</td>
<td>59.48</td>
<td>54.33</td>
<td>113.79</td>
</tr>
<tr>
<td>2250</td>
<td>51.50</td>
<td>43.41</td>
<td>94.91</td>
</tr>
<tr>
<td>1920</td>
<td>39.00</td>
<td>57.18</td>
<td>96.18</td>
</tr>
<tr>
<td>2220</td>
<td>39.25</td>
<td>80.15</td>
<td>119.41</td>
</tr>
<tr>
<td>2380</td>
<td>50.58</td>
<td>74.34</td>
<td>124.92</td>
</tr>
<tr>
<td>2630</td>
<td>69.48</td>
<td>88.64</td>
<td>158.12</td>
</tr>
<tr>
<td>2670</td>
<td>55.14</td>
<td>106.66</td>
<td>161.80</td>
</tr>
<tr>
<td><strong>Average:</strong></td>
<td><strong>52.65</strong></td>
<td><strong>73.78</strong></td>
<td><strong>126.43</strong></td>
</tr>
<tr>
<td></td>
<td>42%</td>
<td>58%</td>
<td>100%</td>
</tr>
</tbody>
</table>

of time, the saturation value may go up to a maximum of 180%. However, in animals with low metabolic rates and high resistance to pollutants (eel, carp), weight increase may be obtained even at saturation values of up to 200% (Saeki, 1958).

Commercial cultivation projects are often conducted without sufficient knowledge of (i) waste production of animals cultured at high population densities, (ii) dimensioning of system components, (iii) environmental pollution potential of intensive culture farms. In a pioneer study, Murphy and Lipper (1970) determined the BOD of the waste produced by two adult channel catfish *Ictalurus punctatus* (Table 2-34). The BOD production measured would—in a raceway containing 100,000 catfish of 0.45 kg each—be equivalent to that of a flock of 150,000 chickens of 0.45 kg each, of a swine operation involving the feeding of 1500 hogs of 45 kg each, or of a fed herd of 480 steers of 453.6 kg each. Knowledge of such data is of importance for system design and environmental protection.
The importance of continuous chemical monitoring of culture water quality is now more widely realized, and reliable monitoring systems are being developed in several institutions. An example is the inter-disciplinary effort of Epifanio and co-authors (1973), who have begun monitoring essential variables (Table 2-35) in a pilot farm designed to raise molluscs from egg to marketing size.

Table 2-35

Chemical monitoring of culture water in a closed-system aquaculture pilot farm (After Epifanio and co-authors, 1973; reproduced by permission of the World Mariculture Society)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Analytical method</th>
<th>Trend in system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>Electrode</td>
<td>Spike, then constant</td>
</tr>
<tr>
<td>Nitrite</td>
<td>Azo dye method</td>
<td>Spike, then constant</td>
</tr>
<tr>
<td>Nitrate</td>
<td>Brucine reaction</td>
<td>Slow accretion</td>
</tr>
<tr>
<td>Reactive phosphorous</td>
<td>Molybdate reduction</td>
<td>Relatively constant</td>
</tr>
<tr>
<td>pH</td>
<td>Electrode</td>
<td>Constant</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>Electrode</td>
<td>Decreasing</td>
</tr>
<tr>
<td>Salinity</td>
<td>Salinometer</td>
<td>Slow increase</td>
</tr>
<tr>
<td>Ca++</td>
<td>Electrode</td>
<td>Decreasing</td>
</tr>
<tr>
<td>Mg++</td>
<td>Electrode</td>
<td>Increasing</td>
</tr>
<tr>
<td>Cl−</td>
<td>Electrode</td>
<td>Constant</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>Winkler</td>
<td>Constant</td>
</tr>
<tr>
<td>Mass suspended matter</td>
<td>Gravimetric</td>
<td>Data not available</td>
</tr>
<tr>
<td>Trace heavy metal</td>
<td>Atomic absorption</td>
<td></td>
</tr>
</tbody>
</table>

* Literature quoted in: Epifanio and co-authors (1973).

Organic substances accumulating near the filter surface usually contain phosphorous compounds such as phospholipids, nucleic acids, sugar phosphates and phytines (inositol phosphates of uncertain origin). Most bacteria produce phosphatases near their cell membranes, which facilitate rapid breakdown of all phosphorous compounds except inositol. In general, decomposition of phosphorous compounds is closely related in speed and efficiency to the mineralization of carbon compounds and nitrogen. Under specific conditions, precipitation of inorganic phosphates may occur; however, this does not seem to affect the filter. Heavy accumulation of inorganic phosphorous compounds and subsequent liberation of large amounts of soluble phosphate may lead to algal bloom in well-illuminated culture systems. Due to their fast decomposition and low initial concentration, phosphate compounds rarely become critical to the filter system's carrying capacity.
(c) Standards for Closed-culture System Design

Based on his experiments, SAEKI (1958) recommends several standards for design of a closed-culture system. The size of the culture enclosure should, in general, be about 10 times that of the volume of animals cultured, and the weight of the filter sand, about 30 times that of the animals. If specific gravity and interstitial coefficient of the sand are known, weight and volume of the water can be calculated. In large systems, turnover rate should be about 2 hrs; in small systems, about 30 mins. Ordinarily, the volume of air used for aeration should amount to about 2 to 3 times that of the circulating water. Speed of water movement through the filter should be about 100 to 700 m day$^{-1}$. According to SAEKI (1963), the approximate normal standard concentrations of nitrogenous compounds in the culture water are 0.3 ppm organic-N, 0.1 ppm ammonia-N, 0.1 ppm nitrite-N, and 10 to 300 ppm nitrate-N. In systems which do not conform to these standards, the carrying capacity must be calculated: Determine exchangeable ammonia per 10 g of sand and the dissolved ammonia in the water; find the ratio of exchangeable ammonia to total ammonia; multiply this value by 60. This pertains to a standard nitrogenous excretion of 50 mg day$^{-1}$ 100 g$^{-1}$ of animal.

For proper management of culture water, SAEKI (1958) recommends adjusting the aeration in such a way that dissolved-oxygen concentrations are maintained at near-saturation levels. Whenever the pH falls below 6 to 7 (or alkalinity below 0.4 meq. l$^{-1}$), lime or magnesia must be added. The standard amount of lime to be added is found by multiplying the gram-days (population weight $\times$ cultivation time) by 0.1/100 which gives the number of grams of lime l$^{-1}$ of water.

(d) Relationships between Metabolic Byproduct Concentrations and Culture-water Reuse

Investigating salmonid-hatchery water-reuse systems, LIAO and MAYO (1972) have developed models for assessing the relationships between metabolic byproduct (metabolite) release and water reuse. Their considerations are presented here in some detail.

In an open fish-rearing system, the metabolite concentration is $M/Q$ at the outlet:

\[
M = \text{release of metabolic byproducts from the fish, and } Q = \text{water flow. At the inlet and outlet of the rearing unit, the concentrations of metabolic byproducts are zero and } M/Q, \text{ respectively.}
\]

If the water inflow is reduced by half, and this half is reduced twice, and if the
metabolites released are completely mixed, the following flow-chart model is obtained:

At inlet and outlet, the concentrations of metabolites are $M/Q$ and $2M/Q$, respectively.

After incorporating a water-treatment process that removes 33% of the metabolic byproduct entering the treatment unit, the effluent metabolic byproduct concentration is $3/2 M/Q$ under steady state conditions:

Since Liao and Mayo (1972) have shown that the metabolite concentration in the rearing unit is a function only of the degrees of water reuse and water treatment, a general model can be derived:
where $C = \text{concentration of metabolic byproduct at outlet of rearing unit}$; $C = 1.0$ in an open system (single-use system); $E = \text{metabolic byproduct removal efficiency}$ of water treatment unit ($\%$); $M = \text{rate of metabolic byproduct release}$; $Q = \text{water-flow rate}$; $R = \text{percentage of water reused}$; $RU = \text{rearing unit}$; $WTU = \text{water treatment unit}$.

According to Liao and Mayo (1972), under steady-state conditions, the metabolic byproduct concentration in the mixture of incoming water and treated effluent should be equal to that at the rearing system inlet:

\[
\frac{(1 - E)CMR}{RQ + (1 - R)Q} = (C - 1) \frac{MR}{Q} \tag{1}
\]

i.e.

\[
\frac{(1 - E)CMR}{Q} = (C - 1) \frac{MR}{Q} \tag{2}
\]

i.e.

\[
CR - CRE = C - 1 \tag{3}
\]

i.e.

\[
C = CR + CRE = 1 \tag{4}
\]

i.e.

\[
C = \frac{1}{1 - R + RE} \tag{6}
\]

Equation (6) is considered important for predicting metabolic byproduct concentration in the rearing unit at any degree of water reuse and water treatment. A graph constructed on the basis of Equation (6) is illustrated in Fig. 2-95.

(e) Conclusions

The capacity of culture systems for supporting aquatic animals must receive critical attention before starting a culture experiment. Many investigators have conducted their research without ensuring that the culture system employed is indeed capable of adequately supporting the test organisms used and suited for pursuing the specific experimental project proposed.

Only a few pioneers have developed methods for assessing the carrying capacity of a culture system. Our present knowledge on dimensions and design of a closed sea-water system, concurrent with the aim to support a certain weight of animals and to offer certain environmental qualities, is based on four authors: Saecli (1958), 1963, 1965), Hiyayama (1965a, b, 1966a, b, 1970) and Liao and Mayo (1972). More work along these lines is urgently required.

The picture that has emerged thus far is still incomplete and the fundament not solid enough for qualified generalizations.
Even if all essential parts of a culture system are reasonably dimensioned, changes are unavoidable, e.g. in the weight of the animal population supported, in the amount or quality of food added, or in the environmental requirements of the growing animals. Modifications in animal food load, temperature, salinity, water-circulation speed or aeration intensity can induce significant alterations in the dynamics of the system, and render part of the original considerations meaningless. Consequently, the carrying capacity of a given culture system must be evaluated in the light of all essential changes anticipated during the experiment. Continuous monitoring of essential system functions and computer control are likely to become a must for many cultivation projects.

![Diagram](image)

**Fig. 2.95**: Relationships between metabolic by-product release and different degrees of water reuse and water treatment in a fish-rearing system. (After Liao and Mayo, 1972; reproduced by permission of Elsevier Scientific Publishing Company.)

(9) Equipment used in Cultivation

The list of equipment used in the cultivation of marine organisms is almost endless. However, a critical examination reveals that many types of equipment can be traced back to a few fundamental designs developed long ago. These have been modified and improved time and time again to suit individual requirements, to comply with financial capacities and to adjust to the limited space available. Some basic equipment has been 'invented' several times. Only in a few cases was it possible to solve problems of priority with certainty and to give credit to the original inventor.

The large diversity of apparatus used in cultivation precludes exhaustive documentation. We present here a selection of useful devices and interesting techno-
BASIC EQUIPMENT

logical solutions to general problems confronting the cultivator. Additional technological and methodological aspects are dealt with, in context with the type of organism cultivated, in Chapters 3, 4, 5 and 6.

(a) Basic Equipment

Collection of Organisms

Of the numerous types of equipment used for collecting aquatic micro-organisms, plants and animals (e.g. Welch, 1948; Schlieper, 1968), very few devices have been tested critically with regard to their performance and efficiency. Most collection equipment involves rough handling and often causes injury. Since healthy organisms are a basic prerequisite for successful cultivation, special attention must be devoted to obtaining intact culture material. Development of new equipment, especially designed for collecting organisms to be used in cultivation experiments, is highly desirable.

Two suction devices for careful collection of non-attached forms are illustrated in Figs. 2-96 and 2-97. They employ an airlift and a pump, respectively. The pump-driven device consists of a battery (enclosed in submersible housing) which powers a 6-volt submersible bilge pump. Water and specimens are drawn into a screened glass trap, in which the organisms collect. Comparable, but larger suction devices have also been operated from on board ships.

Small plankters can be collected from water samplers or pipes delivering raw sea water (airlift or other gentle means of water propulsion) using the aquarium arrangement shown in Fig. 2-98. Many motile organisms aggregate in response to baiting, light or other attractants and subsequently can be trapped, scooped up in a pail or caught by other gentle means. Attached forms have been collected successfully on a variety of substrates (glass, plastics, wood, stones, rocks, etc.) that had been exposed to in situ conditions to allow settlement. For collecting spores of benthonic algae, strings or nets are used.

Overflow Sieves

A number of devices have been developed to avoid loss of organisms through the overflow or drain of culture enclosures. Two small and easy-to-construct overflow sieves are illustrated in Fig. 2-99. A rotating overflow sieve with a revolving cylinder of fine mesh forces small organisms away from the entry of a drain (siphon) located near the cylinder axis (Fig. 2-100). This apparatus and the two overflow sieves mentioned above have been used in numerous modifications. It was not possible to determine the original inventors with certainty.

Aerators

In addition to the airlifts considered above, numerous different devices are used for aerating culture media (see also the section Aeration). Aerators can be classified according to the air pressure applied (normal-pressure versus pressurized-air aerators) or according to the aeration site (surface versus submersed aerators). For cul-
ture purposes, aeration by non-pressurized air has the advantage that exchange equilibria between air and water are established under normal pressure conditions. This simulates natural conditions and excludes the danger of supersaturation.

Fig. 2-96: Diver-operated, airlift-driven suction collector. (Photograph: Dr. G. Lauckner; reproduced by permission of Biologische Anstalt Helgoland.)

Aerators operated under normal air pressure include drippers, sprinklers, sprayers, splashes, cascades, and wave makers. These aerators divide the water into small portions or cause water-surface undulations. They all qualify as surface aerators. Several types simulate natural waterfalls. Injection of an air-water mixture through the water surface by aspirators may use either air of normal pres-
BASIC EQUIPMENT

Fig. 2-97: Diver-operated, pump-driven suction collector. (After Smith, 1973; modified; reproduced by permission of N. V. Boekhandel & Drukkerij voorheen E. J. Brill, Leiden.)

Fig. 2-98: Aquarium arrangement for gentle collection of plankters. (Original).

Unfiltered sea water

to waste

Sure or pressurized air. Practically all submersed air distributors employ pressurized air, released via air stones or other porous material, perforated pipes, etc., which distribute air bubbles into the surrounding water. Substantial air pressures may modify the normal exchange equilibrium between air and water.
CULTIVATION OF MARINE ORGANISMS (O. KINNE)

Fig. 2-99: Overflow sieves. Two designs operated at the intake end of the culture tank’s overflow. The sieve consists of perforated plastic. For retaining very small organisms, the sieve is surrounded by spongyous material. (Original.)

Fig. 2-100: Rotating overflow sieve. (Original.)

A few aerators, which are less well known than the common air stones, perforated tubes or plates and related basic aquarium equipment, receive brief attention below.

Among the surface aerators, multi-step cascaders require more space than single-step cascaders. The aeration efficiency of the latter type can be substantially improved by employing a pipe-lattice cascade (WINGRICH, 1968a, b; ALBRECHT,
Splashes are used for fishpond aeration as air propellers (e.g. Vejvoda, 1973a), rotors (e.g. Vejvoda, 1973b) and related equipment (Fig. 2-101, a-c).

Aspirators usually consist of a pipe tee with an air pipe, through which the flowing water sucks air in, or through which pressurized air enters. Aspirators drive fine air bubbles to water depths of several metres. In the aspirator described by Vincent (1908), water flow and air pressure are adjusted by screws (Fig. 2-101d). Air bubbles can be sent down to a depth of about 4 m. The aspirator can be installed in the water
supply pipe. For salmon rearing, Burrows and Combs (1968) recommend series of aspirators that drive well-aerated water down to a water depth of 1.5 m (Fig. 2-102). Each aspirator has a 1.9-cm air pipe inserted in a 3.8-cm pipe tee, and is designed to deliver about 4701 min⁻¹ at 0.07 atm; this pressure must be maintained, in order to achieve the necessary velocity past the air pipe intake. Each of these aspirators requires 0.4 m² of surface area. For maximum efficiency, the outlet pipe should be about 15 cm above the water surface.

Aeration may cause problems if the tanks are located far away from public electricity sources. Petrol-driven compressors or cylinders of compressed air are usually employed in such situations. Where compressors cause a noise nuisance, compressed-air cylinders are preferred. If these have to be transported across difficult terrain, reduction of weight and increase in efficiency become important. Abram (1971) has constructed an apparatus which achieves substantial savings in air and transportation costs. While standard compressed-air cylinders are charged to a pressure of about 180 atm, fish tanks are usually aerated at about 0.2 atm. Hence, a large portion of the energy used to charge the cylinders is wasted at the pressure-reducing valve. Abram's apparatus utilizes some of this energy in an expansion engine which aerates the tanks at about 0.2 atm. The total volume of air obtained for tank-water aeration is 5 to 6 times that available when an air cylinder is connected direct to the aeration system.

Aerators employed in waste-water treatment have been reviewed by Eckenfelder and O'Connor (1961). Three basic types of aerators are commercially available: (i) Small orifice dispersers that consist of porous materials, or of plates or tubes constructed of silicon dioxide or aluminium oxide grains held in a porous mass with a ceramic binder. Competitive units include saran or nylon-wrapped tubes or bags. (ii) Mechanical or air-shear units, such as the impingement or jet aerator. (iii) Large orifice dispersers such as the sparjer and disfusser. The sparjer contains short tube orifices from which air is emitted at high velocity; water turbulence tends to redivide large air bubbles into smaller ones. The disfusser emits air from around the periphery of a disc. Additional types of recently developed air dispersers include: (i) the hydraulic shear disperser that discharges air into a box.

![Aspirator assembly](image)

Fig. 2-102: Aspirator assembly. (a) Design details of a single aspirator; (b) culture tank with 5 aspirators. (After Burrows and Combs, 1968; modified; not copyrighted.)
through an open pipe; the rising air-water mixture and the downward water flow create turbulence and hydraulic shear which break large bubbles into small ones; (ii) the Venturi disperser in which an air-water mixture is accelerated and discharged; (iii) the Inka system consisting of grating pipes, perforated on the underside, and mounted 0.76 m below the surface on one side of a longitudinal vertical baffle.

A new method for maximum contacting between air and water has been described by DERINGER (1970). It employs a gas-liquid mixer which produces air bubbles of constant size. For sludge aeration, DERINGER proposes a new counter-current process. Mechanical surface aerators and ejector aerators used in large-scale water treatment plants have received attention from SCHUSTER and PÜSCHEL (1969) and KRAUSE (1971), and surface aerators for sewage treatment from RÜB (1971).

![Efficiencies of various aeration methods at 20°C.](image)

ENGELBART (1969) developed and tested line aerators to be used in lagoons serving sewage treatment and sludge stabilization.

The performance and efficiency of aerators have been evaluated, for example, by LANGELIER (1932), MORGAN and BIEWTRA (1960), BAERS and MUSKAT (1962), PASVEER and SWEERIS (1962), GLOPPEN and ROBER (1965), SCHERB (1965), VAVRUSKA (1971), POON (1972) and SCOTT (1972b). VAVRUSKA (1971) tested aerators used in Czechoslovak fish cultures and measured the resulting oxidation capacity. SCOTT (1972b) determined aeration efficiencies of venturis, air stones and sprayers in a 1.8 m³ tank. Maximum efficiency was obtained with venturis discharged through a nozzle near the tank bottom; air stones were 53% as effective;
sprayers directed at the surface were 41% as effective. The Biological Station at Nanaimo (Canada) uses venturis with success in its main sea-water system, maintaining the intake O₂ level at 90 to 92% saturation (Alders, personal communication). For efficiencies of further aerators consult Fig. 2-103.

Fig. 2-104: Airlifts, operated by a series of single large air bubbles (left) and by air stone dispersed air (right). The latter design has a greater water-transport efficiency. (Original.)

**Airlift**

The principle of lifting water in a tube by air injection was invented in the nineteenth century by the German mining engineer Carl E. Löschner. It is based on the weight equilibrium of fluids in communicating vessels, in which the fluids rise to different heights, depending on their density. If a tube is placed vertically in water, and air is injected from below, an air-water mixture is produced in the tube that is
lighter than the water and hence, rises to a certain height. In tubes shorter than the maximal height attained, the air-water mixture overflows, and water is transported. The water-transport efficiency of this air-driven water pump, airlift pump or simply, airlift, depends on the amount of air, bubble size, tube diameter and the ratio transport: height (t): submersion depth (s) of the tube. At a given bubble size and tube diameter, the water-transport efficiency increases with the amount of air, and with a decrease in the ratio t:s. The airlift principle has also been employed for transporting solid bodies contained in a fluid (e.g. sand, gravel, dead fish). The general importance and usefulness of airlifts in cultivating aquatic organisms has attracted much attention (HOEFER, 1913; OWENS, 1921; WARD and KESSLER, 1924; SACHS, 1929; HAGMEIER, 1933; O'BRIEN and GOSLINE, 1935; NEEDHAM, 1937; WIEDEMANN, 1943; Datta, 1948; HUCKSTEDT, 1963a; PLEBSIS, 1964; KRÜGER, 1966; JEBRAM, 1970, 1973; SPOTTE, 1970; KING and KELLEY, 1971; KNOESCHE, 1971).

Typical airlifts used in cultivation are illustrated in Fig. 2-104. According to KRÜGER (1966), maximum lift height is achieved if air bubbles enter the lift tube (5 mm diameter) well above its lower end (compensation for recoil effect). HAGMEIER (1933), SPOTTE (1970) and KING and KELLEY (1971) stress that the water transport capacity is greater if the air entering the lift tube is well dispersed (e.g. by an air stone in small systems or by perforated PVC baffles in larger systems). SPOTTE'S estimations of airlift capacities as a function of design are listed in Table 2-36.

Temporary trapping of ascending air in a tilted glass cup (GUNDELACH in: HAGMEIER, 1933; BÜCKLE in: JEBRAM, 1970; JEBRAM, 1973) produces large air bubbles which are released at intervals and reportedly have high water-transport capacities (Fig. 2-156, bubble cup).

In algal cultures, an airlift circulator has been used for producing laminar water flow (SALSER and MOCK, 1973). The device (Fig. 2-105) facilitates directional water circulation and even distribution of nutrients. With an air flow of 81 min⁻¹, at an air pressure of 0.98 kg cm⁻², the water in the tank (90 x 58 x 60 cm) is circulated at a rate of 500 l min⁻¹. The deflector at the top of the circulator (a section of plastic pipe) determines the flow direction. To achieve circulation at lower water depths, a second deflector is placed into the next lower discharge slot. Skeletonema sp., Thalassiosira sp. and Cyclotella sp. are examples of algae successfully mass cultured with this apparatus. In addition, the circulator has been used for culturing larval and post-larval penaeid shrimp.

Airlifts tend to be troublefree and, in general, seem to represent a lesser mechanical hazard to many plankters than most other water-transport methods (propeller, rotor, paddle-wheel, pump).

**Float Valve and Constant-level Siphon**

A simple float valve, described by BREDER (1964), consists of a valve (or glass stopcock), a suitable-sized chemical flask, a 1-hole cork and some additional parts of plastic or wood (Fig. 2-106). Serving similar ends, but more complicated in construction, is the float-actuated sleeve valve designed by LICKLEY and co-authors (1970). BREDER has also developed a simple constant-level siphon composed of some
Table 2-36

Airlift capacities as a function of design dimensions (side air-inlet method) (After Spotte, 1970; modified; reproduced by permission of Wiley, New York)

<table>
<thead>
<tr>
<th>Pipe diameters</th>
<th>Water-transport capacity (lift tube submerged)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lift tube</td>
<td>40%</td>
</tr>
<tr>
<td>cm</td>
<td>l min⁻¹ *gal min⁻¹</td>
</tr>
<tr>
<td>cm</td>
<td>in.</td>
</tr>
<tr>
<td>2.54</td>
<td>1</td>
</tr>
<tr>
<td>3.17</td>
<td>1 ½</td>
</tr>
<tr>
<td>3.81</td>
<td>2</td>
</tr>
<tr>
<td>5.08</td>
<td>2</td>
</tr>
<tr>
<td>6.35</td>
<td>2 ½</td>
</tr>
<tr>
<td>7.62</td>
<td>3</td>
</tr>
<tr>
<td>8.89</td>
<td>3 ½</td>
</tr>
<tr>
<td>10.16</td>
<td>4</td>
</tr>
<tr>
<td>11.43</td>
<td>4 ½</td>
</tr>
<tr>
<td>12.70</td>
<td>5</td>
</tr>
<tr>
<td>15.24</td>
<td>6</td>
</tr>
</tbody>
</table>

* US gallon = 3.785 l
Fig. 2-105: Airlift circulator for algal and invertebrate mass cultures. (After Salser and Mock, 1973; reproduced by permission of the World Mariculture Society.)

Fig. 2-106: Float valve and constant-level siphon control in and outflow of water in a flow-through aquarium. (After Breder, 1964; modified; reproduced by permission of the author.)
straight glass tubing, a tee, flexible plastic tubing and two plastic supports (Fig. 2-106, right). A comparable device is illustrated in Fig. 2-107.

Interaction of float valve and constant-level siphon controls the water-level height in a flow-through aquarium. Flow speed can be adjusted by changing the diameter of the siphon tubing. Water-level height can be adjusted by varying the height of the horizontal tee arm.

Fig. 2-107: Constant-level siphon. (After Haagmeier, 1933 and Flecher, 1988; modified; reproduced by permission of VEB Gustav Fischer, Jena.)

The constant-level siphon becomes foolproof if the open arm which faces upward is extended by a piece of flexible tubing bent over to form an inverted U (with the open end facing down into the aquarium and ending slightly above the water level). As the water level in the aquarium rises, it occludes the open end of the inverted tube, and the constant-level siphon converts into a normal siphon with two inlets; this increases the flow rate considerably (the amount of increase being related to the length of the drainpipe attached to the horizontal tee outlet). As a consequence, the water level in the aquarium falls rapidly, and the end of the inverted-U tube is
re-exposed, with the system regaining its constant-level feature. If the water inflow is slightly higher than the constant-level siphon will carry off, the water level in the aquarium will be at the mouth of the open tube, and the tube will suck water and air from the surface through a large inflow range. This arrangement removes scum forming on the water surface.

Related equipment with a wide range of applicability are capillary priming siphons (p. 246).

Culture-tank Cleaner

A suction device for cleaning culture tanks (Vincent, 1908) is illustrated in Fig. 2-108. With the piston valve and lower end of the drainage tube below the water
level in the container to be cleaned, the bulb is squeezed with one hand and the piston valve pressed with the other. Placing the pipette tube in the water, first the bulb, and then the piston valve are released, thus starting the siphon. The water flow can be regulated by the piston. Vincent also developed and illustrated a comparable device for cleaning culture ponds. For aquaria, numerous different cleaners that work on the same principle are commercially available.

**Flow-direction Reverser**

Breder (1964) illustrates a pipe valve device which allows water flow rates to be changed from maximum in one direction through zero to maximum in the opposite direction (Fig. 2-109). With valves A₂ and B₁ closed and the others open, maximum water flow leaves pipe A and enters pipe B (arrows). If A₁ and B₂ are closed and the other two open, net water flow is reversed. To pass slowly through a state of no flow to reverse flow, either A₂ or B₁ is opened gradually. This reduces the speed of water movement because of 'back leakage'; after one of the 2 valves has been opened fully, the opening of the other valve further retards net flow; when it too has been fully opened (all valves fully open), there should be no flow through pipes A and B. By beginning to close either A₁ or B₂, the flow begins to reverse; when these two are fully closed, maximum flow in the opposite direction is reached.

**Equipment for Controlling Environmental Factors**

Control of environmental factors is essential for providing definable and/or reproducible culture conditions. An overview of the long list of equipment available for controlling environmental factors reveals: (i) The period of improvisation and make-shift has given way to engineer-designed, commercially available equipment of high
performance and reliability; (ii) control of the important factor light has received insufficient attention; (iii) control of dissolved gases and of organic substances has been sadly neglected.

Accounts on environmental factors have been presented in Volume I. In the following pages, a few examples are presented of apparatus employed for controlling some important environmental factors, especially under conditions of running sea water.

Light

No artificial light source is entirely satisfactory. Most cultivators have employed illumination levels far above those normally encountered in the natural habitats of the organisms investigated. In several marine organisms, natural light may be a prerequisite for normal development. Hence, ecologists interested in the normal performance of their culture organisms must conduct experiments under natural light conditions (wavelength, intensity, direction, diurnal and annual light rhythms). Some animals such as planktonic fish larvae seem to be able to catch their prey with sufficient accuracy and efficiency only when the light rays enter the culture enclosure from a proper direction (usually from above) and at adequate illumination levels.

Some properties of light sources used in cultivation have received attention by Carr (1970) who briefly considers incandescent lamps, fluorescent lamps and discharge lamps.

Among the incandescent lamps, tungsten bulbs give rather continuous broad emission spectra, but these are richer in long wavelengths than sunlight, and have no ultra-violet components. Tungsten lamps produce considerable irradiance in the near-infrared (800-1500 nm) with maximum emission near 900 nm (Fig. 2-110). The exact location of the maximum depends upon the operating temperature of the lamp which, in turn, is a function of the voltage applied. Hence, the illumination characteristics obtained from a 50-W bulb differ from those received from a 500-W
CULTIVATION OF MARINE ORGANISMS (O. KINNE)

2.

bulb. With increasing wattages and operating temperatures, the emission spectrum moves toward the ultra-violet. Since the visible flux of a tungsten bulb decreases with time until the filament burns out, it is necessary to regularly check the illumination actually produced. Tungsten bulbs irradiate over 80% of the input energy as heat. This disadvantage must be compensated for by dissipation or absorption (water filter).

In fluorescent lamps the spectrum, in contrast to natural sunlight, consists of a number of discrete emission bands. In general, these bands do not coincide with the wavelengths of maximum absorption by chlorophyll. ‘Warm White’ fluorescent lamps are often considered more adequate for cultivation purposes than ‘Daylight’ or ‘Cool White’ because they generate less ultra-violet. Emitted by a low-pressure mercury lamp, the ultra-violet produced in fluorescent lamps is absorbed by phosphors (mostly inorganic salts and oxides) within the tube that fluoresce at longer wavelengths. Some control in light quality is possible since variations in the proportions of the different phosphors lead to light of different emission characteristics. In ‘Warm White’ lamps, the emission spectrum shifts toward the longer wavelengths in comparison with the ‘Cool White’ fluorescent lamp. Infrared radiation is absent. In contrast to tungsten bulbs, most energy is dissipated by convection and conduction. Hence, fluorescent lamps cause considerably fewer heat problems, and are usually preferred by cultivators.

Discharge lamps produce ultra-violet, visible and infrared light. A large variety of discharge lamps are available, operating at high or low pressures, and with electrodes of different materials. Discharge lamps can deliver narrow bandwidth light. Carr (1970) discusses the production of defined-wavelength light with the aid of grating monochromators or self-absorption and interference filters, as well as the control of the amount of radiant energy produced (see also Withrow and Withrow, 1956; Altman and Dittmer, 1966).

Light and temperature

Numerous light- and temperature-controlled rooms, cabinets or water baths have been designed and successfully tested. A convenient light-temperature-controlled cabinet for unicellular algae cultures can be constructed in Handy Angle or Dexion, faced with 3.8-cm-expanded polystyrene boards (Droop, 1969). The cabinet is illuminated from below through a double-glazed base. A domestic refrigerator replacement unit maintains temperature levels between 5° and 27°C with 160-W fluorescent lighting 30 cm below the base. Illumination from the side (as in many commercial cabinets) cannot provide equal illumination to all cultures. Temperature control is effected by a mercury–toluene thermometer which, for trouble-free operation over long periods, should control the relay by interrupting a light beam rather than an electrical circuit directly (although satisfactory results can be obtained if the interrupted current is limited to a few hundred micro-amperes). In either case, Droop recommends that the mercury be covered by medicinal paraffin to protect the meniscus.

A rubber-glove reach-in cultivation cabinet for long-term use (2 independent, alternately operating cooling–heating systems) with controlled, fluctuating (diurnal, annual) light and temperature conditions has been constructed by Kinne. Based on the original version (Kinne, 1958a), a more advanced model was devel.
BASIC EQUIPMENT

199

oped in 1964 (Kinne, unpublished). Daily temperature and light schedules are programmed onto the periphery of rotating, exchangeable plastic discs (1 rev. in 24 hrs) and transferred into the control system by contact feelers riding on the rotating disc's periphery. Culture dishes on perforated boards can be handled and observed through a microscope without removing them from the cabinet. The cabinet has been used for cultivating the cnidarian *Cordylophora caspia*, as well as a variety of snails, amphipods and copepods.

For maximum control of environmental factors, the aquarium of the Zoological Museum at the University of Nancy (France) is equipped with electronic light and temperature controls (static relays and thermostats) for individual aquaria, linked to a general control panel (Terver, 1972). Based on the automatic control system developed at the Nancy Aquarium, Genet and Brachotte (1972) describe individual control modules and the use of a thermistance probe (electrical resistance as function of temperature). The control panel, corresponding to the layout of the whole aquarium, receives information from the individual modules, and relays a visual or acoustic alarm to different 'look-outs'.

Temperature

For thermal adjustments in running sea-water systems, the principle of heat exchange has been used with much success. A large variety of heat exchangers are commercially available (e.g. Carbone equipment).

A temperature-control system that delivers constant-temperature (±0·5°C) running sea water to a number of aquaria simultaneously (11 min⁻¹ to each aquarium) was developed by Moore and Gray (1969). The system was planned to: (i) deliver water with temperatures ranging from 2°C to 40°C (using incoming water of 21°C to 31°C); (ii) use raw sea water that contains silt, plankton and other objects which may cause clogging; (iii) incorporate no materials which might be toxic or corrosive; and (iv) run for a year or more with minimum maintenance. The system supersedes earlier designs (Moore and Sanchez, 1967; Moore and Gray, 1968a, b), and employs mixing valves. An apparatus which provides cycles of warmed and cooled water, and which can be programmed for any desired plan, has been described by Nourissin and Veillet (1963).

The temperature-control system for recirculation units designed by Scott (1972a) includes heating, refrigeration, filtration and aeration devices on a caster-mounted frame. Heating (electric resistance) and cooling (hermetic refrigeration compressor) are performed in a stainless steel combination cooler-heater unit. Temperatures can be maintained within ±0·06°C between 2°C and 20°C in 900-l tanks, using a modulating electronic temperature controller. The system is compact and easily movable and has been used for cultivating rainbow trout *Salmo gairdnerii* over an 8-month period (Scott and Gillespie, 1972).

An inexpensive system for providing temperature-controlled, filtered, running sea water (Tenore and Huguenin, 1973) features a Ti heat-transfer panel for water-temperature regulation. The water is filtered by a combination of filter bags and cartridges of various porosities (resulting in 1 μm-filtered sea water with particulate C concentrations of less than 30 μg l⁻¹). The temperature-regulated and filtered water is pumped into an insulated header tank for distribution to experi-
mental trays. Alarm systems are installed for reporting excessive temperature and water-level fluctuations.

Additional information concerning temperature control of culture systems has been published, for example, by Loosanoff (1949), Tighe-Ford (1967a) and Lickey and co-authors (1970). For exact temperature control, water baths are ideal. A large variety of excellent equipment is now commercially available.

Salinity

Salinity control in running sea water is not easy to achieve. The system developed by Moore and Gray (1970) requires a source of sea water, of fresh water and of high salinity water (produced in a glass still). It consists of: (i) a mixing valve for blending water from any of the three sources in desired proportions, (ii) an inductance cell through which the water flows from the mixing valve, and (iii) a Beckmann salinometer and relay system that measures the output of the cell and controls the mixing valve so as to maintain a predetermined mixture. The control system delivers 11 min⁻¹ of water with salinities from about 0% to 35%. It should be possible to insert a differential gear in the salinity shaft and to power this from the torque amplifier with the aim of controlling the system by a programmer. Water of reduced salinity can best be prepared by dilution with uncontaminated rain water.

Fig. 2.111: Plunger jars. Water movement is produced by slowly raising and lowering a plate in the culture container. The counter-balance vessel is periodically emptied by siphoning (a) or by tipping (b). (After Browne, 1898; modified; reproduced by permission of Marine Biological Association of the U.K.)
Where rain water is not available, aged tap water or glass-distilled water are acceptable. Chlorinated tap water should be aerated before use for 3 (20° C) or 4 days (10° C) in order to remove excess chloride.

**Water movement**

Water movement is of basic ecological importance to aquatic life (Volume I: Riedl, 1971a, b; Schwenneke, 1971). In culture systems, water movement is the key to water treatment (p. 100). The equipment employed for generating water movement depends on system size and type of water movement to be created. Common equipment comprises aerators (p. 183), shakers, rotators, stirrers and paddle devices.

*Fig. 2-112: Water-movement generation by shaker, schaukel and piston-pump.*

(After Svoboda, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)

In small culture systems with capacities in the millilitre range, water movement can be produced by temperature gradients, evaporation or capillary forces. In systems containing up to about 1 l of culture medium, simple methods for producing water movement are: (i) to allow single air bubbles to ascend at intervals (e.g. 1 or 2 secs) to the surface; (ii) to create slow pressure fluctuations, e.g. with a rubber ball attached to a pipette communicating with the culture medium (e.g. Hannerz, 1952/53); by alternately depressing and releasing the rubber ball, the pipette rhythmically pours out and sucks in water; (iii) gentle stirring, e.g. by slowly rotating glass rods (e.g. Anthony, 1910; Hagmeier, 1933).

In medium-sized culture systems with capacities between one and several thousand litres, a great variety of equipment has been used for creating water movement. Numerous examples can be found in this and other chapters of the present volume.
A classical technique is the plunger jar (Brown, 1897). It was originally used to keep plankters afloat by gentle water movement, in the absence of bubble aeration. A glass plate with a centre hole is slowly raised and lowered, traditionally by a counterbalancing container, which is periodically filled with water and emptied by siphoning (Fig. 2-111a) or tipping (Fig. 2-111b). The advantage of the plunger jar is its simplicity; its disadvantages lie in the potential damage which may be caused to delicate forms, as well as in the intermittent turbulence caused. Later systems of vanes and paddles moving in the water share the plunger jar's tendency to damage delicate organisms (e.g. Rice and Williamson, 1970). Examples for producing water movement by flow-through techniques have been provided in the section devoted to open sea-water systems.

Shaker, schaukel and piston-pump are illustrated in Fig. 2-112. A space-saving design with stacked boxes is illustrated in Fig. 2-113. The schaukel consists of a long container, forced into slow up-and-down motions by an eccentric cam. Svoboda (1970) found that shaker and schaukel produce water-movement rhythms which are too fast for cultivating the hydroid Aglaophenia plum. Hence, he simulated slow, oscillating water movement in a piston-pump system. In this system, an eccentric cam moves a piston up and down in a cylinder, forcing culture water through a Plexiglas tube in and out of a compensation cylinder. The piston is 60 cm long, contains 50 l water and displaces 40 l of culture medium during maximum stroke length. A pumping cycle lasts 7 secs and produces a maximum water movement velocity of 160 cm sec\(^{-1}\). The velocity can be reduced down to 70 cm sec\(^{-1}\) by shortening the length of the piston stroke. The Plexiglas tube serves as culture enclosure; it is partitioned at both ends by horizontally and vertically arranged Plexiglas plates, in order to avoid undesired turbulences. In the compensation cylinder, these plates can be removed to allow access to the tube. Piston and cylinders are constructed from PVC. Friction and turbulence cause a heating effect (3 C° above room temperature) which must be counteracted through cooling.

Rotators and combinations of rotators and shakers have been used with much success for culturing a variety of marine organisms. In most cases, it turned out to be essential that the rotations be slow. All devices designed and tested enhance both water movement and gaseous exchange. An example of a rotator used for zoo-
plankton cultivation has been provided on p. 233. For cultivating protozoans, Hjelm (1970) developed the culture-bottle rotator illustrated in Fig. 2-114. In this rotator, exponential population growth of *Tetrahymena pyriformis* continued up to about $10^6$ cells ml$^{-1}$. The culture container is centred and fastened on plate $P$, carried by the axles $A$ and $A_1$, which are rotated by a motor (not shown). Rotation speed can be varied between 50 and 400 rpm (normal speed: below 300–350 rpm). The rotator is positioned above a thermostated water bath, and the temperature of the cultures is controlled by pumping water from the bath onto the container through tap $T$. A transparent bottomless plastic box with slits for the axle $A_1$, and for the neck of the container, surrounds tap and container (prevention of excessive water splashing). Air is led into the container through glass tubing in the stopper, which is connected by rubber tubing to the stainless steel tubing $U$ that rotates inside a similar tubing $V$ (connected to the air pump).

![Fig. 2-114: Rotator for protozoan cultures.](image)

Fig. 2-114: Rotator for protozoan cultures. $A$ and $A_1$: axles carrying plate $P$ to which the cultures are attached; $R$: ring-shaped plate; $S_1$: screws holding $P$ and $R$ together; $S_2$: centring screws; $S_3$: screw for fixing position of $A_1$ inside $A$; $T$: tap; $U$ and $V$: stainless steel tubings leading air into the culture. (After Hjelm, 1970; modified; from *Experimental Cell Research* 60, p. 192; reproduced by permission of Academic Press.)

Responses of gammarids to different ambient oxygen concentrations under varying conditions of water movement were observed in the apparatus described by Vobis (1973). The apparatus makes use of the laboratory-stream technique (Fig. 2-115). Water of defined oxygen content is stored in Plexiglas cylinders, and enters the screened animal chambers through dosimeter pumps.

Laboratory streams—as models of small water currents—have been employed by several experimental ecologists. Most studies were concerned with limnic organisms (e.g. Fabricius and Lindroth, 1954; Odum and Hoskin, 1957; Kalleberg, 1958; Ambühl, 1961; Zimmermann, 1961; Warren and co-authors, 1964; Davis and Warren, 1965; Eichenberger and Wührmann, 1966; Brocksen and co-authors, 1968). Objectives, possibilities and constraints of laboratory-stream research have been reviewed by Warren and Davis (1971); the equipment used has received attention from Luther and Mauer (1963), Lauff and Cummins (1964), Whitford and co-authors (1964), Hartman (1965), Gee and Bartnik (1969), Ziegelmeier (1969) and Warren and Davis (1971).
Luther and Maier (1963) developed a small, paddle-wheel-driven laboratory stream (Fig. 2-115a) for orientation experiments on marine invertebrates. This apparatus was rebuilt and modified by Ziegelmeier (1969), who investigated current-dependent tube-building activities in marine polychaetes (Fig. 2-115b).

Fig. 2-115: Small laboratory streams. a: Top view. (After Luther and Maier, 1963.) b: Side view. (After Ziegelmeier, 1969; both reproduced by permission of Biologische Anstalt Helgoland.)

A similar but larger laboratory stream (Fig. 2-116) has been used by Warren and Davis (1971). The laboratory stream of Feldmeth (1970) provides relatively large rearing areas and can be operated with different current speeds. The compact design allows rapidly flowing water courses to be set up in a rather small laboratory area.

In large culture systems with volumes exceeding several thousand litres, pumps and gravity feed are most commonly used for producing water movement. Several
Fig. 2-116: Laboratory stream designed as semi-open system with flow meter and variable-speed motor. (After Warren and Davis, 1971; modified; reproduced by permission of Annual Reviews Inc.)

Fig. 2-117: Thermal gradient plate for cultivating small organisms. (After Thomas and co-authors, 1963; reproduced by permission of American Society of Limnology and Oceanography, Inc.)

Aquaculture farms employ aeration (p. 145) as a means to secure both the necessary oxygen supply and adequate water movement. Numerous makes of sea-water inert pumps are now commercially available and so are highly efficient, large-size aeration units.
Dissolved gases

Running sea water with constant concentrations of dissolved oxygen can be obtained from an apparatus described by Hicks and Dewitt (1970). The apparatus discharges streams of water controllable within 0-2 mg O₂ l⁻¹ over a wide range of oxygen concentrations. Individual streams can be maintained at any flow rate between 175 and 350 ml min⁻¹. The system can be used for running-water-toxicity bioassays, in which constant dissolved-oxygen conditions are desired. Scott (1971) monitored fish-tank oxygen concentrations with a digital recorder, and Peak and co-authors (1971) designed a simple, automatic servo-mechanism for controlling oxygen tension in aquaria.

Environmental gradients

Environmental gradients (e.g. light, temperature, salinity, chemical substances), in which the organisms studied develop differentially according to their requirements or in which they are allowed to select their zone of preference, have been designed by Shelford and Alle (1913), Herter (1925, 1934), Höglund (1951, 1961), Zahn (1960), Jansson (1962), Ganning and Wulf (1966), Staaal (1969), Gottwald (1970), and Dusenberg (1973). For a review on various types of gradient equipment used in the study of fishes consult Fry (1960). Preference experiments yield preliminary information on environmental requirements and are important for designing proper culture conditions.

Thermal gradient plates for cultivating bacteria (e.g. Oppenheimer and Drost-Hansen, 1958; Cannfax, 1962; Landman and co-authors, 1962), algae (e.g. Eppley and McClasr, 1962), and bacteria, algae and foraminifers (Thomas and co-authors, 1963), as well as other small organisms, consist of a metal block with holes for accommodating culture tubes, and have a provision for cooling one part of
the block and heating the other. An example is illustrated in Fig. 2-117. Cold water enters the left end of the aluminium block, warm water the right end.

A light-temperature gradient plate has proven useful for determining combined light and temperature effects on cultured algae (HALLDAL and FRENCH, 1956, 1958; EDWARDS and VAN BAALLEN, 1970; VAN BAALLEN and EDWARDS, 1973). Originally described by EDWARDS and VAN BAALLEN (1970), the plate can be used to accommodate small Petri dishes or other culture vessels. The plate is of aluminium, $14 \times 16 \times 0.76$ mm, and insulated with styrofoam on the bottom and along the edges. Cooling and heating baths are connected to either side of the plate via rubber tubing. The temperature gradient is controlled by thermocouples, which regulate the temperature in the cooling and heating baths. Illumination can be varied over a limited range in wavelength and intensity by choice of the fluorescent lamp phosphor (p. 198) and its distance from the front edge of the plate. Daylength can be programmed with an interval timer. Examples of experimental results obtained with the light-temperature gradient plate are illustrated in Fig. 2-118a and b.

Horizontal gradients of dispersants have been produced by WILSON (1974) in a ‘fluvarium’ which generates a stepwise gradient at right angles to the direction of water flow. WILSON’s apparatus, modified after HÖGLUND (1961), has been used for examining the ability of herring and plaice larvae to avoid oil dispersants.

**Small Recirculation Devices**

A small continuous recirculation tube for growing hydroids (e.g. *Bougainvillia muscos*) has been used by BROWNE (1907). Installed in a bell jar, the device consists of a glass tube (32 mm diameter, 200 mm length) from which water is continuously...
withdrawn by an airlift. The resulting water current facilitates metabolic exchange and provides contact with food organisms (e.g., copepods) added to the culture water (Fig. 2-119). Where required, the assembly could easily be combined with a sand–gravelp filter.

Sorceloos and Persoone (1972) tested and described three recirculation devices for cultivating a variety of aquatic animals (protozoans, rotifers, crustaceans, and larvae of molluscs and fishes): a single-cylinder recirculator and two double-cylinder recirculators.

![Fig. 2-120: Single recirculator. (After Sorceloos and Persoone, 1972; modified; reproduced by permission of Springer-Verlag.)](image-url)

The single-cylinder recirculator (Fig. 2-120) consists of a glass cylinder with an airlift. Continuous recirculation of the culture water prevents sedimentation of cultivated organisms and food particles. Recirculation speed can be regulated via air-inflow volume and height–diameter ratio of cylinder and lift tube.

The double-cylinder recirculator without accessories has an internal glass cylinder with plankton gauze glued to its bottom, which prevents the animals cultivated from passing through the lift tube (Fig. 2-121). The internal cylinder (animal compartment) can be removed, facilitating collection or transfer of the
animals. Particulate wastes can be drained via a stopcock. An overflow allows replenishment or renewal of the culture medium during the experiment.

The double-cylinder recirculator with accessories features a lateral siphon drain and a water-collecting tube (Fig. 2-122). The siphon drain can be operated with stopcock 1 closed, and a given amount of water siphoned off through stopcock 2; with stopcocks 1 and 2 open, the drain becomes a constant-level siphon (p. 191) lowering the water level from a to b. The water-collecting tube causes effects comparable to the ‘wave maker’ mentioned on p. 229. Water is lifted through the

![Diagram of double-cylinder recirculator](image)

Fig. 2-121: Double-cylinder recirculator with animal compartment, overflow and bottom drain. (After SOROELOOS and PERSOONE, 1972; modified; reproduced by permission of Springer-Verlag.)

lift tube into the wider collecting tube until it reaches level c; then siphoning begins, rapidly emptying the tube to level d. The sudden water flow creates a current which cleans the gauze bottom and ensures intermittent, thorough mixing of the culture water. (The most efficient gauze-bottom cleaning can be affected by back-washing.) Air-flow rate and dimensions of elevator and collecting-tube parts determine the length of the time intervals between successive discharges and the water mass involved. SOROELOOS and PERSOONE (1972) drained about 1/10 of the culture-cylinder volume every other minute. The device can be supplied with fresh culture medium by electromagnetic valves, multi-channel pumps, or Mariotte flasks.
Many aspects of population ecology and the dynamics of multispecies cultures and microcosms (Chapter 6) can be studied more adequately in continuous-flow cultures than in batch cultures (see also Chapters 3, 4 and 5). In batch cultures, environmental and nutritive conditions tend to change quickly and extensively; the changes incurred are often unpredictable and largely uncontrollable. Only in continuous cultures can the organisms be maintained under predetermined, feedback-controlled circumstances.

Continuous-culture Devices

Continuous-culture devices consist of: (i) a culture enclosure; (ii) equipment for delivering and removing culture medium (nutrient supply, dilution and removal of metabolic products) and for reducing the population density of the organism cultured; (iii) a mechanism for thorough mixing of culture-container contents; (iv) systems for controlling culture volume, temperature, gaseous contents of the culture;
water, water movement, etc. The essential components of a continuous-culture device are illustrated in Fig. 2-123.

Continuous-culture conditions can be provided (i) by using a constant-volume device and maintaining a constant culture-medium flow, thus allowing the population density to equilibrate (chemostat); (ii) by regulating the medium flow so as to obtain a constant population density (turbidostat).

Continuous cultures usually contain organisms in randomly different states of development. Where material of an identical physiological state is desired, syn-
chronization becomes necessary (see also Chapter 4.1). Synchronization can be effected in two ways: (i) An originally random population is subjected repeatedly to a timed environmental stress that initiates, promotes or slows cyclic processes. (ii) Certain developmental stages (e.g. spores, dividing cells) are differentially filtered, centrifuged or manually selected. Shock treatment through heavy stress can modify functions or structures of the cultured organisms and may produce effects that become apparent only after considerable time. Such consequences of environment-stress synchronization are insufficiently investigated. An ecologically acceptable method is synchronization by defined photoperiod rhythms. However, not all organisms may be sufficiently susceptible to such treatment. A combination of photoperiod and non-detrimental changes in temperature may yield optimum results.

![Flow diagram of a chemostat for culturing rotifers, based on the following assumptions: (i) no algal growth occurs between A and B; (ii) there is no negative slope in the working region of the nutrient concentration/growth-rate relation; (iii) steady-state conditions prevail. (After Droop, in; Conover, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.]

**Chemostat**

In a chemostat, the continuous, defined flow rates of culture medium and nutrients produce a situation in which a limiting factor controls the equilibrium between supply and consumption of life-supporting energy and matter. The principal advantages of the chemostat are: (i) The state of dynamic equilibrium can be altered by introducing controlled changes (e.g. in nutrient supply; medium flow rate, temperature, salinity, oxygen, etc.; addition of toxicants, removal of specimens). Analyses of the consequences of such alterations yield insights into the forces which control intra- and interspecific co-existence. (ii) Results are produced on a statistical basis. Major disadvantages of the chemostat are: (i) ‘unnaturally’ high population densities; (ii) maintenance of logarithmic growth rates and perfect equilibria, which may rarely be realized in marine environments.

Chemostats have been used extensively as a statistical instrument for studying population dynamics in marine micro-organisms and unicellular plants. In mixed cultures of micro-organisms, specific nutritional circumstances may be chosen, so as to analyze the dynamics of competition and co-existence (e.g. Veldkamp and Jannasch, 1972). Chemostat experiments with marine animals have thus far been restricted to osmotrophs and bacteria- or phytoplankton feeders.
Chemostats have received attention from Monod (1950), Novick and Szilard (1950), Herbert (1958), Herbert and co-authors (1965), Droop (1966b, 1969) and Conover (1970). Powell (1965) and Tempest (1970) have provided useful accounts regarding the theory of the chemostat, and Evans and co-authors (1970) have presented a comprehensive paper on chemostat construction, as well as a list of manufacturers of chemostat components.

It is convenient to construct chemostats in two separable parts, a fermenter unit and a control unit, so that one set of control instruments can be used with a range of fermenter units of different capacities (Herbert and co-authors, 1965). The fermenter unit comprises the culture enclosure, medium and antifoam reservoirs, effluent receiver, medium and antifoam pumps, and acid or alkali reservoirs with their associated valves. In order to obtain rapid and complete mixing, vigorous stirring is necessary. Stirring is effected by dispersing sterile air or by mechanical or magnetic means. The culture volume is controlled by an overflow. Samples may be withdrawn directly from the culture enclosure, but as this causes a sudden decrease in culture volume, and a corresponding increase in dilution rate, sample volumes must be restricted to a few percent of the culture-enclosure content. Larger samples can be collected from the overflow, in which case the weir type of overflow is preferable to the external type of overflow, unless the flow rate is large. According to Herbert and co-authors, proper flow control requires: (i) variability of flow rate over a
range of at least 20 to 1; (ii) long-term constancy of a given flow setting within a few percent; (iii) possibility of sterilizing (autoclaving) all parts in contact with the culture medium; (iv) medium passage without biological or chemical contamination. The most reliable flow control is facilitated by employing a properly designed metering pump.

A diagram of the simplest form of chemostat is presented in Fig. 2-124. DROOP (1966, and in: CONOVER, 1970) has used a chemostat for cultivating the rotifer *Brachionus plicatilis* (Chapter 5). The flow diagram of his rotifer chemostat is illustrated in Fig. 2-125. In the rotifer culture compartment, the perfectly mixed medium flows in and out at a constant rate, and the *B. plicatilis* population grows as fast as the food-alga (nutrient) concentration permits, reaching a steady-state in which rates of population growth and wash-out are identical. Maximum stability of the system can be attained by employing the combination of an algal chemostat (autotroph producer) and rotifer chemostat (heterotroph consumer), separated by a sensing device that doses culture medium and food algae in proportions necessary to maintain a constant algal concentration. The dosimeter senses algal density at fixed intervals and decides whether to dose an aliquot of culture medium or an equal aliquot of algal culture. Interval and aliquot size are adjustable, as is algal density. In the long run, this leads to constant medium flow and constant food levels, which can be maintained over months. After each change, e.g. in flow rate, it takes a week or two to reach a new equilibrium. In this way, dynamics of nutrition and population growth can be analyzed (Fig. 2-126).

A diagram of the chemostat used by DROOP (1966) for measuring the parameters of vitamin $B_{12}$ requirement in the chrysomonad *Monochrysis lutheri* is illustrated in Fig. 4-18. The chemostat consists of a medium reservoir, a spherical constant-level culture enclosure (reactor), placed in a photothermostat with 2000 lux continuous illumination and an effluent receiver. Filtered air is pumped through the system for aeration (at the rate of $400 \text{ ml min}^{-1}$), stirring and medium transport. The air is water-saturated to lessen evaporation from the culture, the cotton-wool air filters being gently warmed to keep them dry. Mixing of the culture is aided by the spherical shape of the culture enclosure and the eccentric position of the medium-air nozzle. Ports are provided in the various containers for inoculation, medium replenishment and sampling. The chemostat is made entirely of glass and silicone rubber, and can be autoclaved. The positive air pressure within the system ensures sterility during continuous operation. Aliquots of culture medium are metered into the culture vessel at hourly intervals. This is effected by 2 clamp-type solenoid valves, operated by a timer. Both valves close together. Valve 1 ($SV_1$) remains closed for about 20 secs, Valve 2 ($SV_2$), a little longer. Closing of Valve 1 raises the culture medium in the dosimeter, while the opening of Valve 2—a little later—allows the medium to flow slowly into the culture enclosure, until the level in the dosimeter has dropped to a position determined by the (adjustable) height ($h$) of the junction of the medium and air tubes. According to DROOP, this dosing system has the advantage of reliability over capillary systems, and of economy over peristaltic pumps; but it has the disadvantage that a truly continuous flow is not provided. However, the dosage interval never exceeded one twenty-fifth of the generation time of the organism cultured.

For cultivating tintinnids, a semi-automatic, continuous-flow system was
developed by Gold (1973). The system controls the yield through adjustment of both food input and flow rate (Fig. 2-127). In the case of Tintinnopsis beroidea, maximum sustained yield approached 10^{-3} \text{cells ml}^{-1}. The intermittent addition of food (4 algae: Isochrysis galbana, Platymonas tetrathele, Rhodomonas lens and an unidentified species) was the only function not carried out automatically. While it would be simple to grow I. galbana and the unidentified species at the same temperature as the tintinnid (10^\circ \text{C}), P. tetrathele and R. lens require higher temperatures (10^\circ \text{to} 15^\circ \text{C}), and grow poorly—or not at all—at 10^\circ \text{C}. These different thermal requirements of consumer and food were the principal reason for intermittent food addition. According to Gold, T. beroidea is the first phytoplankton-feeding protozoan cultured in a continuous-flow system.

**Turbidostat**

To the marine ecologist, it is important to study population parameters at individual densities comparable to in situ conditions. This is possible in a turbidostat where the rate of flow is controlled by population density (in a chemostat, population density is controlled by flow rate and hence, is usually much higher than in the sea). In modern turbidostats, a photoelectric monitor detects deviations from a pre-set culture density (turbidity) and initiates (via pump or valve) a compensatory increase or decrease in dilution rate of the culture medium. Apart from the photoelectric control system and some optical requirements with respect to the culture tube, all the components of a turbidostat have functions similar to those of a chemostat.
The apparatus illustrated in Fig. 2-128 can be used either as a chemostat or as a turbidostat; it employs an external optical cell (photometer) in a circulation line, and can be fitted to any conventionally stirred fermenter (HERBERT and co-authors, 1965). Circulation is effected by an external peristaltic pump (Pump 2), drawing culture medium downwards through a vertical pipe inside the fermenter at a rate which allows air bubbles to rise (so that culture medium reaching the optical cell in the photometer is bubble free). Part of the circulation line containing the flow-through cell can be isolated and flushed with 3N NaOH followed by sterile water; such procedures remove wall growth and keep the cell clean for at least 1 month without contaminating the culture medium. The photometer signal is fed to a recorder-controller, operating control contacts when the turbidity reaches a programmed level. The contacts activate a metering pump (Pump 1) which adds fresh medium to the culture container; the volume of the medium added is registered on a second recorder (not shown). The pump is set to about twice the steady-state flow rate corresponding to the optical density selected, so that it is on or off for about
equal periods; this results in a close control of optical density. By switching off the control system and operating the metering pump continuously at a constant rate, the turbidostat becomes a chemostat, with the extra facility of continuous optical-density recording. Turbidostat techniques have been considered, for example, by Bryson (1952), Anderson (1953), Phillips and Myers (1954), Fogg and co-authors (1959) and Droop (1969). A comprehensive account on turbidostat operation, design and application in microbiology has been presented by Munson (1970).

![Diagram of Continuous-culture light thermostat](image)

**Fig. 2-129**: Continuous-culture light thermostat. Diagram. (After Senger and co-authors, 1972; modified; reproduced by permission of Academic Press.)

Light thermostats for continuous and synchronous cultures of photo-autotrophs (bacteria, algae, flagellates, ciliates), which may serve as food organisms for a variety of marine animals, have been employed by a number of experimental ecologists and physiologists (e.g. Pfau and co-authors, 1971; Senger and co-authors, 1972). The apparatus illustrated in Fig. 2-129 is produced commercially by Kniese Apparatebau GmbH, Marburg, FRG.
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

(b) Specific Equipment

Specific culture equipment is referred to in Chapters 3, 4 and 5. A few additional examples follow below.

_Dialysis Cultures_

Introduced by METCHNIKOFF and co-authors (1896), artificial membranes have been used by numerous investigators for cultivating micro-organisms (see review by SCHULTZ and GERHARDT, 1969). In regard to marine organisms, dialysis cultures have thus far been restricted to unicellular phytoplankters (TRAINOR, 1965; JENSEN and co-authors, 1972). JENSEN and co-authors cultivated a variety of unicellular algae under laboratory as well as under field conditions. They found the dialysis technique to be very useful: It produces a high algae yield, facilitates quality assessment of a given volume of sea water for supporting phytoplankton growth, and allows the evaluation of the effects of environmental factors, including pollutants, under close-to-natural conditions.

The apparatus employed by JENSEN and co-authors (1972) is illustrated in Fig. 4-5a. Membrane filters of various porosities or dialysis tubing made of regenerated cellulose were used as dialysis membranes. Most experiments were carried out in dialysis bags (Fig. 4-5b), mounted on a rotating support. Under laboratory conditions, dialysis cultures of a number of algae gave dense populations when grown in running, non-enriched sea water (Table 2-37). In the open sea, satisfactory growth was obtained in most cases.

A number of technical problems remain to be solved. JENSEN and co-authors (1972) list the following: (i) Light penetration through very dense cultures (light tubes may be introduced directly into the cultures); (ii) diffusion-limited growth (porous membrane materials will speed up nutrient support); (iii) inhomogeneity of culture population inside narrow bags (this can, perhaps, be overcome by pumping).

_Accumulation and Isolation of Unicellular and Small Multicellular Organisms_

In freshly collected samples, accumulation of unicellular and small multicellular organisms is achieved by decantation, pipetting or sieving; selective sedimentation; centrifugation; migration initiated by environmental or nutrient gradients; elutriation; and interphase accumulation. Most of these methods may also be used for isolation which, in addition, requires sterilization (p. 102): repeated transfer of individuals through a sequence of culture dishes containing sterile media and antibiotics. For details consult Chapters 4.1 and 6.1. General accounts on sampling, accumulation and separation of meiofauna have been provided by HULNOS (1971), HULINGS and GRAY (1971), ELMGREN (1973) and UHLIG and co-authors (1973).

Decantation, pipetting, sieving, sedimentation and centrifugation do not involve equipment that would require special mention here. Equipment used for migration, elutriation and interphase-accumulation techniques receives brief attention in the following paragraphs.
Migration of mesopsammal (interstitial) microfauna elements from a sediment sample into an accumulation dish can be effected by establishing environmental gradients (e.g. temperature, salinity, dissolved gases). Such 'climate deterioration technique' has been employed by Uhlig (1964b, 1968). Crushed sea-water ice, placed on top of a sediment sample, forces the vagile microfauna to leave the sediment, to pass through a nylon gauze, and to enter a sea water-filled accumulation dish.

Table 2-37

Dialysis cultures of phytoplankters. Approximately 3000 lux (24 hrs); 6°–12° C; non-enriched sea water, passing the test tank at a flow rate of 200 l day⁻¹ (After JENSEN and co-authors, 1972; reproduced by permission of North-Holland Publishing Company)

<table>
<thead>
<tr>
<th>Species</th>
<th>Final density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletonema costatum</td>
<td>2.0 x 10⁷ cells ml⁻¹</td>
</tr>
<tr>
<td>Thalassionema nitzchidoides</td>
<td>2.6 x 10⁶ cells ml⁻¹</td>
</tr>
<tr>
<td>Navicula transiens var. dersa f. delicatula</td>
<td>1.0 x 10⁶ cells ml⁻¹</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>2.4 x 10⁷ cells ml⁻¹</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>2.4 x 10⁷ cells ml⁻¹</td>
</tr>
<tr>
<td>Gonyaulax tamarensis</td>
<td>4.0 x 10⁴ cells ml⁻¹</td>
</tr>
<tr>
<td>Gonyaulax spinifera</td>
<td>3.0 x 10⁴ cells ml⁻¹</td>
</tr>
<tr>
<td>Peridinium trochoideum</td>
<td>3.0 x 10⁶ cells ml⁻¹</td>
</tr>
<tr>
<td>Prorocentrum micans</td>
<td>1.0 x 10⁴ cells ml⁻¹</td>
</tr>
</tbody>
</table>

Fig. 2-130: Crushed, melting sea-water ice forces protozoans and other interstitial organisms to leave the sediment sample, pass through a nylon gauze and to enter the filtered sea water in the accumulation dish. (After Uhlig, 1964; modified; reproduced by permission of Biologische Anstalt Helgoland.)
CULTIVATION OF MARINE ORGANISMS (O. KINNE)

(Fig. 2-130). The effects of the melting ice are primarily due to the resulting salinity gradient, to a lesser degree to a reduction in temperature. The sea-water ice technique is most effective in sandy sediments with a capillary structure. Muddy sediments require treatment of sediment fractions and repeated extraction. UHLE's methods can be amended by subsequent decantation and elutriation (see below); for flagellates and ciliates, the efficiency of the forced migration is close to 100%.

In the migration-tube system developed by PHILPS and FERNANDEZ (1960; see also PHILPS, 1947), motile micro-organisms out-swim their accompanying contaminants and thus facilitate isolation and purification (Fig. 2-131). Nutrient medium is placed in an Erlenmeyer flask with rubber stopper, entry and exit tubes, and plugged with cotton. This whole arrangement is then autoclaved while a clamp prevents the medium from flowing. The Erlenmeyer is shaken thoroughly to aerate the medium, the clamp removed and air is blown through the short entry glass tubing, provided with sterile cotton, in order to fill the tube system with medium. If
bubbles remain in the tubing, they must be removed by sucking the medium back and by again forcing it through until the fluid in the Erlenmeyer and that in the thistle cup are at the same level. A pinch clamp is applied to the rubber tubing and about 2 ml of medium withdrawn from the thistle cup; 0.5 ml of the culture to be purified is then added to the thistle cup and the pinch clamp removed. This procedure ensures against gravity flow of contaminants towards the Erlenmeyer. Protozoans added to the system usually complete the migration within 3 days. At the end of migration, the pinch clamp is re-applied to the rubber tube, and Erlenmeyer, U-tube and rubber tube disconnected from the rest of the system. Samples are then transferred from Erlenmeyer flask to sterile media.

Fig. 2-132: Elution. Small organisms are separated by water current and retained on a sieve. (Based on Boisseau, 1957; from Uhlig and co-authors, 1973; modified; reproduced by permission of Biologische Anstalt Helgoland.)

Elution (Boisseau, 1957) is based on water-current separation. Exposed to a continuous water stream, floating protozoans and other micro-organisms are washed out according to weight (volume) and collected on sieves (Fig. 2-132).
Interphase accumulation has been employed by Gitelson and co-authors (1970) for accumulating and harvesting protozoans. This simple technique makes use of the tendency of many unicells to accumulate at the interphase between air and culture medium or between culture-enclosure bottom and culture medium. By gradually reducing the interphase areas available (neck of Erlenmeyer flask, bottom part of burette), cell densities are attained up to 260 times those of random distribution in log-phase cultures.

![Phytoplankton harvester](http://example.com/fig2-133.png)

Fig. 2-133: Phytoplankton harvester working on the principle of foam separation. Foam tube length ca 70 cm; diameter ca 7.6 cm. (After Epifanio and co-authors, 1973; modified reproduced by permission of the World Mariculture Society.)

For observation and experimentation, protozoans have been immobilized on protamine-coated slides (Marsot and Couillard, 1973). Best results are obtained with 0.015 to 0.06% (w/v) of protamine sulphate. Glass microscope slides are cleaned with 95% ethanol and a 1.5-cm circle of melted paraffin is painted on, thus forming a shallow compartment. The circle is then filled with a few drops of freshly
prepared protamine solution and 15 mins allowed for adsorption to the glass. Subsequently, the remaining solution is drained off and the slides rinsed with culture medium. Finally, a drop containing washed protozoans is mounted under an untreated coverslip. Protamine is possibly just one of the many positively charged polyelectrolytes which could arrest negatively charged cells, histones being an obvious alternative. Immobilization of protozoans can also be obtained in the 'roto-compressor chamber' (Biological Institute Philadelphia, USA).

**Phytoplankton Harvester**

Algal food for phytoplankton-feeding animals must be added without large amounts of nutrient medium. Epifanio and co-authors (1973) have developed a

![Diagram](image-url)

*Fig. 2.134: In situ* sporophyte culture system with germanium dioxide disperser (GD) for control of diatom contamination. (a) Single unit (normal positioning is horizontal); CS: culture slide; SE: secondary water-entry port; V: vacuum pump. (b) Double unit assembled under *in situ* conditions. (After Pace, 1973; modified; reproduced by permission of Walter de Gruyter & Co., Berlin-New York.)
phytoplankton harvester (Fig. 2-133) which allows the collection of undamaged, relatively nutrient-medium-free algal cells (*Platymonas* sp., *Carteria* sp., *Nannochloris oculata*, *Phaeodactylum tricornutum*). The harvester operates on the principle of foam separation (p. 140).

![Diagram of phytoplankton harvester](image)

Algal cells are airlifted into a column of foam produced by a high-capacity air stone (ca 2.5 cm in diameter; very fine pores). Trapped in the foam, the cells are carried into a collector, while the culture water trickles back into the harvester tube, from where it is siphoned back to the culture tank. From the collector, the algal cells are harvested via an effluent tube. The harvester has no moving parts; this reduces the chance of possible malfunctioning. Prefoaming of the cell suspension in the airlift increases the harvesting efficiency.

In situ Sporophyte Culture System

For cultivating early sporophytes of *Macrocystis integrifolia* under field conditions, *Pace* (1973) used a Perspex cylinder containing 6 glass dishes (37 × 25 mm) held end-to-end by a double border of plastic tubing (Fig. 2-134). The dishes serve as attachment substrate for the sporophytes. A germanium dioxide (GeO₂) disperser is attached to the inflow end of the cylinder. It consists of a screened glass funnel which amplifies and directs the ambient water current through an aspirator-type vacuum pump. A plastic bottle containing 1 g of crystalline GeO₂ is attached to the
pump’s suction nozzle. The GeO₂ slowly dissolves in the water current entering via a secondary entry port. The rate of dissolved GeO₂ admission to the culture cylinder can be adjusted by changes in size or position of the secondary entry port.

![Diagram](image)

Fig. 2.136: Planktonkreisel. Inset indicates direction of water-jet outlet relative to container wall (a) and to substrate surface (b). (After Greve, 1968; modified; reproduced by permission of Springer-Verlag.)

Counteraction of diatom contamination in algal laboratory cultures had been demonstrated by Lewin (1966). Pace (1973) showed that GeO₂ treatment (5 m water depth, 1 knot current speed, high ambient diatom abundance) significantly
suppresses the diatoms. Inhibition of diatoms was almost complete during periods of reduced diatom settlement. Comparison of test and control slides revealed no adverse effects of the GeO₂ inhibitor on the developing sporophytes of *Macrocystis integrifolia*.

![Diagram](image)

**Fig. 2-137:** Water transport system of planktonkreisel. (After Greve, 1968; modified; reproduced by permission of Springer-Verlag.)

### Spray Apparatus for Cultivating Seaweeds

Some macroscopic algae have been reported to grow well under a continuous shower of sea-water medium (HANIC, personal communication in: Chapman, 1973). HANIC constructed an apparatus (Fig. 2-135), in which the medium is recirculated and sprayed continuously over the alga placed on the bottom of a Plexiglas container.

### Apparatus for Cultivating Plankters

The planktonkreisel for cultivating carnivorous zooplankters (Greve, 1968) consists of a round culture enclosure (30 to 50 cm high), an inside sand-gravel filter
and a water transport system (Figs 2-136, 2-137). It is made of glass and plastic materials. The kreisel can be operated as a closed- or semi-open sea-water system.

Fig. 2-138: Double cuvette for culture experiments on plankters. Parallel compartments (Cuves 1 and 2) with identical water quality facilitate comparison between controls and experimentalss. (After Guezv, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)

In the closed-system arrangement, an airlift transports the water upwards until it falls into the collecting tube, where remaining air bubbles are released before the water re-enters the culture compartment via a jet. The jet outlet must be placed close to the outer wall of the round container, and arranged at angles shown in the inset of Fig. 2-136. The resulting water movement and the specific configuration of the sand surface facilitate the plankters' re-entry into upwardly directed water currents. The speed of water rotation is a function of airlift intensity, and can be modified over a rather wide range (1 rotation per 20 secs to 4 mins). In the semi-
open-system arrangement (Fig. 2-137), new sea-water enters the collecting tube through the water-supply tube (which ends below the water level of the culture compartment). In the collecting tube, the new sea water mixes with the filtered sea water. Constant water-level height in the collecting tube (which must be somewhat higher than that in the culture compartment to provide gravity feed) can be

Fig. 2-139: Kreisel for seston-feeding plankters. Seston recircles through the perforated PVC cone while zoo-plankters remain in the culture compartments and thus avoid air contact in the lift. When filled, the bottom-weighted wave maker tilts around its eccentric axis (open circle), emptying into the culture compartment; downwards and upwards movements of the beaker are limited by restriction points (filled circles). After Grieve, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)
achieved by connecting a constant-level overflow (Fig. 2-107). In the planktonkreisel, GREVE cultured the North Sea plankters *Pleurobrachia pileus*, *Beroe gracilis* and *Sagitta setosa* from egg to egg. More delicate forms (species of *Bolinopsis* and *Oikopleura*) may be injured by the fast water stream leaving the jet. Increasing the number of jets from 1 to 8, and widening the jet openings from 2 to 4 mm diameter avoided such damage and provided more uniform water-movement.

The double cuvette (GREVE, 1970) employs vertical water rotation, and has its water-transport system located outside the culture enclosure (Fig. 2-138). Cuvettes 1 and 2 are separated above the sand, but communicate in the gravel layer and thus harbour identical water bodies. This fact, as well as photographic recording, facilitates comparative studies on plankton populations (e.g. inter- or intraspecific population dynamics), using one cuvette for controls, the other for experiments. GREVE'S original apparatus measures 40 × 50 × 10 cm.

A kreisel designed for cultivating seston-feeding plankters (GREVE, 1970) allows seston particles to pass through a perforated PVC cone, and to re-enter the culture compartment via lift tube and collecting tube (Fig. 2-139). Most zooplankters tested avoid passing through the cone; they remain in the culture compartment and hence, are protected from contacting the bubbles in the airlift. The seston (e.g. phytoplankton) remains well distributed in the water column, and can be grazed on by the zooplankters cultivated. In order to simulate wave action, a wave maker can be installed on the kreisel; it consists of a bottom-weighted beaker, which tilts around an eccentric axis (open circle) when filled with sea water, and straightens up again when empty. The intervals at which the wave maker empties into the culture compartment can be regulated by the speed of water inflow.

In the 'Meteor'-plankton-cuvette (Fig. 2-140), newly caught plankton can be maintained for extended periods of time aboard ships (GREVE, 1975a). The all Perspex cuvette consists of a cylindrical culture enclosure (30-cm diameter, 12-cm width) with a small cylinder (10-cm diameter) on top, and a combined water-movement-aeration system. The small cylinder prevents culture water from splashing out of the main cylinder if the ship rolls. Temperature-controlled water enters (1) and leaves (2) a Perspex box which surrounds the culture enclosure and serves as water bath. Inside the culture enclosure, the water is kept in gentle rotation, entering through a jet and leaving through a large gauze-covered opening.

The taumeltisch (Fig. 2-141) features a cardanically suspended table which is slowly swivel-tilted by wheels (W) attached to a tilted, motor-rotated frame directly beneath the table (in the figure, the frame is shown detached from the table's subsurface). Culture dishes (not shown) are placed on the table and kept in continuous motion. The taumeltisch has been used successfully for cultivating a variety of plankters.

In the double cylinder (Fig. 2-142), two joined Perspex cylinders are separated by a sheet of gauze. The latter allows larval or juvenile zooplankters to leave the adult compartment and to enter the larval compartment. From here, the larvae can be removed via a light-trap siphon. A second, finer gauze sheet prevents escape with the recirculating water. The water returns to the culture enclosures via airlift and header tank. GREVE'S (1975b) double cylinder facilitates continuous cultivation over several successive generations.
Fig. 2.140: ‘Meteor’-plankton-cuvette. 1: entry, 2: exit of temperature-controlled water. (After Greve, 1975a; modified; reproduced by permission of Elsevier Scientific Publishing Company.)

Fig. 2.141: Taumeltisch. W: wheels fastened to tilted, rotating plate; the wheels roll over the table’s subsurface. M: motor. (Based on Greve and Heinberg, unpublished.)

Fig. 2.142: Double cylinder. From adult compartment, larvae can move through gauze into larval compartment from where they are collected via a light-trap siphon. Sp: sampling port. (After Greve, 1975b; modified; reproduced by permission of Biologische Anstalt Helgoland.)
The *in situ* kreisel (Fig. 2-143) allows cultivation of macroplanktonic and benthonic seston feeders on the sea bottom (JATZKE, 1970). The perspex apparatus may be from 0.5 m to several metres high, and is served by skin divers or aquanauts. The *in situ* kreisel is positioned parallel to prevailing water currents. Water rotation inside the kreisel remains constant, even in the presence of alternating ebb and flood tides, since water coming from left or right is deflected in opposite directions. The incoming water carries small plankters and detritus through an outer and inner screen (gauze or perforated plate); it is then forced to circulate by current deflectors, rotate upwards and finally leave through the top screen. Where excessive wave action prevails, a turbulence protector reduces extreme pressure changes. In the southern North Sea, JATZKE raised a number of invertebrates, e.g. *Beroe* sp., *Pleurobrachia pileus*, larvae and subadults of *Hormarum gammarus*, *Eupagurus bernhardus*, and *Galathea squamifera*. It turned out that the outer screen requires periodic clearing. For plankton cultivation in the sea, plastic bags (p. 76) have proven very useful. In large plastic bags, installed in wind-protected sea areas, plankton populations can be sustained under close-to-natural conditions. The plastic-bag concept is likely to develop into a powerful ecological tool.

Culture tanks for ctenophores and medusae (Fig. 2-144) have been constructed by WARD (1974). He used 2 standard 80-l aquaria (30 × 60 × 46 cm) in line with a 360-l polyethylene reservoir tank (not shown in the figure). The inside corners of each aquarium are partitioned off by glass plates (Silastic aquarium sealant). Two plates facilitate water distribution via 1-mm × 5-mm notches (spaced 2.5 cm apart along the edge of each plate); the other pair assists in returning water from the aquarium to the reservoir. The culture water is recirculated continuously (6 l min⁻¹), creating a strong circular current. The water percolates through a gravel filter (3 to 6 cm deep), and siphons into a pair of overflow vessels, from where it drains back into the reservoir. The temperature-controlled reservoir is aerated vigorously with filtered (glass wool, carbon) air. WARD maintained several hundred large adult *Mnemiopsis* sp. (>5 kg ctenophore biomass) in this culture system for up to 4 weeks with no apparent physical deterioration. *Mnemiopsis* cydippid larvae (1 to 2 mm in diameter) have been raised to adult size. In addition, *Beroe ovata* and the medusae *Cyanea capillata* and *Chrysaora quinquecirrha* have been maintained successfully in the system for extended periods of time.

The planktonrotor (PAFFENHOFER, 1970) consists of a tilted aluminium frame holding a glass jar (culture enclosure). The upper axis of the frame is attached to a universal joint; the lower axis penetrates the loop of a drive shaft, which is rotated slowly by an electric motor (Fig. 2-145). The jars (3000-, 4000- or 8000-ml capacity) rotate twice each minute. While aerators, stirrers, and related equipment operated in the culture enclosure, may cause physical damage to the organisms cultivated, the slow rotations of the planktonrotor apparently cause no harm. PAFFENHOFER offered chain-forming diatoms to the zooplankters cultured; the diatoms remained evenly distributed, and did not sink to the bottom of the planktonrotor. The zooplankters normally remained afloat and rarely contacted the glass walls or the water surface. The copepods *Calanus helgolandicus*, *Pseudocalanus elongatus*, *Temora*
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

Fig. 2-143: *In situ* culture for cultivating macroplanktonic or benthonic seston feeders in the sea. Left: vertical; right: horizontal section. Tidal current is shown to enter from left. (After JATZEK, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)

Fig. 2-144: Culture tank for ctenophores and medusae. Sea water enters two opposite corner compartments formed by notched glass plates. Forced through the notches by a 1-cm head pressure, the water circulates and, after passing from the gravel-filter bed into a pair of return compartments (RC), siphons into 2 overflow vessels (OV) from where it returns to the reservoir. (After WARD, 1974; modified; not copyrighted.)
Eongicornia and the appendicularian Oikopleura dioica have been reared and bred successfully in the plankton rotor (Paffenholzer, 1970, 1973 and personal communication; see also Chapter 5.1).

![Diagram of plankton rotor](image)

Fig. 2-145: Plankton rotor. Placed in a tilted frame and fastened by a belt, a glass jar containing the plankters is slowly rotated around a universal joint by a motor driven shaft. (After Paffenholzer, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)

A closed culture system for planktonic copepods (Fig. 2-146) has been developed by Zillieux (1969a, b). He used the ciliate Euplotes vanus as culture partner. E. vanus controls bacterial growth and consumes accumulated debris. The copepods graze on the ciliates, as well as on phytoflagellates provided as primary food sources. The culture system facilitates foam separation and sustains high population densities of copepods (e.g. Acartia clausi and A. tonsa) at 15° C in artificial sea water (‘Triton Marine Salts’). From the foam tower, sea water flows by gravity through a glass-wool filter and enters the lower reservoir, where it remains until a level switch...
(L) activates an induction-driven pump. The water is then forced through a 15-μm cellulose acetate cartridge filter (F₁) in series with a 0.45-μm pleated membrane cartridge filter (F₂) and into the upper reservoir, from where it enters the bottom of each culture tank. Flow rate is governed by pinch valves located on the delivery tubes. Through removable screens, the water passes into troughs which extend the width of the tanks, and flows out to re-enter the foam tower. The screens consist of 'Nitex' No. 200-74 nylon screen cloth (rated porosity = 74 μm) imbedded in an acrylic frame. A screen of 155 cm² area sufficiently distributes the outflow of 125 ml min⁻¹ per tank, so that no undesirable concentration of copepods occurs in its vicinity; at this flow rate, the screen restricts passage of the smallest nauplii, but still allows food algae to pass freely. The compressed air supplied to the foam tower is first dried, passed over activated charcoal, and then rehydrated by bubbling through a column of distilled water.

The culture tanks (100-l capacity) consist of 1-cm thick, solvent-welded acrylic plastic; the tubing is of Pyrex glass, silicone rubber or nylon; the pump surfaces in contact with the sea water are of polypropylene (except for the titanium shaft). Polyethylene and polyvinyl chloride are also used in the system. According to ZILLIOUX (1969a), none of these materials had any demonstrable effect on the copepods when tested separately. (See, however, Chapter 7.) Constant overhead illumination (approx. 1500 lux from a 40-W, cool-white fluorescent tube) keeps the copepods generally well distributed; however, some aggregation occurs at the upper tank corners; this facilitates harvesting.
The closed culture system described above has been improved by ZILLIUX and LACKIE (1970). The 3 major improvements are: (i) increase of foam-tower efficiency by removing cellular particulates prior to foaming; (ii) modification of foam-tower design to facilitate rapid removal of foam; (iii) easy switch-over from recirculating closed system to flow-through open system (Fig. 2-147). Cellular particulates are reduced by installation of the glass-wool filter before (not after) the foam tower. The improved foam tower is described on p. 143. The two 100-l culture tanks have been subdivided into eight 25-l containers. Switch-over from a closed to an open system was considered desirable for providing more natural water conditions. At

Fig. 2-147: Improved version of culture system illustrated in Fig. 2-146. Pump and cartridge filter are under the bench. (After ZILLIUX and LACKIE, 1970; reproduced by permission of Biologische Anstalt Helgoland.)

first, water was pumped from the mouth of an estuary into a column with 2 nylon screens. While this filter column worked fairly satisfactorily, it allowed foreign animals such as hydroids, tunicates, small copepods and molluscs to enter the culture tanks. Thus, the filter column was replaced by a filter-settler (Fig. 2-145). Flow
Fig. 2.148: Filter-settler designed for open-system operation of the culture system shown in Fig. 2.147. Cover, supporting frame and insulation omitted. (After Zilliox and Lackie, 1970; reproduced by permission of Biologische Anstalt Helgoland.)

Fig. 2.149: Recirculation system used for cultivating planktonic crustacean larvae. Side view. Airlift-operated countercurrents keep the plankters nearly evenly dispersed. (After Sandifer and co-authors, 1974; modified; reproduced by permission of Biologische Anstalt Helgoland.)
rates stated in the figure are essential for proper filtration and settling; they are controlled by hosecock clamps on tubing connections. Continuous, slow water flow through the base of each of the 3 compartments avoids the rapid oxygen depletion which often occurs in static sediments. Mesh openings of the 3 removable successive nylon screens are 250 µm, 74 µm and 37 µm, respectively. The 37-µm mesh allows passage of microflagellates suitable for the cultured copepods. Sea water is delivered to the upper reservoir at the rate of 750 ml min⁻¹; the final delivery rate to the culture containers is limited to 500 ml min⁻¹; an overflow port added to the reservoir assures maintenance of a constant supply level. Insulation (e.g. foam neoprene rubber) of the exterior of the filter-settler and the upper reservoir maintains the culture water at nearly natural temperatures. Unwanted accumulations of organic contaminants must be removed periodically.

Sandifer and co-authors (1974) have described a system for cultivating planktonic decapods (Figs 2-149, 2-150): Although a cylindrical shape might offer certain advantages, they adopted a rectangular shape for the culture enclosure because of the configuration of the building used. The tank has a sloped bottom; the water enters at the shallow end and flows across the bottom; at the deep end, an airlift (pumping rates 400- to 600 l hr⁻¹) produces a surface countercurrent. The water can be continuously recirculated through an attached (or separate) gravel filter. The water circulation established in the culture enclosure (arrows) maintains food particles in suspension and keeps the plankters nearly evenly dispersed. Sandifer and co-authors have used this closed system successfully for culturing the prawn *Macrobrachium rosenbergii* and postulate that it may be suitable for other species (see also Sailer and Mock, in press; Zielinski and co-authors, in press).
A pelagic egg incubator for shipboard use has been constructed by Krakatitsa and Sapin (1971). The temperature-controlled device has been employed successfully for incubating eggs of various fishes, e.g. Sardinella jimbfriata, Stolephorus zollingeri, Anchoviella sp., Fistularia villosa as well as eggs of members of the families Carangidae, Serranidae, Sparidae and Mullidae.

An apparatus for measuring feeding rates in cultured Calanus helgolandicus has been described by Richman and Rogers (1969). The apparatus (Fig. 2-151) is similar to that developed by Paasche (1967) for cultivating algae. Cylindrical Pyrex test tubes (65 x 500 mm), which easily accommodate 1000 ml of culture medium, serve as culture containers. Each tube is fitted with an airlift that recirculates the culture medium and keeps the food diatoms (Ditylum brightwellii) in suspension. Air is pumped down the inlet tube and collected by a funnel. The water thus transported falls back through a 100-μm Nitex net held by a pair of concentric Teflon rings. The air pressure is adjusted to produce a water-flow rate sufficient to wash the diatom cells through the netting without damaging the copepods' faecal pellets, which are retained and subsequently collected. A second, lower net with 500-μm netting allows...
SPECIFIC EQUIPMENT

both faecal pellets and diatoms to pass, but not the copepods. The whole double-net assembly can be pulled out of the culture container, thereby collecting all copepods on the lower net. Samples of culture medium are removed at 6- to 10-hr intervals via the sampling tube, and diatom-cell counts are made with a Coulter Counter.

Mass cultivation of brachyuran larvae under controlled conditions of light, temperature, salinity, water movement and food (nauplii of Artemia salina) has been achieved by SASTRY (1970a, b). In the prototype system, larvae of Cancer irroratus and Panopeus herbstii were raised from eggs (obtained from freshly caught ovigerous females and developed in large finger bowls containing filtered sea water treated with penicillin) to the first crab stage; however, only a few juveniles were obtained (SASTRY, 1970a). Improvement of techniques (SASTRY, 1970b) resulted in the mass culture system illustrated in Fig. 2-152. In contrast to the prototype, the improved version has provisions for removal of waste materials (exuviae), and can be used with several culture enclosures simultaneously. Each culture enclosure (ca 40-l capacity; Plexiglas) has separate water in- and outlets. The apparatus can be operated both as a closed and an open system. Sea water from the reservoir (or from the sea) is pumped (P₁) into a header tank from where it enters the culture enclosure(s). The flow into each enclosure can be regulated by an inline PVC ball valve. From the culture enclosure the water is pumped (P₂) through: (i) a PVC cylinder containing activated carbon, (ii) 2 Orlon-wound cartridge filters of diminishing pore size (15 µm, 0-5 µm), (iii) a sterilizer (2 parallel 91-cm long ultra-violet lamps); finally, the water is returned to the reservoir (or led to waste). A timer regulates the photo-

Fig. 2-152: Culture system for rearing brachyuran larvae (mass cultures). Improved version. EC: electronic controls for liquid-level controller and pump P₁. (After SASTRY, 1970b; modified; reproduced by permission of Verlag Birkhäuser.)
period (fluorescent lamp). The sea-water volume in the culture enclosures was maintained between 25 l and 27 l, by controlling the operation of the fill pump (P₁). The platinum electrodes of the level controller are positioned in the reservoir to maintain a minimum water level. If the water level drops below this minimum, P₁ is stopped; it is re-activated by rising water in the reservoir. The culture-enclosure drain pump (P₃) operates continuously. Streptomycin (2 ml l⁻¹) is added to the sea water in the reservoir. During operation, pH values remained between 7.9 and 7.7, and oxygen concentrations near the saturation level (aeration in header tank).

Construction details of the culture enclosure are shown in Fig. 2-153. Larvae of *Cancer irroratus* were introduced into the apparatus immediately after hatching; they received daily 'large quantities' of freshly hatched *Artemia salina'. Few
mortalities were noted during zoeal development, but loss of larvae may have occurred due to cannibalism. Mortality was high during megalopa development (no details given; see also Chapter 5.1).

A simple apparatus for cultivating decapod larvae in an open sea-water system is illustrated in Fig. 2-154. Perspex cylinders (10 to 15 cm in diameter, 15 to 30 cm long; covered at the lower end by nylon mesh) serve as floating culture containers (Rice and Williamson, 1970). A floating, compartmented plastic box with a perforated bottom accommodates single individuals.

Attempts to rear phyllosome larvae of scyllarid decapods remained rather unsuccessful until special equipment had been developed (Dexter, 1972). Figure 2-155
2. CULTIVATION OF MARINE ORGANISMS (O. KINNE)

illustrates DEXTER's closed recirculation system (132-l capacity). Thorough, but gentle aeration is provided by a 2-cm-diameter PVC tube of 1.2 m length, into which a series of small holes had been drilled. A stainless steel rod, sealed in rubber surgical tubing and placed inside the air tube, holds it to the tank bottom. Main features of

Fig. 2-156: Recirculation system for small sessile and hemisessile animals feeding on plankton. Water-current speeds (0 to 50 cm sec⁻¹) are produced by a combination of airlift and bubble cup (bubble pump.) BC: bubble cup; R₁: water-intake regulation; R₂: air-intake regulation; T: thermometer. All measurements in cm. (After JERRAM, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)
the system are: ultra-violet disinfection, intensive filtration (2 inside corner filters, 1 outside filter), and oyster-shell fragments scattered over the tank bottom for pH control. Culture enclosures are kept afloat by styrofoam sheets (30 cm × 10 cm × 1 cm). They consist of Plexiglas tubing (2 cm, 2.5 cm, 4 cm, 5.5 cm or 6 cm in diameter) cut into 3.2 cm lengths; small windows are made in the sides of the 4 larger tubes, and nylon netting (mesh size 253 μm) is glued (silicone cement) to bottom and windows. The small containers are used for Stage I and Stage II phyllosome

Fig. 2-157: Rearing apparatus for sessile plankton feeders. Continuous horizontal water rotation keeps food plankters in suspension and carries them past the bryozoan colonies on the slides (S), placed on a perforated board. BC: bubble cup; T: thermometer. All measurements in cm. (After Jebb, 1973; modified; reproduced by permission of Institut für Meereskunde, Kiel.)
larvae; the larger ones, for older larvae. Dexter leached the culture enclosures in fresh water for at least 48 hrs before use. The fibre-glass-coated plywood tank is covered with black Plexiglas sheeting to reduce evaporation and to prevent foreign material from falling into the tank. Details on larval growth, survival and nutrition are presented in Chapter 5.1.

**Apparatus for Cultivating Sessile Plankton Feeders**

Equipment for cultivating bryozoans has been developed by Jerram (1970, 1973). The system illustrated in Fig. 2-156 accommodates small sessile and hemisessile plankton feeders. Water-current speeds produced by an airlift-bubble cup combination can be regulated from 0 to about 50 cm sec\(^{-1}\). Water entering the round culture enclosure is forced into circular movement. Exact gauging of current speed remains a problem. The 'rearing apparatus for sessile plankton feeders' (Fig. 2-157) has a U-shaped perspex culture enclosure, thus facilitating vertical water rotation. The necessary gravity feed is supplied by the right tubular container, in which the water level is maintained about 16 cm higher than in the culture enclosure.

Several plankton feeders have been provided with natural plankton (open flow-through system or hand feeding). Such a procedure may introduce additional culture members (including parasites or young stages of predators) which can harm the cultured animals.

A growing tank for the juvenile hard clams Mercenaria mercenaria (2 to 4 cm in width) has been developed by Epifanio and co-authors (1973). Constructed of plywood, the 2.6-m long, 1.3-m wide, 0.6-m deep tank holds juvenile clams on screened baskets (Fig. 2-158). The recirculated culture water flows from top to bottom of the tank. Two flow directors are installed to achieve uniform water flow. The clams are fed algal suspensions obtained from the phytoplankton harvester illustrated in Fig. 2-133. The water of the clam growing tanks is emptied into the main filter twice a day, and the tanks are supplied with freshly filtered sea water.
Organisms attached to solid substrates can be cultivated in a variety of culture systems already described. However, sediment dwellers require special arrangements. Devices for culturing sediment dwellers are constructed in such a way that a flow of aerated water penetrates the sediment either from top to bottom, from one side to another or from bottom to top (e.g. Fig. 2-159). In the flow-through device illustrated, water enters the header tank, flows down the central tube, spreads along the bottom, penetrates the sediment and leaves through siphons. The speed of water penetration is regulated by water-level height in the header tank. The water-level height selected is maintained by a float valve and a constant-level siphon (p. 191).

A micro-aquarium for mud-living nematodes has been described by von Thun (1966). The micro-aquarium consists of 2 thin glass slides (26 x 76 mm, or 18 x 58 mm), glued to glass pieces of 1-mm thickness (Fig. 2-160a). It contains enriched seawater agar as substrate (Mayer, 1956). For interstitial sand dwellers, von Thun uses shallow micro-aquaria with sand and sea-water circulation (Fig. 2-160b). Both arrangements allow microscopic observations of mud or sand-living micro-organisms. These small and narrow containers are better suited for short-term observation than for long-term cultivation.
Apparatus for Cultivating Intertidal Plants and Animals

Rhythmic fluctuations in tidal dynamics are a primary characteristic of intertidal habitats. Under laboratory conditions, fluctuations in tidal level have been achieved in various ways, e.g. by raising and lowering of attachment plates (Fig. 2-161); changes in overflow height (Figs. 2-162, 2-163); raising and lowering of a water reservoir (Fig. 2-164); rhythmic siphoning (Fig. 2-165) or capillary-priming siphons (Fig. 2-166); valve operations (Fig. 2-167); timer-controlled pumping (Figs. 2-168, 2-169); see also the thalassiotron (Fig. 4-30).

Organisms attached to solid substrates, such as bryozoans or barnacles, can be exposed to tidal variations by raising and lowering their substrate, allowing for defined periods of immersion and submersion. Using attached algae as objects, TOWNSEND and LAWSON (1972) developed the apparatus illustrated in Fig. 2-161. An electric motor, attached alternately to 4 wheels of different diameters (gear-box)—completing 1 revolution in about 12 hrs, 6 hrs, 3 hrs or 1.5 hrs—lowers the substrate plates into, and lifts them out of, the water-filled culture enclosure. Each wheel axis connects to metal arms on either side of the apparatus, and each arm is fitted with a pin, which has a metal loop with a nylon thread attached. The pin can be moved up and down the arm to facilitate proper positioning of the plate in the culture enclosure (top of plate barely covered with water in low position; bottom of plate just above water surface in high position). When the 12-hr gear is engaged, the metal arm raises and lowers the plate every 12 hrs. Culture enclosures and pulley systems may be arranged in a series on one side of the apparatus, or on both sides, utilizing both arms.

Changes in overflow height have been employed by DE BLOK (1964) who constructed an apparatus (Fig. 2-162) in which the flexible overflow tubing is raised and lowered by motor-driven rod inclinations. For proper functioning, maximum overflow rates must be higher than the constant water-input rate. In the culture enclosure, the resulting tidal dynamics can be varied by changing: (i) the motor's speed or the diameter of the cogged chain-operated wheel, (ii) the attachment position of the motor-wheel combination on the stand, or (iii) the overflow point (OP) on
the rod. In order to be able to discriminate between effects of hydrostatic pressure and water-level height, De Block used closed culture containers (Fig. 2-163). Air and water entered the enclosure at defined pressures.

The principle of raising and lowering a reservoir tank relative to the culture enclosure has been employed by Micallef (1967). The reservoir, filled with sea water, is connected by rubber tubing to the static culture tank (Fig. 2-164). A motor oscillates the reservoir in a circular path above and below the static tank, slowly filling or emptying it in the process. Designed for laboratory operation, the apparatus is portable. Tidal dynamics can be controlled by altering the speed of the motor and/or by changing the set of gears.

Providing the rate of water supply is constant and less than the rate of drainage, the siphon arrangement shown in Fig. 2-165 provides a simple but effective means for rhythmic alternation of high and low-water conditions. When the water level...
in the regulator jar reaches the upper bend portion of the siphon, the water begins to drain from all jars. Tidal dynamics can be adjusted empirically by modifications in water flow, and in the dimensions of siphon tubing and jars.

Capillary priming combined with intermittent siphoning (Fig. 2.166a) has been employed by Plessis (1964). The narrow upper curved part of the siphon exerts capillary action. With the long arm of the siphon not reaching into the water of the lower tank (into which the water is drained), the water in the upper tank ascends freely in the short arm, and—aided by capillarity—reaches the highest point of the curvature before the upper tank overflows. The water in the upper tank is then

Fig. 2.162: Tidal system. Changes in overflow height cause rhythmic water-level fluctuations in the culture enclosure. OP: overflow point. (After De Blok, 1964; modified; reproduced by permission of Netherlands Institute for Sea Research.)
siphoned off to the level of the short-arm opening (providing the inflow is smaller
than the outflow through the siphon, and the siphon intake has a larger diameter
than the curvature). The curvature of the siphon must be smaller in the downward

Fig. 2-163: Tidal system. Modification of the apparatus illustrated in
Fig. 2-162. Water-level fluctuations are achieved at constant hydro-
static pressures. Air escapes through a tube which ends near the
bottom of an open vessel in which tidal water movements are generated
in the same manner as described for Fig. 2-162. Water escapes near
the bottom of the culture enclosure via a fixed overflow level. (After
De Blok, 1964; modified; reproduced by permission of Netherlands
Institute for Sea Research.)

than in the upward direction, and the material must be wettable glass, not plastic.
The culture enclosure (Fig. 166b) receives a slow flow of water through a pipe in its
sand bottom (not shown in figure). The intermittent siphon causes rhythmic fluctua-
tions between high and low water. Air escapes or enters at the top.
Valve operations for producing tidal changes have been employed by THORNTON-FORD (1967a, b) for cultivating barnacles attached to Tufnol panels. In his tray tidal system, sea water from a constant-head tank flows through a valve (Fig. 2-167).
Fig. 2.166: Tidal system for cultivating intertidal animals. (a) Intermittent siphon with capillary priming; (b) culture container. High and low indicate tidal range. (After Plessis, 1964; modified; not copyrighted.)

Fig. 2.167: Valve for controlling tray tidal system. An electric motor is mounted behind the Tufnol cam. The head of the plunger assembly which seats against the perspex body (P) is made of nylon. (After TICHELL-FORD, 1967a; modified; reproduced by permission of Laboratory Practice.)
into a series of interconnected, shallow plastic trays and then to waste. A tidal effect is produced by cyclic opening and closing of the valve over 6-hr periods. The Tufnol cam is turned clockwise by a geared electric motor which completes 1 rev. in 24 hrs. High and low quadrants of the cam bear upon a roller on top of a plunger assembly. The high cams push the assembly down, shutting off the water flow. This position is held for 6 hrs, while the water in the trays drains out. The low cam releases the spring, allowing the water to flow again.

For cultivating the intertidal midge *Clunio marinus*, *Neumann* (1968) employed a tidal system (Fig. 2-168), consisting of 2 pumps (K-T ‘Tauchpumpen’ LP 60 made of PVC), two culture enclosures (a and b; 50 x 33 cm; 130 cm high) of 6-mm trans-

![Fig. 2-168: Tidal system for cultivating the intertidal chironomid *Clunio marinus*. a, b: culture enclosures; C1, C2: cultures in glass dishes; V: valves. The timer activates the pumps alternately. (After *Neumann*, 1968; modified; reproduced by permission of Springer-Verlag.]

parent PVC plates, PVC tubing, 4 PVC valves and a PVC overflow. Sea water is pumped alternatively into the 2 containers to a maximum level of 1 m above low water. A timer governs the pumping cycle and allows simulation of the tidal rhythm in the midge’s habitat. The valves make it possible to regulate the degree of tidal change per unit time. The difference of 1 m in water height is effected at an almost constant rate in 2 hrs, and maximum low or high-tide conditions (wavy horizontal lines) are maintained for 4.25 hrs. To avoid critical choking of the pumps, a portion of the water transported is returned to the container being emptied. In this way, turbulence is provided in both culture enclosures. The cultures (C1 and C2) are in dishes resting on the bottom of the containers. *Neumann* placed his tidal system in a room maintained at 20°C and provided controlled photoperiods.

The tolerance of salt-marsh plants to flooding has been examined by *von Weihe*
and Dreyling (1970) in the system illustrated schematically in Fig. 2-169. Paraffined clay pots with a perforated PVC plate and coarse gravel at the bottom served as culture enclosures with a quartz gravel–peat (3%) mixture as substrate. Sea water enters the culture enclosure from below; it is periodically forced into the pots, and allowed to drain from them by a timer-operated pump combined with a constant-level overflow arrangement. In this way, flooding periods can be controlled and varied.

Apparatus for simulating tidal cycles have also been employed by Martin and Reid (1935), Aleem (1949), Freudenthal and co-authors (1963) and Morton (1970).

Fig. 2-169: System for examining flooding and salinity tolerances of cultured salt-marsh plants. (After von Weire and Dreyling, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)

Holding and Experimental Tanks for Fishes

Numerous reports are available on design and construction of holding and experimental tanks. Two examples of successful designs must suffice.

Holding tanks for flat fishes (e.g. Pleuronectes platessa) have been developed by McGregor (1973). The culture water is aerated, cooled, recirculated, filtered and treated with ultra-violet irradiation (Fig. 2-170). One cooling trough (275 x 70 x 30 cm deep) contains 6 separate holding tanks (Fig. 2-171); it is constructed of 20-mm Douglas fir plywood, with cross struts of 10-mm brass rods providing additional strengthening; it is made watertight by an inside lining of black polythene sheeting. Five plaice of 10- to 20-cm length can be kept in each holding tank. The tanks are not self-cleaning; hence, uneaten food and faeces must be removed by siphoning. The unit can be assembled inexpensively and requires little attention unless the
temperature of the mains suddenly changes. A temperature-controlled refrigeration unit would eliminate this problem.

For juvenile Pacific salmon (genus *Oncorhynchus*), plastic holding tanks have been developed by Alderdice and co-authors (1966). Their design (i) provides water circulation for maintaining cultured fish in a state of steady swimming, (ii) avoids projections or surfaces which tend to cause injury, and (iii) allows easy cleaning.

(Fig. 2-172). In addition, the tanks are self-cleaning and minimize accumulation of solid wastes. They have been produced in 3 sizes (80-, 197- and 780-l capacity) (Fig. 2-173). The material used is glasscloth and polyester resin, built up on an inexpensive plywood mould. The mould provides a very smooth finish for the tank interior. The first resin coat on the mould is a colour coat, which forms the inner surface of the tank. Glass cloth and subsequent resin coats are built up to a thickness of about 0.32 cm. The resulting tank is strong enough to hold its shape without distortion when filled with water.
Drain connections are made by setting a 3-8 cm plastic elbow on the mould in the drain position, and laying up the tank around it. The central drain consists of 2 concentric pipes. The inner waste pipe is fitted to the drain connection and extends to within 3-8 to 8-9 cm of the lip of the tank; the outer drain pipe extends to within 1-3 cm of the tank lip, and has a serrated or slotted bottom to allow drainage from the lowest tank area. Water and faces rise through the slots between the 2 concentric pipes and leave by the inner waste pipe. It has been found convenient to set up the holding tanks in boxes of plywood mounted on suitable stands. Temperature control in the tanks is assisted by filling the space between the tank and the plywood box with insulation, such as expanded mica. Tank tops consist of 2 sections: a small acrylic sheet (fixed in place and drilled to receive a water inlet pipe and auxiliary equipment) and a large, reinforced polyester-resin panelling with a hole over the drain (allowing inspection of the waste overflow). Part of the top panel may be painted black to provide some cover for the fish. Water enters the tank through a rigid plastic elbow, capped at the lower end, and fixed in a peripheral position (Fig. 2-172). A series of 6 to 8 vertically spaced, small holes drilled in the pipe serve as horizontal jets, creating circular water movement. For experiments on long-term

![Diagram of cooling trough with 6 holding tanks](image)
energy budgets of cultured fish, the holding tank has been equipped with controlled water recirculation (Brett and co-authors, 1969; Brett and Higgs, 1970; Brett, 1971; Brett and co-authors, 1971). For description of the experimental tank, it is convenient to consider sequentially 3 different components (Fig. 2-174).

(i) Water adjustment. Using manually controlled PVC diaphragm valves, primary control of incoming-water temperature is effected by cross-mixing heated and chilled water. The tank is flushed at a rate of 2 volume exchanges hr⁻¹. The incoming water (Fig. 2-175) passes through a flow meter (F) into the top of a stripper column (S); there it cascades over porcelain saddles counter-current to a rising air flow, in order to avoid deviations from normal air saturation of vital gases. The water passes thence to a bubble trap, whose tubular outlet is inserted through the Plexiglass top of the tank, discharging below the water level (Fig. 2-174). A slight constriction of this tube causes the trap to fill just to the overflow point, assuring bubble-free water input. Since the bubble trap is attached to the removable tank lid, flexible tubing is used for both the tubes leading immediately into, and overflowing out of, the detachable trap. When a closed water circuit is required, the tank drain is closed off to induce complete filling and then, the flow-control valve on the line leading to the trap is turned off; the backed-up water in the stripping column rises to the overflow.

Fig. 2-172: Holding tank for fishes. Left: complete assembly with plastic tank in a plywood box; upper part of the central drain extends above the water surface; water-inlet pipe enters tank through fixed piece of sheet acrylate. Right: a second tank is turned upside down to illustrate tank shape and drain connection. (After Alderdice and co-authors, 1966; reproduced by permission of the Fisheries Research Board of Canada.)
and exits to waste. Adjusted new water may be re-introduced by simply opening this flow-control valve again.

(ii) Tank. Incoming water circulates in the tank at a mean velocity of about 15 cm sec\(^{-1}\), and overflows from a central pipe (an outer pipe of clear Plexiglas, higher than the overflow, forces water through bottom slots, carrying faeces and unused food rapidly to waste; Fig. 2-174). Recirculated water enters through input jets in the tank wall; it produces a vertical water ‘fan’ yielding fairly smooth water movement in the tank. Water is drawn through a rosette of fine holes drilled in the opposite tank wall, and pumped back to an imbedded, perforated pipe. The force of input jets is throttled by a valve in series with a pressure gauge.

To switch into closed circuit, the ‘pressure off-set clamp’ is actuated, pinching off a rubber sleeve at the drain. The water level rises in the tank, impinging on the undersurface of the Plexiglas top. The latter is sealed by jam locks around the periphery, compressing an O-ring seal (upper insert in Fig. 2-174; see also Fig. 2-175). Since the tank is tilted slightly, the rising water displaces all air, and finally forces it out through corner holes in the (slightly higher) front edge of the top. Once the tank is filled to overflowing, the flow-control valve is turned off, leaving the water in the bubble trap to seek a lower level in equilibrium with the flooded top. Sealing-off corks are then inserted in the small flood holes and in the feeding funnel, and a
water sample is taken for oxygen determination (from valve near heat exchanger).

(iii) Recirculation and control. On a platform below the tank are mounted the basic parts for recirculation and control of the culture water (Figs 2-174 and 2-176). A recirculation pump forces the water through a heat exchanger, past a pressure gauge, over temperature and oxygen sensors, and thence into the rising jet pipe. The heat exchanger incorporates a coil, through which chilled water is continually passed at a rate which just overcomes all heat input. A 300-W heater with thermistor sensor is activated by a proportional thermoregulator for precise temperature control (±0.1 °C both in open or closed circuit). The temperature is recorded via a thermograph bulb housed in the sensor chamber (lower inset in Fig. 2-174).
A row of six fully-equipped environmental control tanks is shown in Fig. 2-177. The front control panel and the flow-control valves are readily accessible. Insulated polyethylene lines below the tanks carry chilled water to the heat exchangers. The fish are allowed to temperature-acclimatize in the tanks for 1 month; during this period, food-ration control is applied. The next 6 weeks are devoted to weekly weighings, and 2 weeks (3rd and 5th) to metabolic-rate determinations. Energy expenditure is determined during a continuous 24-hr programme, with each tank cycled on a regular open-closed-circuit schedule (equal on and off periods of 2, 3 or 4 hrs, depending upon the time required to draw down 20 ± 5% of the culture water's oxygen content). The period is set by checking from O₂-probe readings. Energy lost to excretion is determined indirectly (food input = growth + metabolism + excretion) or by direct measurements on faeces trapped at the tank drain in a fine-meshed screen and on ammonia + urea contents of the water (Brett and co-authors, 1971).

Fig. 2-175: Experimental tank. Water adjustment. Labelled offset jam lock is in open position, the other jam locks seal the Plexiglas top of the tank. F: flow meter; S: stripper column. (After Brett and co-authors, 1971; reproduced by permission of the Fisheries Research Board of Canada.)

Feeding Equipment used in Commercial Fish Culture

In aquaculture, the application and distribution of feeds, is one of the most labour consuming processes. Traditional manual feeding has little chance in modern intensified fish culture. Cost of labour, inaccurate rationing, need for regular feeding schedules also on non-work days—these are the main reasons for the development and world-wide application of new mechanical, automatic feeding equipment. Mechanical feeders are also used for a variety of experimental purposes. The per-
performance and efficiency of a large number of feeders have been evaluated by Berka (1973). His review includes addresses of manufacturers. Berka (1973) subdivides the feeders available into stationary and mobile equipment. Stationary equipment is installed in individual culture enclosures; mobile equipment is used for serving a number of cultures consecutively. The development of both stationary and mobile feeders has been greatly facilitated by the introduction of dry, pelleted or granular feeds.
Among the stationary feeders, the equipment illustrated in Figs 2-178, 2-179 and 2-180 depends on controlled energy supply. The feeder shown in Fig. 2-178 employs an electromagnet, that shown in Fig. 2-179, pressurized air. In the latter case, with the magnetic valve (MV) closed, feed pours out to form a small heap in the distributing tube (1) under the discharge opening. As the magnetic valve is opened, compressed air drives the heap along the distributing pipe, together with further feed poured from the container. As soon as the magnetic valve is closed again, the air flow stops and a new feed heap forms, preventing further pouring from the container until the valve is opened again. The air-tight distributing tube is adjustably

fitted into a guide tube; its free end points to the water surface of the fish enclosure. The compressed-air container makes it possible to maintain a certain air-pressure level while the valve is open. This is important, especially when the feeders are placed at a longer distance from the air compressor. Valve operation is timer controlled; during one action, the valve can be kept open for periods varying from 0.1 to 10 secs. Water flow has also been used to operate a variety of stationary feeders, depending on energy supply. For remote areas lacking power lines, battery-powered fish feeders have been constructed (e.g. CARNES and LOUDER, 1970).

Of the stationary feeders operating without man-supplied energy, the most important ones are demand feeders or self-feeders. Unlike the automated feeders, which apply feed even in periods when the fish are not hungry or consume only part of the
feed provided, demand feeders (Fig. 2-181) apply feed only upon determined action by the fish. Hungry fish move a bait lever and thus, release a certain amount of feed from the food container. The fish quickly learn how to push the lever, usually within a day or two. Demand feeders provide parallelism between appetite and food provision, and between diurnal fluctuations in the fishes' locomotory and feeding activities. They further allow a certain control of the fishes' physiological condition: no or too slow decrease of feed-container contents signals that something is wrong, e.g. the presence of disease, parasites or oxygen depletion.

In practical fish farming, successful operation of demand feeders depends on adequate adjustment of the bait rod. When the feed container is full, it is more difficult for the fish to move the rod because of higher feed pressure. For this reason GRIZZEL (1969) and BERKA (1973) recommend making a perforated partition in the middle of the container to reduce the maximum pressure. The bait rod may be moved also by strong water movement or wind. Such undesired interference can be compensated for by placing the feeder in a circular breakwater submerged 12 to 15 cm under the water surface (BERKA, 1973). Some fish are more playful than others and may press the bait lever without being hungry. Increase in the minimum thrust required for feed release may discourage such unwanted activities. Numerous different types of demand feeders are on the market. Three examples are illustrated in Figs 2-182, 2-183 and 2-184.
Since a certain minimum thrust is required for operating the bait rod, fry and small-sized fish cannot handle bait-rod demand feeders. For these cases, demand feeders have been developed that do not require the fish to contact a bait rod. Instead, removal of all feed from a submerged plate activates the feeder. The weight of the food resting on the plate controls the pouring of further feed.

Demand feeders can also be used for catching fish (Stramel, 1970). The feeder is placed in a construction of wire netting with 6 entry funnels for fish on 3 sides (Fig. 2-184). On the fourth side, an outlet funnel leads into a trap (fish pen). The fish caught are carried with the trap to the side of the pond and collected.

Mobile feeding equipment comprises, for example, feeding devices mounted on a pick-up car or a boat. From the car, blowers spread the feed over distances of up to 20 m; from the boat, the feed is distributed by controlled washing out through a bottom slot. In the USSR, vehicle-mounted feeders are sometimes combined with equipment for water aeration and fertilization (Tjuktajev, 1969). In the USA, fish-cage culture is becoming increasingly popular. According to Berka (1973), the cages are usually arranged in rows extending over several kilometres. Feeding and servicing are carried out from barges which pass over the cages. A 2-km line of fish cages can be serviced within 3 hrs. Computer programs take into account stocking density, fish weight and mortality, and determine the preferred harvesting time. The computer programs are continuously adjusted according to weekly fish samplings.

For small-scale experiments, a mechanical food dispenser has been designed and successfully tested by Atron (1972). The food dispenser works at a low voltage and
can be used either for automatic feeding or demand feeding (Figs 2-185, 2-186, 2-187). It can be also operated without electricity by using a clockwork time switch and a car battery. In essence, the food dispenser consists of a solenoid, a food hopper

![Diagram of automatic fish feeder](image1)

**Fig. 2-180:** Automatic fish feeder employing the endless screw mechanism. The screw (enlarged view of screw portion on top) is slowly rotated by an electric motor and transports feed to the fish tank. Timing of the motor controls amount of feed and feeding schedule. (Based on FRICK, 1963; Patent FRG No. 1168314.)

![Diagram of demand feeder](image2)

**Fig. 2-181:** Demand feeder. Working principle. (Drawing Dr. F. LANDLESS.)

and a PVC slide. The keyhole shape of the hole in the PVC slide (Fig. 2-185) is necessary to prevent jamming when the dispenser is used with large pellets. The dimensions of the feeder can be easily adjusted to experimental requirements. The feeder has been used for long periods requiring little attention except for routine cleaning. Experimental fish fed with the automatic feeder were plaice *Pleuronectes platessa,*
salmon smolts and parr *Salmo salar*, as well as larvae of several species of flat fish. The demand-feeder version has been used only for rainbow trout *Salmo gairdnerii*.

For offering fine-particle food to fish larvae, Barnabé (1974) used net bags (the larvae take food pieces through the net) or a screened airlift—a so-called 'feeding hopper'. One screen at the bottom, the other at the top of a tube just below the water surface, the feeding hopper sieves the food and distributes it in the culture tank from one point. This feeder enables the fish to be trained to obtain their food in a restricted area, thus eliminating or reducing the need for them to search throughout the entire water column.

(c) Conclusions

Development of adequate equipment is a key factor for successful cultivation. Numerous devices are commercially available. The home-aquarist industry, the swimming-pool industry and the manufacturers of equipment used for domestic and industrial waste-water treatment offer a large variety of apparatus that can be used directly, or after some modification, for cultivating aquatic organisms. However, in many cases, the cultivator still depends on self-invented or self-made
equipment. The requirements of marine organisms are often very specific as are the conditions for conducting experiments.

We need better and more equipment for gentle collection of test organisms, for controlling environmental factors under fluctuating and multivariate conditions, for cultivating planktonic forms, including nannoplankters, and sediment dwellers. Special attention must be devoted to accommodating suspension feeders and larvae of marine animals under ecologically acceptable circumstances.
CONCLUSIONS

6 BA Nylon screws

2 BA Tapped hole

Rigid PVC Tube

Food hopper (port only)

Food inlet hole

Fig. 2.185: Fish feeder for small-scale experiments. Exploded view. (After ADRON, 1972; reproduced by permission of Conseil International pour l'Exploration de la Mer.)

Fig. 2.186: Fish feeder for small-scale experiments. General layout circuit of control box. (After ADRON, 1972; reproduced by permission of Conseil International pour l'Exploration de la Mer.)
2. CULTIVATION OF MARINE ORGANISMS (G. KINNE)

Acknowledgements. It would not have been possible to write this chapter without assistance from my colleagues and associates at the Biologische Anstalt Helgoland. For advice and criticism, I am particularly grateful to Drs. M. GILBRICHT, W. GREVE, W. GUNKEL, M. HOPPENHEIT, K. LÜNING, K. MOEBUS, H. ROSENTHAL and D. SIEBERS. The help of M. BLAKE, V. CLARK, J.-K. HOLTZMANN, H. L. NICHOLS and H. WITT is very much appreciated. I should also like to thank Drs. D. F. ALDERDICE and J. R. BRETT (Fisheries Research Board of Canada, Biological Station, Nanaimo, British Columbia, Canada) and Dr. T. J. PANDIAN (Madurai University P. G. Centre, Palni, India) for going through sections of the manuscript.

Literature Cited (Chapter 2)


LITERATURE CITED


2. CULTIVATION OF MARINE ORGANISMS


LITERATURE CITED


2. CULTIVATION OF MARINE ORGANISMS


DELAUNAY, H. (1913). Sur quelques faits particuliers à la répartition de l'Azote dans la lique
LITERATURE CITED


FRANZ, J. and GARNEAUX, A. (1971). Entkeimung mit Ozon unter besonders hohen Anforde-


GALTSOFF, P. S. (1937). General methods of collecting, maintaining, and rearing marine in-

GANNING, B. and WOLF, F. (1866). A chamber for offering alternative conditions to small motile aquatic animals. Ophelia, 3, 151–160.


TUTTLE, W. D. (1967). Stabilization of air bubbles at the air–sea interface by surface-active

2. CULTIVATION OF MARINE ORGANISMS


2. CULTIVATION OF MARINE ORGANISMS


LITERATURE CITED


sludge process.


2. CULTIVATION OF MARINE ORGANISMS


2. CULTIVATION OF MARINE ORGANISMS


The effect of...


LITERATURE CITED


SILLIS, J. B. (no date). Saran screen fish barriers. U.S. Fish Farming Experimental Station, Stuttgart, Arkansas, 3 pp. (mimeograph).


2. CULTIVATION OF MARINE ORGANISMS


Sobow, N. N. (1931). Oceanographical Tables, Hydro-meteorol. Committee, Oceanographic Institute, Moscow, U.S.S.R.


2. CULTIVATION OF MARINE ORGANISMS


LITERATURE CITED


2. CULTIVATION OF MARINE ORGANISMS


3. CULTIVATION OF MICRO-ORGANISMS

3.1 BACTERIA

K. GUNDERSEN

(1) Introduction

Bacteria differ significantly from other organisms. They encompass more different metabolic types than any other group; but morphological diversity and number of different species are small. They are easier to culture than most higher forms of marine life; but it seems to be more difficult to cultivate bacteria under conditions that are ecologically meaningful; i.e. under conditions that allow extrapolations of results obtained in the laboratory to situations prevailing in oceans and coastal waters.

In the sea, the total number of organisms in a millilitre of water rarely exceeds a few thousand. Actually, their numbers are so small that statistically one would have to examine hundreds of microscopic fields to be able to see one bacterium. In marine sediments and in productive waters bacterial numbers are considerably higher but, in general, their densities in marine environments are low and are in direct proportion to the nutrient content.

Fortunately—and unfortunately—many marine bacteria are easy to grow if properly fed. Nutrient-rich media and optimal cultivation conditions may readily produce millions of organisms in a millilitre of culture overnight. We always tend to optimize and are never happier than when we have found out how to grow dense liquid cultures and large pretty colonies on agar media. But can meaningful ecological studies really be made with this kind of culture?

The bacteria of the sea are adapted to live at very low nutrient levels and at low temperatures; this certainly does not promote abundant and rapid growth. Chemostat studies of cultures grown at ocean levels of nutrients have permitted the calculation of specific growth rates as low as 0.005 $\text{hr}^{-1}$, corresponding to a generation time of 200 hrs (JANNASCH, 1969). When occasionally more concentrated nutrients appear, e.g. particulate excretions from animals, dead bodies of plankton or other organisms, the nutrient situation may, at a first glance, look similar to the high-nutrient laboratory culture. However, it might be somewhat dangerous to draw a parallel between the two situations. Laboratory cultures comprise pure cultures of enormous population densities or, at best, enrichment cultures of a highly selective nature. In the sea, colonization and growth on particulate matter occur as a dynamic system of numerous positive and negative interactions between mixed microbial populations, and each participating species may rarely reach a population density of more than a few hundred or thousand individual cells.

Population size is important in this connection because gene mutations are known to occur with an average frequency of about one in every $10^8$ cell divisions. A
semispherical bacterial colony of 1 mm diameter (a rather small colony) contains about $5 \times 10^8$ individual cells when these have a size of about 1 $\mu$m$^3$. It is easy to see that potential genetical events alone reduce somewhat the reliability of predictions made from laboratory observations.

There is ample evidence of changes in characteristics of marine bacteria during prolonged cultivation in nutrient-rich laboratory media, e.g., changes of morphology, loss of pigmentation, loss of ability to reduce nitrate, loss of requirement for sea water, etc. Direct proof that changes occur during transition from low to high nutrient conditions was furnished by Jannasch (1968) who showed that some of the major original growth characteristics were lost when marine bacteria, isolated and grown in the chemostat at low nutrient levels, were transferred to a peptone-yeast extract medium.

What is the purpose, then, of cultivating marine bacteria? Can we with the so-called 'pure culture technique', developed almost 100 years ago for the cultivation of pathogenic bacteria, learn something about the microbial systems of the marine environment? To some extent the technique has been useful. It has been possible to enumerate bacteria and to find out about their distribution in oceans and coastal waters; bacteria causing fish diseases have been isolated and studied; the presence or absence of many of the known physiological groups of bacteria in the sea has been demonstrated; and many important nutritional, biochemical, and taxonomic studies on marine bacteria been made.

If we consider the many unsolved problems in marine microbial ecology it becomes obvious that a well-developed cultivation technique is far from enough and that we have neglected to work out new methods and techniques to tackle ecological problems. After at least 70 years of research we still do not know the laws that govern bacterial growth, and metabolism in the sea. We do not know whether bacteria significantly contribute to the productivity of the sea and exactly what rôle they play in the food chains, including mineralization. Bacteria are claimed to be responsible for many of the major characteristics of ocean waters but we still do not know to what extent the oxygen content of the seas is controlled by bacterial activity or where, and at what rate, nitrate regeneration takes place. We do not know whether hydrostatic pressure is a significant factor in bacterial metabolism or whether barophilism is the rule rather than the exception in the depths of the sea, etc.—just to mention a few significant problems facing the microbial ecologist.

Admittedly, the difficulties and expenses of working in areas as large, complicated and diverse as oceans and coastal waters are considerable. It shall also be admitted that some new and meaningful trends in marine microbial research have become apparent, and that new tools and techniques have become available and are being used to a larger and larger extent.

This chapter on the cultivation of marine bacteria contains several examples of the traditional bacteriological techniques which the author, for reasons which are obvious from the statements made in this introduction, does not consider fully relevant in ecological research. A few newer techniques, of which a couple are still in their infancy of use and perfection, will be described and may, hopefully, inspire marine microbiologists to some badly needed inventiveness in this important field.
(2) Growth Requirements

(a) Organic Nutrients

Carbon Sources

A large number of organic carbon compounds can be utilized by heterotrophic marine bacteria as nutrient and energy source. They include simple sugars (pentoses, hexoses, disaccharides, sugar-alcohols, etc.), polysaccharides (cellulose, starch, glycogen, agar, pentosans), organic acids, alcohols, and aldehydes, lipids, hydrocarbons, etc. Although several bacterial species may grow equally well with a number of these carbon sources, singly or in combination, others may have more specific requirements. Many marine pseudomonads, for example, do not utilize glucose but will grow well with fructose and galactose (Colwell and Liston, 1961). In cultivation, the most used carbon sources have been glucose, glycerol, acetate, and succinate. Although many of the sugars may not support growth they may still be fermented and thus serve as a source of energy.

The special carbon requirements of various physiological groups of bacteria will be dealt with in a later section (p. 326ff; see also Volume II: Pandian, 1975; Schlegel, 1975.)

Carbon-Nitrogen Sources

The most luxuriant population growth of heterotrophic marine bacteria occurs on complex media containing peptone or similar mixed proteinaceous materials, including yeast extract. Many attempts have been made to replace peptone with chemically defined compounds. MacLeod and co-authors (1954) conducted an extensive nutritional study on 33 marine bacteria and found that these organisms would all grow in a salt medium with 18 amino acids, several vitamins, and glucose or glycerol as major carbon source. The amino acids, which could replace peptone, and the vitamins used by MacLeod and co-authors are listed in Table 3-1.

A similar investigation was made by Skerman (1963) of bacteria from neritic and oceanic waters and from sediments. A fairly high proportion of the microorganisms required amino acids or amino acids and vitamins for growth. Alanine and proline were found by Colwell (1968) to be particularly good for use in isolation media.

Chitin, a polyglucose amine, can be utilized by many marine bacteria as a source of carbon and nitrogen, but vitamins are usually also required.

Vitamins

The vitamin requirements of marine bacteria have been investigated by MacLeod and co-authors (1954), Burkholder (1963), and others. Burkholder found that a surprisingly high proportion of bacteria from various sources (water and sediments from tropical, temperate, and subarctic regions) required one or more vitamins of the B-complex when grown in chemically defined (holidic) media. Biotin was re-
Chemically defined (holidic) medium for cultivation of marine heterotrophic bacteria (After MACLEOD, 1968; reproduced by permission of Academic Press)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (mg l⁻¹)</th>
<th>Component</th>
<th>Amount (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine*</td>
<td>500</td>
<td>L-Tyrosine</td>
<td>100 mg</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>200</td>
<td>L-Valine</td>
<td>100 mg</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>200</td>
<td>Glucose</td>
<td>3 g</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>100</td>
<td>p-Aminobenzoic acid</td>
<td>1 mg</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>500</td>
<td>Biotin</td>
<td>10 μg</td>
</tr>
<tr>
<td>Glycine</td>
<td>100</td>
<td>Cobalamin</td>
<td>10 μg</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>100</td>
<td>Folic acid</td>
<td>10 μg</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>100</td>
<td>Niacin</td>
<td>1 mg</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>100</td>
<td>Pantothenic acid</td>
<td>1 mg</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>200</td>
<td>Pyridoxal</td>
<td>1 mg</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>100</td>
<td>Thiamine</td>
<td>1 mg</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>100</td>
<td>Tris† buffer, pH 7.5</td>
<td>0.1 M</td>
</tr>
<tr>
<td>L-Proline</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Threonine</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*If DL forms only are available, twice the amounts of the L forms should be used.
†Tris = Tris (hydroxymethyl) aminomethane added as the HCl salt.

The above organic components can be dissolved in sea water, artificial sea water or an appropriate mixture of salts. A salt solution consisting of NaCl, 0.2M; KCl, 0.01M; MgSO₄, 0.05M and CaCl₂, 0.01M, will substitute for sea water for the growth of many bacteria. Both sea water and the artificial salt mixtures require the further addition of an iron salt (e.g. FeCl₃, 0.1mM) and inorganic phosphate (e.g. (NH₄)₂HPO₄, 0.33mM) for good growth.

Adjustments of pH can be made with NH₄OH prepared by boiling a solution of commercial NH₄OH and passing the NH₃ gas evolved into glass-distilled water.

Required by 505 strains, thiamine by 501, cobalamin by 153, nicotinic acid by 97, pantothenic acid by 15, and riboflavin by 1 strain. The vitamins used by MACLEOD and co-authors are listed in Table 3-1.

In mixed cultures of marine bacteria, vitamins usually need not be added, as many bacteria actually excrete vitamins (GANDHI and FREITAS, 1964; STRICKLAND, 1965).

Ascorbic acid is often used in media for anaerobic bacteria but its function is that of a reducing agent and not as a vitamin as such.

(b) Mineral Requirements

Nitrogen, Phosphorus and Sulphur

All organisms require nitrogen, phosphorus and sulphur. Bacteria which can utilize sugar or other nitrogen-free carbon sources can satisfy their nitrogen requirement by simple ammonium salts, e.g. NH₄Cl or (NH₄)₂SO₄, which generally are better than nitrates. Phosphates and sulphates are used readily. Whereas sulphates are abundant in sea water and rarely need to be added to culture media, most
natural waters are low in inorganic nitrogen and phosphorus, requiring the addition of these nutrients in quantities determined by the amount of carbon and other nutrients.

The chemo-autotrophic nitrifying bacteria use ammonium and nitrite as energy and nitrogen sources and are favoured by rather low concentrations (Volume II: SCHLEGEL, 1975). Nitrogen-fixing bacteria use elemental nitrogen as a nitrogen source and can grow on simple carbon compounds. The photosynthetic nitrogen fixers, which are anaerobes, need the addition of nitrogen gas if grown anaerobically in hydrogen gas.

Photosynthetic sulphur bacteria and chemo-autotrophic sulphur bacteria have a requirement for reduced inorganic sulphur, the former as electron donor, the latter as a source of energy. The photosynthetic bacteria use sulphide, the chemoauto-trophs either sulphide, elemental sulphur, or thiosulphate.

**Calcium and Magnesium**

The specific requirement for magnesium and calcium by marine bacteria has been demonstrated by many workers. The two metals are necessary for the normal development and function of the cell wall (HIDAKA and SAKAI, 1968). BRISOU and VARGUE (1963) found that a halophilic Achromobacter required a minimum of 0.5 mM of calcium and magnesium per litre for growth whereas a halophilic Vibrio sp. would grow with as little as 0.1 mM litre$^{-1}$. In media deficient in the two metals lysis would occur in the early growth phase. Extreme halophiles have been found to require 100 to 500 mM of magnesium for optimal growth and stability (BROWN and GIBBONS, 1955).

The two metals can, to some extent, substitute for each other. MACLEOD and ONOFREY (1956b) found, for example, that in media low in calcium, a requirement of from 4 to 8 mM of magnesium existed for maximal growth, but if 2.5 mM of calcium was added, the magnesium requirement was reduced from 8 to 0.04 mM. In general, the calcium-magnesium requirements of marine bacteria are higher than of terrestrial bacteria. Both calcium and magnesium exist in fairly large amounts in natural sea water (Mg, 0.054 M; Ca, 0.01 M) and should not become limiting when natural sea water is used in media.

**Sodium and Potassium**

The specific need for sodium by a marine bacterium was first demonstrated by RICHTER (1928). It has since been established that all Gram-negative marine bacteria require sodium for growth (MACLEOD and co-authors, 1954; MACLEOD and ONOFREY, 1956a; TYLER and co-authors, 1960). MACLEOD (1968) states, that of the 95% of Gram-negative rods found in the sea, the majority require sea water or salt mixtures on initial isolation. Every one of these bacteria examined in detail has been found to require sodium for growth whereas the Gram-positive species, which make up the remaining 5% of the organisms, have not been found to have this specific requirement. It is now generally believed that true marine bacteria can be distinguished from terrestrial species by this criterium.

The concentration of sodium required is in the order of 0.2 to 0.3 M; at lower
concentrations (0.1 M and thereunder) the organisms will lyse after an initial outgrowth. Sodium, in these organisms, cannot be replaced by lithium, potassium, rubidium, or cesium.

Requirement for potassium in marine bacteria has also been established but not as well investigated as the sodium (Payne, 1960; Hidaka and Sakai, 1968).

Iron and Trace Elements

Iron has been found to be required in small amounts (0.1 mM) for most bacteria investigated by MacLeod (1968). For chemo-autotrophic iron bacteria a ferrous salt serves as energy source. Manganese, cobalt, zinc, copper, and boron are necessary elements for most organisms and are functional in various enzyme systems. Whether marine bacteria have any specific requirement for these metals is not well known but they are all present in small amounts in natural sea water. Molybdenum is required by nitrogen-fixing bacteria and is usually added, together with iron and other trace elements as EDTA chelates, to nutrient media.

Carbon Dioxide

Carbon dioxide is the only carbon source for the chemo-autotrophic and photoautotrophic bacteria and as such constitutes a principal nutrient (Volume II: Schlegel, 1975). As a considerable reserve of carbon dioxide is present in sea water, mostly as bicarbonate (about 0.14 g l⁻¹ at pH 8 and salinity 35%), it rarely becomes limiting. However, for intensive cultivation, additional carbon dioxide, usually in the form of sodium or potassium bicarbonate, is added.

Small amounts of carbon dioxide are also fixed by many heterotrophic bacteria but as sufficient amounts are produced by the same organisms in their respiration no addition is necessary.

(c) Sea Water

Natural Sea Water

In the definition of MacLeod (1968) all true marine bacteria require sea water on initial isolation. However, sea water from the open ocean and sea water from an estuary are rarely the same although the proportion of many of the mineral components may be comparable. In addition to wide fluctuations in the total salt content (salinity), the content of particulate and dissolved organic matter is likely to vary considerably (see also Volume I: MacLeod, 1971).

Freshly collected sea water has a slightly bacteriostatic effect (Zobell, 1946) and should not be used directly for the cultivation of bacteria. So-called ‘aged’ sea water is water which has been filtered through glass wool or similar coarse filter to remove larger organisms and particles and stored for several weeks in glass or plastic containers in the cold. During this ‘aging’, a good deal of the organic matter is biologically decomposed and transformed into minerals and bacterial cells. The cells slowly settle leaving a clear water low in organic matter and with a slightly lower pH than the original (see also Chapter 2).
Artificial Sea Water

Because the composition and quality of sea water used to cultivate bacteria may have a pronounced effect on the growth characteristics, the need to use sea water of known and standardized composition has long been advocated. Many formulae, simple and complex, have been suggested and used. The composition of an artificial sea water used by BURKHOLDER (1963) to study nutritional requirements of marine bacteria is shown in Table 3-2. A somewhat simpler formula is indicated in Table 3-1 (see also Chapter 2).

Osmotic Requirements

The total salt content of 'normal' sea water (35%; Volume I, Chapter 4) is about 1.09 M, corresponding to an osmotic pressure of 23.1 atm. Of this, sodium makes up 0.46 M, magnesium, 0.06 M, potassium, 0.01 M, calcium, 0.01 M, chloride, 0.54 M, sulphate, 0.027 M, and bicarbonate, 0.002 M. The range of salinity tolerance by marine bacteria varies but, in general, Gram-negative bacteria (whether from the sea or from other sources) are rather sensitive osmotically compared to Gram-positive bacteria (MacLeod, 1971). Many Gram-positive cocci and bacilli tolerate 20 to 25% sodium chloride (corresponding to 3.5-4.3 M, or an osmotic pressure of 78-96 atm), whereas Gram-negative organisms rarely grow above 10% salt. TYLER and co-authors (1960) found, for example, that of 15 marine bacteria tested, all tolerated 4.7% sodium chloride (0.8 M); 9 would grow at 8.2% (1.4 M) but none at 15.2% (2.6 M). The extreme halophilic bacteria (which actually are Gram-negative pseudomonads) grow optimally at about 25% sodium chloride (4 M).

Many marine bacteria can tolerate low salinities better than high. Actually, ZOBELL and MICHEH (1938) showed that a large number of cultures isolated from the sea grew almost equally well in freshwater medium and in sea-water medium after a few months of cultivation. For this reason, sea water is often diluted with distilled water for use in media in proportions 1:1 or 3:1 to prevent an increase in salinity as water evaporates during cultivation.

Sometimes marine bacteria show a very narrow range of salinity tolerance. For example, KADOTA (1968) demonstrated that a group of marine sulphate-reducing bacteria were restricted to a salinity range corresponding to about 2 to 4% NaCl, clearly distinguishing them from a group of sulphate-reducers of terrestrial origin which were inhibited at about 2% NaCl (Fig. 3-1).

Particulate Matter

Marine bacteria have no requirement for particulate matter; but, like many soil bacteria, their population growth and metabolic activity is often stimulated by the presence of particulate matter in culture media. Fine sand, clay, precipitated calcium carbonate, cellulose fibres, etc., have been reported to stimulate bacterial growth in liquid media. The effect is probably purely physical as ions and dissolved organic matter tend to adsorb to and concentrate on the surface of solids providing a more concentrated and improved nutrient situation. The effect is most pronounced
in nutrient-poor media. The stimulation of bacterial growth by particulate matter has been investigated by ZoBell and Anderson (1936), Gillbricht (1961), Krumbein (1971), and others.

![Diagram](image)

**Fig. 3-1:** Population growth of sulphate-reducing bacteria in culture media as a function of NaCl concentration. Marine strains: *Desulfovibrio aestuarii*; freshwater strains: *D. desulfuricans*. (After Kadota, 1968; modified; reproduced by permission of New York Academy of Sciences.)

(d) Hydrogen-ion Concentration

The pH requirements of marine bacteria vary somewhat with the organism; but most heterotrophic bacteria isolated from sea water seem to grow best in the pH range 7.5 to 7.8. Very few bacteria will grow at all at a pH exceeding 8.5 (ZoBell, 1941a). The lower limit again varies considerably; a pH of 5.5 arrests the growth of most bacteria.

Control of pH in bacteriological media and special pH requirements of various physiological types of bacteria are dealt with on p. 314 and p. 326, respectively.

(e) Composition of Culture Media

**Complex (Oligic and Meridic) Media**

Peptone-type media have been the choice of marine microbiologists for growing bacteria from the sea. The most popular medium during the last three decades has undoubtedly been the so-called 'Medium 2216' and various modifications of it. Medium 2216 was the result of testing a large number of potential nutrients and was found (ZoBell, 1941a) to be the medium which consistently yielded the highest and most reproducible plate counts. The medium has the following composition: Peptone, 5 g; ferric phosphate, 0.1 g; agar, 15 g; aged sea water, 1 l; pH 7.5 to 7.8. The medium is still widely used and is now commercially available under the
Table 3-2
Composition of culture media used for isolation, maintenance and vitamin tests of aerobic, heterotrophic bacteria from the sea. pH adjusted to 7.2 (After Burkholder, 1963; reproduced by permission of Charles C. Thomas, Publisher)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Artificial sea water</th>
<th>Isolation and maintenance</th>
<th>Vitamin† tests</th>
<th>Minus amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>To 1 l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>23.476 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>3.917 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.192 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.664 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KBr</td>
<td>0.096 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.026 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>10.610 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SrCl₂·6H₂O</td>
<td>0.040 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.469 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsicase</td>
<td>2 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soyton</td>
<td>2 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine mud extract*</td>
<td>100 ml</td>
<td></td>
<td>7 mg</td>
<td>7 mg</td>
</tr>
<tr>
<td>Natural sea water</td>
<td>900 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(NH₄)₂(SO₄)₃·6H₂O</td>
<td>15 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difco agar†</td>
<td>To 1 l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artificial sea water</td>
<td>To 1 l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casamino acids (-vitamins)</td>
<td>5 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>2 g</td>
<td></td>
<td>2 g</td>
<td></td>
</tr>
<tr>
<td>Na succinate</td>
<td>2 g</td>
<td></td>
<td>2 g</td>
<td></td>
</tr>
<tr>
<td>Cystine</td>
<td>100 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>100 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>50 mg</td>
<td></td>
<td>50 mg</td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>5 mg</td>
<td></td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Cystosine</td>
<td>5 mg</td>
<td></td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Guanine</td>
<td>5 mg</td>
<td></td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Thymine</td>
<td>5 mg</td>
<td></td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>5 mg</td>
<td></td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>5 mg</td>
<td></td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>1 μg</td>
<td></td>
<td>1 μg</td>
<td></td>
</tr>
<tr>
<td>Cobalamin</td>
<td>1 μg</td>
<td></td>
<td>1 μg</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>600 μg</td>
<td></td>
<td>600 μg</td>
<td></td>
</tr>
<tr>
<td>Pantothenate</td>
<td>800 μg</td>
<td></td>
<td>800 μg</td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>500 μg</td>
<td></td>
<td>500 μg</td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>800 μg</td>
<td></td>
<td>800 μg</td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>0.5 g</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mud extract is prepared by autoclaving 1 kg of wet marine mud together with 1 l of sea water for 20 mins and filtering; preserve under toluene and store in a refrigerator.
†Difco purified agar, 15 g l⁻¹, was used in making media for vitamin tests in plates.
‡ Vitamins are omitted from this medium in appropriate fashion, depending upon the experimental design.

The trade name 'Marine Agar' (Difco Laboratories, Detroit, Michigan, USA).

One of the disadvantages of Medium 2216 and other similar rich media is that some fast-growing bacteria (pseudomonads, cytophages, etc.) usually have formed
large and sometimes spreading colonies at the time their slow-growing counterparts start to form visible colonies. BURTON (1968) found that this could be avoided by diluting the standard media. By doing so, he observed that a 1:10 dilution gave twice as many bacterial colonies as undiluted medium but the colonies were considerably smaller. Other investigators have had similar experiences. TYLER (unpublished) has observed that agar plates prepared with 0.5% gelatin as the only organic nutrient gave more but smaller colonies than media prepared with peptone and other complex proteins. Chromogenesis was also found to be better on gelatin agar plates.

A wide variety of complex organic materials have been used for isolation and pure culture media of marine bacteria: beef extract, fish meal, enzymatically digested casein, soy meal, corn meal, blood, yeast extract, casamino acids, soil extract, marine mud extract, etc. In addition, various sugars and other well-defined organic materials have been added for maximum growth. For composition of various selective media consult p. 314.

Defined (Holistic) Media

Chemically defined media have been used for photo- and chemo-autotrophic bacteria which have no organic requirements but have not been too popular for the cultivation of heterotrophic bacteria (see also Volume II: SCHLEGEL, 1975). Several useful holistic media have, however, been described. Components of two such media are listed in Tables 3-1 and 3-2.

(f) Oxygen and Other Electron Acceptors

In a study of the relation to oxygen of 150 bacteria isolated from various depths in the Pacific Ocean, OYE and GUNDERSEN (1970) found that between 30 and 40% of all bacteria were aerobic and required oxygen for growth. About 60% were facultative anaerobes and would grow in the absence of oxygen provided other electron acceptors were available; 5% of the bacteria were micro-aerophilic, growing best in the presence of low concentrations of oxygen. No bacteria from the water column were obligate anaerobes; but such forms are not uncommon in anoxic waters, in sediments and in the littoral zone.

The amount of oxygen required by aerobic bacteria varies considerably: the average is about 200 $\mu$g $\text{mg}^{-1}$ dry weight (corresponding roughly to 10$^9$ cells) hr$^{-1}$. One litre of sea water at 35$^\circ$S and 25$^\circ$C contains, when in equilibrium with air, about 7 mg of oxygen, or 7 $\mu$g ml$^{-1}$. This amount would therefore be used up by a population of 3.5 $\times$ 10$^7$ bacteria ml$^{-1}$ in 1 hr, provided sufficient respirable material were available. ZOBELL (1940) found that the rate of oxidation of organic matter in sea water was not affected by the concentration of oxygen in the range of 0.3 to 12.74 ml l$^{-1}$ but was influenced by the concentration of organic matter.

A surprisingly large proportion of heterotrophic marine bacteria are capable of growing in the absence of oxygen by turning to a fermentative type of metabolism or by using nitrate as electron acceptor, if available. The efficiency of the anaerobic type of metabolism is significantly lower and population growth is correspondingly slower. If nitrate is used as electron acceptor, the reduction product is often nitrite;
but many heterotrophic marine bacteria reduce nitrite further to nitrogen gas, ammonia, and apparently also to some unknown intermediate.

Sulphate is used as electron acceptor by some obligate anaerobic bacteria (sulphate-reducers) with the production of sulphide. Obligate anaerobic photosynthetic bacteria use either carbon dioxide (green and purple sulphur bacteria) or organic materials as electron acceptors (purple non-sulphur bacteria.)

(g) Light

The ecological significance of light for marine bacteria has been dealt with in Volume I: GUNKEL (1970).

![Diagram of pigment absorption spectra](https://example.com/diagram.png)

**Fig. 3-2:** Pigment absorption spectra (relative values) of purple and green photosynthetic bacteria and a green alga. (After Stanier and co-authors, 1970; reproduced by permission of Prentice-Hall, Inc., Englewood Cliffs, N.J.)

Among the marine bacteria only photosynthetic forms are known to require light. To these organisms, light is the only source of energy (the non-sulphur purple bacteria can also use organic energy sources when grown in air in the dark). The light-absorbing pigments—bacteriochlorophyll $a$ or $d$ in the purple bacteria and bacteriochlorophyll $b$ or $c$ in the green bacteria—have characteristic absorption spectra which differ in major respects from pigment spectra found in other photosynthetic organisms. As seen in Fig. 3-2, a substantial portion of absorption occurs in the far-red and infra-red parts of the spectrum at wavelengths which are readily absorbed by sea water. All photosynthetic bacteria can be cultivated in artificial light provided it contains a fair amount of longer wavelengths.

The lethal effect of ultra-violet light and high intensities of other wavelengths on bacteria is well substantiated. The frequent occurrence of pigmented bacteria
in the sea, particularly in the upper water column, has led to the assumption that the pigments (mostly carotenoids) of such bacteria serve as photoprotective agents in a manner similar to the photoprotection demonstrated in the yellow non-photosynthetic bacterium *Sarcina lutea* (Mathews and Sistrom, 1959). Carotenoid-less mutants of *S. lutea* are quickly killed when exposed to direct sunlight in air, whereas the pigmented wild-type is not. Photokilling is due to photo-oxidation and, therefore, both light and air (oxygen) are necessary. However, no conclusive proof has been presented to substantiate a mechanism of photoprotection in marine bacteria except in extreme halophiles (Larsen, 1962).

Moderate light intensities are not known to affect marine bacteria; but it could well be that some light-killing occurs when bacteria from deep dark water are, during sampling, suddenly exposed to strong sunlight. For example, the marine nitrifying bacterium *Nitrosoctys oceana* is sensitive to sunlight and will only grow well in subdued light and in darkness (Gundersen, unpublished).

(b) Temperature

The temperature relationships of marine bacteria have been fairly well investigated and have been discussed in detail in Volume I: Oppenheimer (1970).

(i) Hydrostatic Pressure

Hydrostatic pressure (Volume I, Chapter 8), has not yet been shown to be a general requirement for growth of marine bacteria. However, many demonstrations of pressure effects on permeability of cell membranes, cell-wall formation, as well as on density, viscosity and other physical properties of the cytoplasm and of sea water show that pressure is a factor of ecological significance in oceans and coastal waters. The possibility even exists that the deep sea harbours bacteria for which pressure is an absolute requirement, but this will not be known until sampling and cultivation equipment is developed which will permit manipulation of deep-sea samples without interrupting decompressions (see also ZoBell, 1970; Volume I: Morita, 1972).

(3) Culture Methods

The methods used in the cultivation of marine bacteria are not unique. With a few exceptions they represent methods developed in the early days of medical bacteriology which are now used widely in the entire field of microbiology. This section discusses some of the most frequently and most widely used types of culture media and techniques for the cultivation of marine bacteria.

(a) Basic Culture Techniques

Solid Media

Many useful morphological and biochemical characteristics of bacteria can be studied best on agar plates, e.g. size and shape of colonies, pigment formation, motility and swarming, production of exoenzymes. Agar plates are also used gen-
erally as isolation medium. When one or two drops of sea water, sediment suspension, or liquid enrichment culture are spread evenly over the surface of a selective agar medium only those bacteria which will grow on that particular medium, and under the conditions of incubation will form colonies. Dispersal over the surface reduces competition for nutrients and other interactions between different colonies so that slow-growing bacteria may form colonies under the same conditions as those growing more rapidly.

Bacteria may also be isolated from pour-plates. These are prepared by adding the inoculum to a volume of sterilized agar medium (while still molten) in a test tube, inverting the tube several times to ensure good dispersal and pouring the content into a sterile Petri dish where the agar gels. Subsequent population growth appears in form of lens-shaped colonies within the agar which can readily be picked up with an inoculation needle or a Pasteur pipette for transfer to fresh medium.

GUNNEL and co-authors (1961) have pointed out that whenever sea water is mixed with warm agar for pour-plates, roll-tubes, etc., a risk of heat-killing exists. Many marine bacteria are injured by prolonged exposure to $30^\circ$ C or higher temperatures (ZOBELL and CONN, 1940). It is recommended, therefore, that the agar always be cooled to $42^\circ$ C before adding the inoculum and that the agar volume be kept small (10 ml or less) to facilitate fast cooling.

Tubes are used for a variety of purposes, for example as agar slants for preservation of cultures. Agar shake tubes are employed mainly for isolation of anaerobic bacteria, e.g. sulphate-reducers and photosynthetic bacteria. Narrow, long test tubes, preferably with screw caps, are filled to about $4/5$ with melted nutrient agar and autoclaved. When the agar has cooled to about $42^\circ$ C, the tubes are placed on a water bath of the same temperature. A few drops of inoculum are taken from a liquid enrichment culture and added to one or two tubes which are at once mixed by inversion. If necessary, dilutions are prepared by transfer to further agar tubes. Immediately after inoculation the tubes should be cooled. When colonies have appeared, the glass tube is cut open with a diamond cutter and colonies removed with a Pasteur pipette. Roll tubes are used for aerobic bacteria; in this technique, the agar is permitted to gel while the tube is slowly being turned horizontally along its long axis resulting in a large internal surface area.

Agar, at a concentration of 1 to 2%, is the most preferred gelling agent for bacteriological media. In semi-solid media, mostly used for studying bacterial motility, agar is used in a concentration of 0.2 to 0.5%. Agar-digesting bacteria, which are not uncommon in the sea, may sometimes ruin agar cultures by completely liquefying the medium. Gelatin, at a concentration of 10 to 12%, is a good gelling agent; but it is readily attacked by a large number of proteolytic bacteria. A biologically inert gelling agent is silica gel which in many respects is the ideal solidifier for sea-water media. Still, silica plates are little used because they are laborious to prepare, compared to agar media. A workable and convenient method for the preparation of silica plates has been described by INGELMAN and LAURELL (1947).

**Liquid Media**

Liquid media are mostly used for determination of growth rates, for production of large bacterial crops, and in the enrichment technique. In the latter case, a liquid
selective medium is inoculated and incubated under a given set of conditions. Since any primary inoculum contains a variety of organisms, those for which the conditions are most suitable will outgrow the others. The efficiency of the enrichment can be controlled by manipulating such factors as temperature, salinity, pH, nutrient composition, and oxygen tension. Antibiotics and other inhibitors, known not to affect the bacterium for which selected, may further enhance the efficiency of selection. Isolation of the bacterium can normally be achieved by serial dilution through a series of tubes of liquid medium or by plating on a solid medium.

Diagnostic Media

For the purpose of identifying marine bacteria, a variety of diagnostic media are required. Such media are usually made from a basal sea-water medium containing all necessary components not to be assayed. To the basal medium is then added whatever substance is required for the determination of nutrient requirements (e.g., carbon and nitrogen sources, vitamins), substrates for biochemical tests, nitrate for nitrate-reduction, etc. Standardized procedures for diagnostic analyses have been described in Pelczar (1957). A simplified scheme for the identification of marine Gram-negative bacteria has been proposed by Shewan (1963); but the standard reference work for bacterial classification is still Breed and co-authors (1957). However, the fundamentals of bacterial taxonomy are presently in a state of revision (Mandel, 1969), and particularly the marine microbiologist is likely to encounter frustration in attempting to identify an unknown bacterium.

Adjustment of pH

The natural buffering system of sea water becomes highly inadequate when large amounts of organic matter and other substances are added. Although the original pH may not be significantly changed by such additions, bacterial activity rapidly results in production or removal of sufficient hydrogen ions to drastically change the pH. In most cases it is necessary, therefore, to add pH-buffers to sea-water media. Phosphate buffers are not practical as they tend to form insoluble phosphates with metal ions in sea water. More useful are tris buffers (tris-hydroxymethylaminomethane and hydrochloric acid) with a range of pH 7.2 to 9.0. Tris buffers can be used at concentrations of 50 to 100 mM and are non-toxic to most bacteria.

Precipitated calcium carbonate is an efficient buffer for use in cultures of nitrifying bacteria, serving at the same time as a neutralizer for the acid produced and as a carbon source. The particulate nature of calcium carbonate is in itself stimulatory to bacterial growth by providing large solid surfaces.

A suitable pH indicator for sea-water media is bromthymol blue (blue in alkaline, yellow in acid solution). One ml of a 0.04% solution of the indicator (in 50% ethanol) 1⁻¹ of medium will add a clear blue colour to media above pH 7.4.

Sterilization

Media can be sterilized in various ways, for example by autoclaving. Most workers seem to use 'hard' autoclaving, i.e. 120°C for 20 mins, which usually results in precipitation of solids from sea water. Jones (1967) analyzed such precipitates by
emission spectrography and neutron activation and found that the precipitates consisted of small amounts of a large number of elements, notably Mg, Si, and Ca. Calcium carbonate, with an aragonite structure, was identified consistently. Natural sea water contains very few spore-forming bacteria and can, therefore, be sterilized by simple pasteurization which will not result in precipitation. If organic materials are to be dissolved in the sea water base it is safer to sterilize these separately in a small volume of distilled water and to add the organics to the pasteurized sea water afterwards.

Sea water and sea-water media can also be sterilized by filtration through membrane filters, asbestos filters, or through fritted glass or porcelain filters. When using filters for sterilization one should realize that minute reproducible bodies of many marine bacteria may pass through filters of about 0.5 \( \mu m \) porosity (Anderson and Heffernan, 1965). Certain types of membrane filters contain small amounts of detergents (Triton X-100 and similar products) to increase their wettability and filtering efficiency. Such filters have bacteriostatic properties (Cahn, 1967).

U.v.-irradiation of sea water in quartz vessels renders it sterile and also results in almost complete oxidation and mineralization of any organic matter present (Armstrong and co-authors, 1966; see also Chapter 2). Hamilton and Carlucci (1966) investigated possible toxic effects of u.v.-irradiated sea water but found that it could safely be used for cultivation of marine bacteria and diatoms. However, brief autoclaving was required to make it comparable to artificial sea water and charcoal-filtered natural sea water for use in vitamin \( B_{12} \) assays.

### Preparation of Inocula

The use of aseptic sampling techniques during the collection of material for enrichment and isolation cultures is, of course, mandatory in bacteriology. The sampled material may sometimes contain a large number of organisms and need to be diluted to serve as a suitable inoculum. This would, for example, be the case with most sediments, intestinal material from marine animals, eutrophic water, etc., which can best be diluted in sterile sea water or enrichment medium. Other types of samples, e.g. water from the open ocean, are mostly low in organisms and some concentrating might be needed. This can be done by centrifugation (not practical on board ship) or by filtration. Bubble scavenging in a foam tower has been used with some success in concentrating bacteria from sea water (Carlucci and Williams 1965). Aggregates of bacteria can to some extent be broken up, and bacteria attached to particulate matter be released by treatment with surface-active agents (Jones and Jannasch, 1956).

Membrane filters have been found extremely useful in marine microbiology to concentrate bacteria from sea water. After filtration of a measured volume of sea water or a suspension of other material under aseptic conditions, the filter membrane can either be transferred to a liquid enrichment medium or be placed directly on a nutrient agar plate and incubated. In the latter case, the filter pad is kept moist by contact with agar, and nutrients and waste products diffuse through the filter pores. Pure cultures are readily obtained when colonies have grown out and can be transferred to fresh medium for further cultivation.
Aerobic and Anaerobic Cultivation

Most bacteria isolated from oceans and coastal waters will grow, and mostly grow best, in the presence of oxygen. To secure an adequate supply of oxygen in cultures, various types of flasks and bottles which provide a relatively large liquid--air interface are preferred. The efficiency of aeration can be considerably increased by the use of a rotary or reciprocal shaking machine or by blowing sterile-filtered compressed air through cultures via an airstone. In large-scale culture vessels, so-called fermenters, aeration is optimized by simultaneously forcing air through the culture and stirring.

Anaerobic culture techniques require special glass or stainless steel jars (Brewer jars) in which the air can be replaced by nitrogen, hydrogen, or other biologically indifferent gas (hydrogen gas in some cases functions in anaerobic metabolism). Cultivation of anaerobic bacteria has been considerably facilitated by the observation that bacteria can be protected from the toxic effects of oxygen by adding strong reducing agents to the medium. Sodium-thioglycollate at a concentration of 1 g l⁻¹, or traces of cysteine or sodium-sulphide (0.1 g l⁻¹) in media make it possible to handle anaerobic cultivation under field conditions when the more bulky jar technique is impractical.

Measuring Bacterial Growth

A variety of methods can be used to determine bacterial growth (population growth):

(i) Direct microscopic counting of cells in a bacterial counting chamber;
(ii) Plating of a known volume of culture (or a dilution) on an agar medium and counting the colonies after incubation;
(iii) Filtering a known culture volume (or dilution) through a membrane filter; incubating on agar medium, and counting colonies;
(iv) Serial dilution (usually 1:10) in liquid medium, incubation, and computation of cell number in the original culture from observations on growth; the most dilute culture which has supported growth must have contained at least one cell;
(v) Measuring the turbidity of a liquid culture in a colorimeter or spectrophotometer; to obtain actual cell numbers, turbidity readings must be calibrated against direct counts or plate counts;
(vi) Determining the dry weight of cells after filtration or centrifugation and subsequent drying in an oven at 105°C;
(vii) Determining a characteristic cell component (e.g. protein, ATP) or a metabolite (e.g. organic acid, carbon dioxide). This method does not necessarily correlate with the actual cell mass or number, but under standard conditions and after calibration it is often found convenient.

Maintenance of Cultures

Maintenance of marine bacteria cultures requires regular transfer to fresh medium to prevent drying out of the medium and death of the organisms. Stock cultures are usually kept on agar medium (slants) at low temperature (0°-4°C) and are trans-
ferred every 1 to 2 months. If overlaid with sterile paraffin oil, transfers can be delayed for several months.

The best method of preserving cultures is by lyophilization; but special precautions must be taken to prevent dehydration of the cells due to concentration of the sea-water medium (evaporation). A critical osmotic state will occur before evaporation is complete; it may result in collapse of the cells. OHY AND GUNDERSEN (1970) found that several suspending media used commonly in lyophilization of non-marine bacteria were unsuitable for marine forms. They used a medium consisting of 200 g dehydrated skim milk in a mixture of 50 ml of aged sea water and 950 ml of distilled water at pH 7.5. Young bacterial cells grown on marine agar (Difco) were used. Ampules were kept under vacuum for 6 hrs, sealed and stored at 0°C. Bacteria which did not survive lyophilization in the skim milk medium usually could be lyophilized in a medium consisting of 5 g peptone and 5 g yeast extract in 250 ml of sea water and 750 ml of distilled water.

**Stock Culture Collections**

Numerous strains of marine bacteria have been deposited with many of the large culture collection (ATCC, NCTC, and NCIB). For storage of stock cultures of marine bacteria and other micro-organisms, the National Collection of Marine Bacteria (NCMB) was established in 1947 at the Torry Research Station in Aberdeen, Scotland. The collection presently comprises more than 2000 strains of marine bacteria which are available to researchers for a nominal charge.

(b) Special Culture Techniques

**The Chemostat**

The chemostat has proved to be the most useful cultivation device for the study of bacterial growth under nutrient-limited conditions (see also Chapters 2 and 4). Basically, the chemostat is a continuous culture system by which a controlled steady-state condition can be established and maintained. Fig. 3-3 shows the features of a chemostat used by JANASCH (1967) to study growth of marine bacteria at very low nutrient levels characteristic of the open-ocean ecosystem.

When steady-state conditions have been achieved, the nutrient concentration in the culture vessel ($\bar{s}$) is dependent on the growth rate ($\mu$) but independent of the limiting nutrient in the reservoir ($s_0$). The population density ($\bar{n}$) is proportional to the limiting nutrient, thus:

\[ s_0 - \frac{\bar{n}}{y} + \left( \frac{D}{\mu_{\text{max}} - D} \right) \]

and

\[ \bar{s} = K_s D/\left( \mu_{\text{max}} - D \right), \]

where $D$ is exponential dilution rate, $\mu_{\text{max}}$ is the maximum growth rate at excess concentration of the limiting nutrient, $K_s$ the saturation constant, i.e. concentration of the limiting nutrient giving rise to one half the maximum growth rate, $y$ the yield coefficient, i.e. the average amount of limiting nutrient consumed per unit
weight of cell material produced (JANNASCH, 1967). Some basic and important information on the control exerted by nutrient-poor ocean water on bacteria growth, and on the peculiarities of the bacteria which can exist under such conditions, have been obtained by JANNASCH (1968, 1969).

The chemostat has also been used for isolation of marine bacteria with low nutrient requirements (JANNASCH, 1964; HAMILTON, 1968). HAMILTON used an artificial sea-water medium enriched with 50 to 100 µg of glucose l\(^{-1}\) in addition
to a nitrogen and phosphorus source and inoculated the system with natural sea water. The medium was pumped through the chemostat at a flow rate of 50 to 100 ml 4 hrs$^{-1}$. After 4 to 7 days, the culture vessel contained one or two bacterial species which would multiply at the low nutrient concentration whereas others, with higher requirements, had been washed out. Interestingly, the culture vessel would also contain one or two species of bacteria which were not able to grow at the low nutrient concentration per se but had established themselves in a form of balanced co-existence with the low-nutrient organisms.

Apart from these investigations it seems that the chemostat has, unfortunately, been little used to study growth and nutrition of marine bacteria.

**Hydrostatic Pressure Cultivation**

The basic equipment for cultivating marine bacteria under elevated pressures was developed by ZoBell and Oppenheimer (1950); it is, with various modifications and improvements (Landau, 1970), still used in many laboratories (Volume I: Morita, 1972).

The equipment consists of a heavy stainless-steel cylinder with a screw cap and a high-pressure needle valve. Bacterial cultures, contained in small glass tubes with neoprene stoppers, are placed in the cylinder cavity which is completely filled with hydraulic fluid (mostly a 50:50 mixture of glycerol and water). The cylinder is connected through hydraulic tubing to a pressure pump by which the desired pressure is produced. Most equipment is constructed to withstand at least 1000 atm, the hydrostatic pressure prevailing in the greatest depths in the ocean (Volume I: Kinne, 1972).

There have been some problems connected with the cultivation of bacteria in this type of pressure equipment. Because of the relatively small volume of culture liquid which can be used, and because of the necessity of closing the culture tubes with tight stoppers, aerobic bacteria rapidly use up the small amount of oxygen available. Also, waste products, particularly carbon dioxide from respiration, accumulate and tend to produce secondary effects.

To overcome oxygen exhaustion, Berger and Tam (1970) substituted the culture tube with a selectively permeable membrane bag which permits gas exchange between the external hydraulic fluid and the culture while retaining water and solutes.

**Controlled Model Ecosystems (Microcosms)**

Kawai and co-authors (1964, 1965, 1968) have shown that it is possible to determine quantitatively the growth and metabolic activity of several different physiological types of marine bacteria in a closed model ecosystem (Chapter 6). They used an aquarium with fish, equipped with a sand bed resting on a screen a few centimetres above the bottom (Fig. 3-4). An air-water lift maintained a circulating current of aerated water in the aquarium and permitted continuous water percolation through the sand bed. Chemical and bacteriological changes occurring in sand and water were monitored regularly during a period of several months.

The analyses showed that the bacterial populations of both filter sand and culture
water changed markedly during the early stages of cultivation, but stabilized somewhat after 1 to 2 months (Fig. 2-5, c and d). A comparison of axenic cultures of nitrifying bacteria with the accumulation of nitrite and nitrate in the model ecosystem showed that nitrification in the sand bed proceeded in a manner almost identical to the axenic culture. (For a detailed account on axenic cultures consult Chapter 5.11).

A similar system, the model sea-bed, was devised by Liston (1964) who studied the microbial degradation of gelatin, agar, cellulose, chitin, and other substrates in water percolated through the system.

The Biochemical Potential Technique

This technique is, in principle, an enrichment technique but its purpose is not the ultimate isolation of bacterial cultures. Instead, it measures the integrated response to enrichment of the entire population of an isolated sample of an ecosystem. To

![Fig. 3-4: Model ecosystem. Aquarium used to study bacterial growth and activity. (After Kawai and co-authors, 1968; modified; reproduced by permission of the authors.)](image)

...make such measurements meaningful in an ecological sense, it is important that the quantity of added substrate be kept within reasonable ('natural') limits, that a minimum of physical disturbance be made to the sample, and that the duration of the experiment be as short as possible. Labelled substrates and sensitive chemical methods are essential in this technique.

A classical example of the biochemical potential techniques is the method for determining primary production by phytoplankton in water bodies (Stemann Nielsen, 1952). Here, the uptake of carbon-14-carbonate is used as a measure of potential light-dependent growth, but the method also gives information on the carbon uptake in dark reactions by phytoplankton and by heterotrophs.

Vaccaro (1969) attempted to determine the heterotrophic potential for organic materials in ocean water by enrichment with carbon-14 labelled carbohydrates, amino acids, and organic acids. But only by preconditioning the populations to the nutrients during a 24-hr period prior to the experiment was he able to derive uptake rates on which mathematical analyses could be used successfully. In short-term experiments (3 hrs), uptake of any nutrient was irregular and unpredictable and not suitable to Michaelis-Menten kinetic analysis. Vaccaro believes that the organic
Fig. 3-5: Model ecosystem. (a) and (b): changes with time of chemical parameters in the water; (c) and (d): growth of various physiological types of marine bacteria in the sand bed. COD: chemical oxygen demand. (After Kawai and co-authors, 1964; reproduced by permission of the authors.)
carbon turnover rates in the open ocean are too low to be measurable even with the sensitive isotope technique, but that more success is likely to be obtained in estuarine and coastal waters where the heterotrophic activity is more intense.

That this may be correct was demonstrated by Harrison and co-authors (1971) who used a technique similar to Vaccaro’s (1969) to determine the heterotrophic potential of sediment. Instead of measuring nutrient uptake (glucose and acetate), these authors determined the amount of carbon-14-carbon dioxide produced from the nutrient and showed that a linear response could indeed be obtained within an experimental period of less than 15 mins.

![Graph showing changes in nitrate content](image)

Gundersen and co-authors (1972) measured the nitrifying and nitrate-reducing potential in a profile of the water column in the Pacific Ocean by enriching water samples with 14 mg of NH₄—N l⁻¹ or with 14 mg of NO₃—N plus 1 g of glycerol l⁻¹ and determining the changes which occurred in the nitrate content of samples after a relatively long incubation period (30 days). The result of this experiment is shown in Fig. 3-6. The nitrifying potential was evidenced by an increase in the amount of nitrate over the initial, whereas the nitrate-reducing potential was shown by an overall decrease in nitrate in the incubated samples.

(c) *In situ* Techniques

In chemostats and model ecosystems (microcosms), experimental conditions, to a considerable extent, can be kept under the experimenters control (see also Chapter
6). The situation becomes more complex and uncontrollable when attempting to study bacterial growth in nature. Brock (1971) has discussed the many difficulties encountered by the microbial ecologist working in the field, but has also pointed out and given examples of several workable methods for measuring microbial growth and activity in situ. A few techniques which are applicable to marine systems will be discussed here.

Submerged Slide

The submerged-slide technique was initially developed by Cholodny (1930) for examinations of the microflora of soils; the technique has since been modified and has been much used in aquatic research (e.g. ZoBell and Allen, 1933; Kriss and Markianovich, 1954; Sobokin, 1963).

Almost any insoluble material will, when immersed in a solution such as natural water, adsorb ions and various polar and non-polar substances (Marshall and co-authors, 1971; Sechler, 1972). The physico-chemical properties of the original solid may change rapidly as a complex microlayer is formed over its surface. If microscopic plankton organisms, including bacteria, are also present in the water, they too may settle on the surface, as a result of electrostatic attraction, by chemotaxis, or simply by chance collision and adhesion. Provided the surface constitutes a favourable substratum for an organism it may proliferate and eventually give rise to a small population.

Because of adsorption, it is likely that biological events occurring on the surface are more dependent on chemical and biological characteristics of the water than on the original nature of the surface itself (except for toxic or biodegradable materials); therefore, microscopic examinations of glass slides, subsequent to a proper period of exposure to water, are representative of the conditions in the water itself. On this premise, Kriss and Markianovich (1954) used the submerged-slide technique to determine bacterial growth rates and biomass increases in various seas. Their results were astonishing as they were able to show that, in some cases, a doubling of bacterial biomass would occur in 24 hrs.

The validity of these findings can be questioned as it has long been known that solid surfaces per se are promotive to bacterial activity and growth (ZoBell and Anderson, 1936; Volume I: ZoBell, 1972) hence micro-biological events are likely to become accelerated when occurring on the surface of a glass slide. Various interactions between microbial populations, such as synergism, predation, antagonism, etc., as well as minute local changes in pH, oxygen tension, etc., may also selectively modify the biotic equilibrium.

Used with care, however, and particularly as a supplement to other techniques, the submerged-slide technique may well be a useful tool in marine microbial ecology.

The Peloscope

The peloscope is a clever little invention byPerfiliev and Gare (1969); it can be used to study the microflora of the upper layer of sediments in lakes and marine environments. It consists of an assembly of flat capillaries attached to a frame. When inserted into the interface sediment–water and left undisturbed micro-
organisms will grow within the capillaries in zones corresponding to the natural zonation of the sediment. When removed, the capillaries can be studied under the microscope and can be broken up and the content used to inoculate nutrient media for further enrichment.

Fig. 3-7: In situ incubator, ISIS (1 litre; Kahl Scientific Instrument Corp., El Cajon, California, USA) used in a primary production experiment with one light bottle (left) and one dark bottle (right). Bottles are interchangeable, facilitating installation of 2 dark bottles, 2 light bottles, or 1 light bottle plus 1 dark bottle. Bottle lids are cocked open during descent through the water column and forced to snap shut by two lateral rubber springs when triggered by a messenger. Bottles can be drained through valves in the lower lids; air vents are on top lids. (Photograph by R. B. Hanson, University of Hawaii.)

In an experiment,Perfiliev and Gabe (1969) were able to demonstrate 8 microzones over a vertical distance of 2 mm in the sediment of a lake. The upper zone consisted of photosynthetic diatoms, followed by a zone of iron bacteria (Ochrobium sp. and Gallionella sp.), a predatory bacterial zone (Dictyobacter sp.), zones of nitro-
gen-fixing bacteria, filamentous bacteria, etc. The portion of the capillaries which represented the deepest part of the sediment exhibited considerable reducing potential with *Desulfovibrio* sp. and precipitates of iron sulphide.

![Fig. 3.8: In situ incubator (2 litre) for determining rates of nitrogen fixation in coral heads and sediments. Nutrients are added and samples removed through valves and inlets (closed with serum bottle stoppers) in the top lid of the experimental chamber. The lower compartment (air filled) contains batteries, a magnetic stirrer, and lead weights for anchoring. (Photograph by R. B. Hanson, University of Hawaii.)](image)

**In situ Incubators**

Most important are experimental ecological studies at the site of events. By removing samples and specimens from the environment for analysis one inevitably disturbs physical and biological equilibria and may not be able to make valid observations. *In situ* incubators attempt to avoid this by permitting remote intended manipulations within the ecosystem. Fig. 3.7 shows an incubator used for experimental work in the water column. It was originally designed for *in situ*
determination of primary production (GUNDERSEN, 1973), but has proved useful for
a number of other ecological studies based on the biochemical-potential technique
(p. 320). Nutrients—e.g. carbon-14 labelled carbonate, amino acids, algal hydro-
lysate, etc.—are released into one or two bottles (if one only, the other serves as a
control) as they close by breaking the glass ampoule which contains the nutrient.
Closing is triggered by a messenger sent along the hydrographic cable from the
supporting ship. During incubation, the in situ incubators will remain at the depth
at which they were closed, suspended under the ship or under a drifting or anchored
buoy. At the end of the incubation period, the incubators are recovered and their
contents analyzed.

Another type of in situ incubator, developed by HANSON (unpublished) for deter-
mination of nitrogen fixation in corals and sediments, is shown in Fig. 3-8. The use
of this incubator requires that the experimenter be capable of diving to the depth
of incubation, placing the coral head specimen, a sample of sediment, or whatever
is being studied, inside the incubator and manipulating the enrichment and periodic
sampling with the help of syringes and other tools he can bring with him.

Both types of in situ incubators are used in marine ecological research at the
University of Hawaii.

(4) Physiological Types

(a) Heterotrophs

Most bacteria in oceans and coastal waters depend on organic material as carbon
and energy sources and participate in the transformation of material which, in the
end, becomes fully oxidized and decomposed to mineral components (Volume II:
PANDIAN, 1975, SCHLEGEL, 1975; Volume IV: SOROKIN, 1977). A small fraction of
the organic matter is transformed into more complex compounds, humus, which in
colloidal or particulate form remains suspended or precipitates, and only very
slowly undergoes chemical and biological degradation.

Micro-organisms, either immediately or after a period of adaptation, act upon
almost any organic material of biological origin made available through excretion,
death of other organisms, or disposal by man. The greater part of these scavengers
are heterotrophic bacteria which belong taxonomically to many different families
and genera. From an ecological point of view it makes more sense to deal with these
bacteria as physiological types rather than as taxonomic entities. In many cases, one
and the same bacterium may appear as two or more physiological types depending
on nutrient availability and physical conditions. For example, many aerobic and
facultatively anaerobic pseudomonads belonging to the genera *Pseudomonas*,
*Vibrio*, *Aerobacter*, and *Beneckea*, are capable of utilizing a wide array of nutrients,
e.g. simple sugars, starch, agar, chitin, gelatin, lipids, etc. (STANIER and co-authors,
1966; BAUMAN and co-authors, 1971) and of using either oxygen or nitrate as
electron acceptor; they may, therefore, qualify as 'aerobic chitin-digesters' or as
'facultative anaerobic nitrate-reducers', depending on the medium and the cultural
conditions applied. Another case is the so-called 'sulphate-reducing bacteria' and
the 'denitrifying bacteria'. Strictly, most of these are regular heterotrophic bacteria
which use sulphate or nitrate as final electron acceptors. The reduction products which result (sulphide and nitrogen gas) are the only justifications for classifying them as we do.

In this section, culture requirements of a few of the most studied and, therefore, better-known groups of heterotrophic marine bacteria will be considered. It is not possible to include in this review every detail of procedure and technique used in the cultivation of the various bacteria; the reader is referred to the individual papers cited for additional details. Information on culture media and special cultivation techniques can also be found in Rodina (1972).

Proteolytic Bacteria

Proteins, polypeptides, and amino acids are perhaps the best general source of nutrients for marine heterotrophic bacteria. Merkel (1965) has developed an elegant method for the detection and isolation of marine proteolytic bacteria. As a base medium he uses either nutrient agar or a 0.5% peptone in sea-water medium with 2% agar to which a chromoprotein is added. Proteolysis is detected as zones of decoloration surrounding active colonies. Various chromoproteins, e.g. phycocyanin and allophycocyanin, are obtained from the red alga Porphyra sp. by extraction with 0.07 M phosphate buffer and subsequent concentration by ammonium sulphate precipitation. A direct correlation exists between loss of chromophore colour and protein degradation.

Cellulose-decomposing Bacteria

Cellulose-decomposing bacteria of the genera Cytophaga, Sporocytophaga, Cell vibrio, Clostridium, and others can be isolated from timber, cotton lines and fish nets, and other cellulose-containing materials from the sea. Many marine sediments have been found to harbour populations of cellulose-decomposing bacteria. These organisms are often also associated with diatoms and other plankton organisms, and cellulose bacteria have been isolated from plankton tows. Decomposition of cellulose is accomplished both aerobically and anaerobically; in the latter case with frequent formation of methane and hydrogen gas.

Enrichment cultures are prepared in simple sea-water media with a source of cellulose as the only carbon constituent. As nitrogen source, NH₄Cl or NaNO₃, 0.5%, is added together with K₂HPO₄, 0.1%. To neutralize any acidity resulting from cellulose decomposition CaCO₃, 2%, has often been found useful. A small amount of yeast extract or vitamin solution enhances activity.

Isolation of organisms is best accomplished on agar media of the same composition with a filter-paper pad or a slurry of cellulose powder spread on top. When inoculated with a dilution of a liquid enrichment culture and incubated for 5 to 10 days, the presence of cellulose-decomposing bacteria is evidenced by the formation of pink, red, or orange—often slimy—spots on the cellulose. With very active strains the filter paper may become perforated. Pigment formation by cellulose-decomposing bacteria is enhanced by growing the bacteria on potato slant.
3. CULTIVATION OF MICRO-ORGANISMS (K. ONUDERSEN)

Agar-digesting Bacteria

Agar (a mixture of polysaccharides extracted from certain red and brown algae) is utilized by about 1 to 2% of the aerobic heterotrophic marine bacteria as carbon and energy source, but as the same bacteria utilize also many other carbon sources, e.g. starch and cellulose, they have no absolute requirement for agar. Agar-digesting bacteria are also common in soil. Most of these bacteria belong to the genera *Vibrio*, *Pseudomonas*, and *Cytophaga*. Often the agar is decomposed with considerable gas production.

Stanier (1941) used 0.1% agar in sea water for enrichment cultures with KNO₃ or (NH₄)₂SO₄, 0.1%, as the only mineral addition. For isolation on solid media a small amount of peptone must be added.

Alginic acid (a polyuronide occurring abundantly in marine algae) is readily used as carbon source by many marine bacteria.

Chitin-digesting Bacteria

The insoluble polysaccharide chitin is a constituent of the exoskeleton of crustaceans; it occurs in many other marine organisms, and in cell walls of fungi. Chemically, chitin is a long-chained polymer of glucosamine and thus contains nitrogen. It can be utilized as nutrient by many marine bacteria and actinomycetes, isolated from marine sediments, plankton tows, intestinal content of fishes, and sometimes from the water column. Enrichment cultures are made in sea water, chitin-peptone medium containing precipitated chitin 0.5%, yeast extract 0.1%, and peptone, 0.5% (Okutan, 1966).

After about two weeks of incubation, dilutions of the enrichment culture are spread on agar plates of the same composition with 2.5%, agar. The presence of chitin-digesting bacteria is evidenced by dissolution of the chitin around active colonies.

Lipid and Hydrocarbon Decomposers

Substantial amounts of triglycerides and related lipids are produced by marine phytoplankton, particularly during periods of nitrogen starvation. Lipids also occur abundantly in most other organisms in the sea.

Bacteria capable of decomposing lipids have been found in all parts of the ocean, and it appears that a large percentage of common marine pseudomonads and other heterotrophic bacteria produce extracellular lipase and hydrolyze and use a variety of natural oils, fats and waxes as nutrients, when these are finely emulsified with the water and a source of nitrogen and phosphorus is available.

Seki (1967) found that from 10 to 100% of marine heterotrophic bacteria isolated from coastal waters and sediments in Japan and from the Pacific Ocean had the capacity to decompose lipids. Lipolytic bacteria were easily detected and isolated from agar plates containing the dye spirit blue, and with olive oil as a substrate. The basal medium contained NaNO₃, 0.01%, or peptone, 1%, as a nitrogen source and FePO₄, 0.01%, in aged sea water at pH 7.8. The olive oil was prepared as a 20% emulsion with Tween 80 and 2.5 ml added per 100 ml of basal medium. Following isolation, the bacteria were grown aerobically in flasks of the same medium. Most of the lipid was decomposed within 24 hrs and yielded about 10⁵ cells ml⁻¹.
Of considerable practical interest is the biological degradation of accidentally spilled oils and petroleum. Many kinds of marine bacteria have been found to oxidize petroleum hydrocarbons with the formation of organic acids, esters, alcohols, carbon dioxide, and bacterial protein. Such bacteria are common in coastal areas, harbours, and around off-shore oil wells where oil spills are more or less chronic, but are rare in the open ocean. With sufficient oxygen supply, and with a source of nitrogen and phosphorus available, oil degradation may proceed at a rate of $0.02$ to $2 \text{ g m}^{-2} \text{ day}^{-1}$ at $24^\circ$ to $30^\circ$ C. Many papers have been published on the subject of oil degradation, and the topic has recently been reviewed by ZoBell (1969; see also Volume V). Solt and Bens (1972) have investigated the cultural requirements of oil-decomposing species of Arthrobacter and Achromobacter. The bacteria decomposed various crude oils and mixtures of hydrocarbons in a basal medium containing $\text{NH}_4\text{Cl}$, $0.1\%$, $\text{KH}_2\text{PO}_4$, $0.02\%$, together with trace elements and vitamins.

Marine bacteria capable of oxidizing gaseous hydrocarbons, notably methane, have long been known. The methane-oxidizing bacterium Methanomonas carbonatophila was grown by Hutton and ZoBell (1949) in a sea-water medium in Söhngren-vessels in a mixture of methane, oxygen and carbon dioxide. The medium was enriched with $\text{NH}_4\text{Cl}$, $0.1\%$, and $\text{K}_2\text{HPO}_4$, $0.05\%$; optimum pH was found to be $6.5$. Culture requirements and growth characteristics of various methane-oxidizing bacteria have been reviewed by Silverman (1964).

Within the major nutritional groups of heterotrophic bacteria dealt with above are numerous interesting physiological types of which many are important members of marine ecosystems. However, as each of these types, and even individual species, require their own special media and conditions for cultivation it would be impossible to include them all in this chapter. The reader is referred to the following papers and reviews: Halophilic bacteria (Larsen, 1962), psychrophilic bacteria (Morita, 1968), luminescent bacteria (Solf, 1970), denitrifying bacteria (Sreenivasan and Venkataraman, 1966; Rhodes and co-authors, 1963), sulphate-reducing bacteria (Starkey, 1960; Postgate, 1965), nitrogen-fixing bacteria (Maruyama and co-authors, 1972).

(b) Photosynthetic Bacteria

Cultivation of this small but interesting group of marine and freshwater bacteria requires light, anaerobic conditions and a suitable electron donor. Purple and green sulphur bacteria use reduced sulphur compounds, mostly in the form of sulphide, as electron donor; they are capable of living autotrophically with carbon dioxide as sole carbon source. Purple non-sulphur bacteria use simple organic materials instead of sulphide but they too can grow autotrophically.

After initial enrichment, photosynthetic bacteria can be plated with relative ease onto agar medium or grown in shake-tubes from which pure cultures can be obtained after several days of incubation. Carbon assimilation in these bacteria is highly efficient. Some of the purple non-sulphur bacteria growing on acetate in light may transform as much as $90\%$ of the carbon into cell material, and large cell crops may easily be obtained in batch culture under optimal conditions.
Purple Bacteria

Thiorhodaceae (purple sulphur bacteria). This group uses hydrogen sulphide as electron donor; the oxidation product, elemental sulphur, is deposited intracellularly as oily refractive globules. Oxygen is strongly inhibitory to growth. A few species require growth factors (vitamins) but otherwise grow in a purely mineral medium at pH 7.0 to 7.5. Enrichment and other cultivation techniques for various purple sulphur bacteria have been described by Schlegel and Pfennig (1961), and more recently by Trüper (1970; see also Volume II, Chapter 2). Trüper isolated and cultivated 36 strains of purple sulphur bacteria from marine sources in a medium prepared from four different mineral stock solutions with vitamin B₁₂ as the only growth factor. The bacteria revealed salinity optima ranging from 10 to 30%.

Athiorhodaceae (purple non-sulphur bacteria). Most of our knowledge of this group of bacteria stems from the classical study of Van Niel (1944). Three genera of the family are now recognized, Rhodopseudomonas, Rhodospirillum, and Rhodomicrobium, the latter multiplying by budding. Several marine isolates have been described. These bacteria can all be grown anaerobically in light with acetic acid, lactic acid, or some other simple organic compound as electron donor. If the electron donor is more reduced than the cell organic matter, carbon dioxide is used as carbon source; if more oxidized, the electron donor is also the carbon source. Some of the purple non-sulphur bacteria can use molecular hydrogen as electron donor in photosynthesis with carbon dioxide as the only carbon source. In the dark, these bacteria behave as heterotrophs by obtaining energy from aerobic respiration of organic compounds.

B-vitamins are required for growth by most species. As vitamin requirements are species specific, selective enrichment is possible. For example, Scher and co-authors (1963) could isolate Rhodospirillum palustris from cultures containing p-aminobenzoic acid, and R. rubrum from cultures with biotin.

Marine purple non-sulphur bacteria can be grown in light in sea-water medium consisting of Na-lactate, 0.5%, K-acetate, 0.04%, yeast extract, 0.5%, and K₂HPO₄, 0.5%, at pH 7 to 8.

Green Bacteria

Isolation technique and media used for green bacteria have been described by Van Niel (1931) and still seem to be used by most workers. Green bacteria thrive in a rather high concentration of hydrogen sulphide, 0.1%, at a pH of about 7— as opposed to purple sulphur bacteria which require lower sulphide concentrations and somewhat higher pH levels. These differences can be used to separate the two groups of photosynthetic bacteria which frequently occur together in nature.

In shake-tubes under anaerobic conditions in light, disc-shaped, yellowish-green colonies of about 1 mm diameter appear after 7 to 10 days of incubation. After isolation, the bacteria can be grown in liquid medium in glass-stoppered flasks. FeCl₃ is required for growth in purely mineral media.

The cultural and biochemical characteristics of the two species of green bacteria Chlorobium limicola and C. thiosulfatophilum have been extensively investigated.
by Larsen (1953). Trüper (1970) isolated and investigated 11 strains of Chlorobium from various marine and brackish-water sources. These organisms would grow in the same medium as the purple sulphur bacteria at pH 6.8.

(c) Chemo-autotrophic Bacteria

All chemo-autotrophic bacteria (Volume II: Schlegel, 1975) use inorganic materials as source of energy and reducing power (electron donor) and carbon dioxide as carbon source. Many of these organisms play an important role in the transformation of inorganic matter in the sea, e.g. the nitrifying bacteria which are instrumental in the regeneration of nitrate (Volume IV: Conover, 1976; Sorokin, 1977). Because members of this group, as the phytoplankton and the photosynthetic bacteria, use carbon dioxide as their only carbon source, they also contribute to some extent to the net production of organic matter in the sea. Many of the chemo-autotrophic bacteria are difficult to cultivate in laboratory media.

Sulphur Bacteria

Tilton and co-authors (1967a, b) isolated marine strains of Thiobacillus thiooxidans and T. thioparus from the Caribbean Sea and the Atlantic Ocean using membrane filtration and thiosulphate agar, and by enrichment in 20-l carboys. Both enrichment medium and agar medium contained per litre of sea water: NH₄Cl, 0.1 g; FeCl₃·6H₂O, 0.001 g; K₂HPO₄ or KH₂PO₄, 0.2 g; and Na₂S₂O₃, 10 g. Oxidation of thiosulphate proceeded much more rapidly when the initial pH was adjusted to 5.6, than at the original pH of 7.2. In the acid medium the final pH would be close to 2 as a result of sulphuric acid production. Most strains also oxidized elemental sulphur and all of them were aerobic and required sea water for growth. If transferred to nutrient broth, marine agar, or to a medium containing 0.01% yeast extract, no growth would occur.

Seven strains isolated by Adair and Gundersen (1969a) from estuarine, neritic and oceanic environments in the Pacific Ocean could, in contrast to the Caribbean and Atlantic strains, easily be adapted to a heterotrophic existence if the thiosulphate was replaced by 0.5% yeast extract or peptone. These facultative autotrophs grow well in the presence of glucose, galactose, mannose, and glycerol (Adair and Gundersen, 1969b). The Pacific strains were initially isolated from a medium consisting of (NH₄)₂SO₄, 0.1 g; NaHCO₃, 0.2 g; MgSO₄·7H₂O, 0.1 g; CaCl₂·2H₂O, 0.02 g; K₂HPO₄·3H₂O, 0.1 g; FeSO₄·7H₂O, 0.003 g; and Na₂S₂O₃·5H₂O, 2.5 g in 1 litre of aged sea water. The thiosulphate was sterilized separately and added as 5 ml of a 50% solution l⁻¹. A solid medium was made by adding 15 g of agar. Initial pH was 8.0.

The rather delicate, micro-aerophilic sulphide-oxidizing bacterium Thiovulum majus has been cultivated from marine materials by Larivière (1963) in a sea-water medium consisting of NH₄Cl, 0.005%; KH₂PO₄, 0.005%; and Na₂CO₃, 0.005%, at pH 8. Hydrogen sulphide and oxygen were supplied at optimal concentrations throughout the propagation period. Pure cultures were obtained by growing the bacteria in controlled gradients of oxygen and sulphide in sea water.

The Beggiatoas are large sulphide-oxidizing bacteria whose chemo-autotrophic
nature is questioned; representatives of this genus are sometimes found in large colonies in the littoral zone and in mud flats. *Beggiatoa* sp. was successfully grown by Burton and Morita (1964) in a semisolid agar medium with yeast extract, 2.0 g; Na-acetate, 0.5 g; and CaCl$_2$, 0.1 g in tap water or sea water. Burton and Morita believe that the difficulties often experienced by others in growing this organism were due to the accumulation of peroxides in the cultures. They overcame this problem by adding a small amount of catalase to the cultures and obtained a marked increase in cell yield.

**Nitrifying Bacteria**

In spite of numerous attempts by various workers to demonstrate the existence of nitrifying bacteria in the sea, it was not until the early sixties that a true marine ammonium-oxidizing organism was isolated in pure culture (Watson, 1962). The organism was named *Nitrosocystis oceanus*; its isolation was followed by the isolation of other nitrifying bacteria which proved that there was a definite biological basis for nitrate-formation in the oceans.

Watson (1962) demonstrated successfully the presence of *Nitrosocystis oceanus* by using large volumes of sea water for the original enrichment. Five to 10-l samples of sea water were enriched with ammonium salts and phosphates and incubated for several months with occasional aeration. When nitrite and nitrate began to accumulate, subcultures were made in 1-l medium of the following composition: $(NH_4)_2SO_4$, 13.2 g; MgSO$_4$.7H$_2$O, 200 mg; CaCl$_2$, 20 mg; K$_2$HPO$_4$, 114 mg; chelated iron, 130 µg Fe; Na$_2$MoO$_4$.2H$_2$O, 1 µg; MnCl$_2$.4H$_2$O, 2 µg; CoCl$_2$.6H$_2$O, 2 µg; CuSO$_4$.5H$_2$O, 20 µg; ZnSO$_4$.7H$_2$O, 100 µg. Subsequent cultivation in 14-l fermenters in the same medium was successful (Watson, 1965); pH was kept constant at 7.5 by adding aliquots of a 2 M K$_2$CO$_3$ solution.

Kimata and co-authors (1961), who studied nitrification in sea-water aquaria, stressed the importance of using low nutrient concentrations for cultivation of marine nitrifying bacteria (Watson was successful with rather high concentrations). They used per litre of medium only 30 mg of $(NH_4)_2SO_4$ for ammonium-oxidizers and 30 mg of KNO$_3$ for the nitrate-oxidizers in addition to 2 mg K$_2$HPO$_4$ and 0.2 mg of chelated iron. The pH was kept at 8.4 by addition of K$_2$CO$_3$. If the nutrient concentration was increased to 300 mg l$^{-1}$ growth was inhibited. Small amounts of sand were found to accelerate nitrification. Carlucci and Henneuse (1965) also recommended the use of low nutrient concentrations for marine nitrifying bacteria and found optimal growth of *Nitrosocystis oceanus* with 50 µg at N l$^{-1}$ equivalent to 6.6 mg of $(NH_4)_2SO_4$.

Gundersen (1966) and Carlucci and McNally (1969) were able to grow *Nitrosocystis oceanus* on mineral agar medium when the oxygen in the gas phase was reduced to 1/10 of atmospheric pressure. The adverse effect of oxygen on the growth of nitrifying bacteria was further investigated by Gundersen and co-authors (1966) and Carlucci and McNally (1969) who demonstrated that oxygen would inhibit carbon-dioxide fixation by *N. oceanus*, even at the partial pressure of oxygen in air, but that the oxidation of substrate was unaffected or even accelerated. Successful growth of batch cultures was obtained only when initial stirring was slow and aeration not started until a cell density of about $10^6$ ml$^{-1}$ had been reached.
The stationary phase was usually reached after 7 to 10 days with a final density of $10^9$ cells ml$^{-1}$ (Gundersen, unpublished).

Based on the use of membrane filters, Feinstein (1968) devised a two-step method for the enumeration of ammonium-oxidizing marine bacteria. Filters, through which measured volumes of sea water had been sucked, are placed on sterile glass-wool pads soaked with a solution of ammonium sulphate and incubated for several days. During the incubation, autotrophic nitrifying bacteria will oxidize a substantial part of the ammonium to nitrite and form minute colonies on the membrane. In the second step, the membranes are first freed of nitrite by several transfers onto sterile cellulose-absorbent pads saturated with sea water and then incubated for 5 to 30 mins on the surface of a solid medium containing $(NH_4)_2SO_4$, 0.15%. During the second incubation, the nitrifying bacteria will have produced sufficient new nitrite to be detectable as red spots when the agar surface—after removal of the filter pad—is flooded by nitrite reagents. Each red spot corresponds to a colony of nitrifying (nitrite-forming) bacteria. The method has the serious pitfall that many marine heterotrophic bacteria (present together with the nitrifiers on the membrane filter) are capable of attacking the membrane filter matrix (cellulose nitrate) and thereby releasing nitrite-positive materials (Gundersen, unpublished). This somewhat minimizes the value of an otherwise very elegant technique.

Iron and Manganese Bacteria

These are chemo-autotrophic and heterotrophic bacteria which are found in iron- and manganese-rich fresh and marine waters and in sediments. The chemo-autotrophic *Thiobacillus ferrooxidans* derives energy by oxidizing ferrous iron to ferric iron at pH values below 3. Such an acidity probably does not exist in the marine environment; hence it is questionable whether this bacterium is active there.

On the other hand, the stalked iron bacterium *Gallionella* sp. has been isolated repeatedly from marine sediments and deposits. *Gallionella ferruginea* was shown by Kucera and Wolfe (1957) to grow well in a medium of $NH_4Cl$, 0.1%; $K_2HPO_4$, 0.05%; and $MgSO_4$, 7$H_2O$, 0.02% with sulphide added as almost insoluble FeS. Growth would occur in a narrow zone of optimal concentrations of oxygen and ferrous iron provided as counter-gradients in agar slants overlaid with water. Under aerobic conditions, addition of carbon dioxide to the gas phase stimulates growth considerably.

Various species of *Gallionella* have been demonstrated in ferromanganese deposits on the sea floor (Buttewitch, 1928); but their participation in the formation of ferromanganese nodules is still uncertain. Alexander (1961) points out that the bacterial oxidation of the organic material to which the ferrous iron is chelated may often be confused with bacterial action on the iron itself. At the normal pH of sea water, and with oxygen present, ferrous iron oxidizes spontaneously to the ferric state and precipitates.

Jones (1965) scraped aseptically the surface of 30 ferromanganese nodules recovered from the Pacific Ocean and was able to isolate several different bacteria. One organism, which was isolated from all nodules, would precipitate a material with an X-ray diffraction pattern similar to that of ferromanganese nodules when grown in a liquid medium with a shark's tooth added. Jones cultivated these
bacteria in a sea-water medium consisting of NH\textsubscript{4}-citrate, 0.05%; glycerolphosphate, 0.01%; MgSO\textsubscript{4}.7H\textsubscript{2}O, 10\textsuperscript{-3} M; and FeSO\textsubscript{4}, 10\textsuperscript{-5} M. The taxonomic relation of the bacteria was not determined.

Hydrogen Bacteria

Numerous bacteria which can utilize molecular hydrogen have been found in the sea (ZoBell, 1947). Some of these are obligate anaerobes which use sulphate as final electron-acceptor (Sisler and ZoBell, 1951). The bacteria usually grow well in mineral sea-water medium with negligible amounts of organic matter and use carbon dioxide as carbon source and molecular hydrogen as energy source. The true autotrophic nature of anaerobic hydrogen bacteria (mostly referred to as 'sulphate-reducing' bacteria) has been questioned (Postgate, 1965).

The presence of aerobic hydrogen bacteria (‘Knallgas’ bacteria) in the sea has not, to the author's knowledge, been reported but a likely locality for such bacteria would be at the mud-water interface over strongly anaerobic sediments, under oxygenated water.

(d) Actinomycetes

Since actinomycetes are often considered bacteria and because some progress has recently been made in the cultivation of these organisms from marine materials they will be briefly mentioned.

Actinomycetes are practically absent from open-ocean waters, but have been isolated from nearshore waters and from marine sediments. They can be demonstrated on agar plates only after a long period of incubation (4–6 weeks). Weyland (1969), who carried out a rather extensive survey of marine actinomycetes in sediments from the North Sea and the Atlantic Ocean, was able to isolate a large number of strains of Nocardia, Micromonospora, Microbispora, and Streptomyces from six different media. A typical medium used by Weyland contained peptone, 0.05%; yeast extract, 0.01%; chitin, 0.1%; FePO\textsubscript{4}.H\textsubscript{2}O, 0.001%; agar, 1.5%, in 75% sea water at pH 7.5.

(e) Bacteriophages and Bdellovibrio

Many marine bacteria—including species of Pseudomonas, Vibrio, Micrococcus, Photobacterium, Cytophaga and Flavobacterium—have been found to be susceptible to bacteriophages (Kriss and Rurina, 1947; Spencer, 1963; Kakiyoto and Nagatomi, 1972). A bacteriophage isolated by Spencer (1955) from the North Sea was lytic to Cytophaga marinaflava; it was shown by Valentine and co-authors (1966) to be a particle with hexagonal head and a tail with base plate but without tail sheaths. Marine bacteriophages apparently differ from phages of other origin with respect to their temperature and ion requirements. Thus, Spencer (1963) found that several phages which would lyse marine bacteria had a specific requirement for sodium and magnesium, and that no plaques were formed at temperatures exceeding 30° C.
The significance of bacteriophages in the marine environment is not known. It is a common observation that many marine bacteria which form colonies on an isolation medium do not survive the transfer to fresh medium. Bacteriophages could well play a role here.

Bacteriocins are substances produced by bacteria; they exert a specific inhibitory effect on other bacteria, usually closely related species. Bacteriocins have been found to be produced by the marine bacterium *Pseudomonas* sp. which is active against an *Arthrobacter* sp. (Sieburth, 1966). Some evidence has been produced that bacteriocins are defective bacteriophages (Sandoval and co-authors, 1965).

*Bdellovibrio bacteriovorus* is a very small (0.2 μm) vibrio-like organism described by Stolp and Starr (1963) which is pathogenic to many bacteria, including marine bacteria. According to Mitchell and co-authors (1967), rapid die-off of *Escherichia coli* in sea water could in part be attributed to *B. bacteriovorus*; typical plaques were formed when the host organism after treatment with sea water was plated on nutrient medium.
3. CULTIVATION OF MICRO-ORGANISMS

3.2 FUNGI

3.21 LOWER FUNGI, ASCO- and DEUTEROMYCETES

J. SCHNEIDER

(1) Introduction

In contrast to other micro-organisms, fungi of marine habitats have received full attention from marine ecologists only in the last 20 to 30 years. The occurrence of these important heterotrophic organisms, however, had already been established about 100 years ago by Montagne (Johnson and Sparrow, 1961). In 1905, Petersen systematically collected marine fungi, especially Phycomycetes. But these and similar investigations initiated no more than passing interest. Nearly 30 years later, Sparrow (1934, 1936) published his work on algae-inhabiting phycomycetes from the coast of Denmark, and in 1944, Barghoorn and Lindner drew attention to the Ascomycetes and Deuteromycetes. This was the beginning of a new era: the fungi were conceived for the first time as integrated constituents of the marine environment (see the historical review by Johnson and Sparrow, 1961).

Numerous publications testify that fungi of nearly all taxonomic classes populate the sea (Johnson and Sparrow, 1961). However, most of the species described belong to—and most of the papers published concern—the following taxonomic groups:

(i) Chytridiomycetes* (including marine species of Chytridium, Olpidium, Phlyctochytrium, Rhizophydiurn, Rhizidium, Rhizophlyctis, and others);
(ii) Oomycetes* (including the genera Ectroegella, Leptolegnia, Thraustochytrium, Lagenidium, Haliphlyctis, Alkinsiella, and others);
(iii) Ascomycetes (especially Halosphaeriaceae, an ecological—not a taxonomical—group related to the Sphaeriales) including the yeasts;
(iv) Deuteromycetes (Fungi imperfecti).

Related organisms, probably parasitic fungus-like forms—e.g. Labyrinthula coenocystis and others (Schmoller, 1960; Schneider, 1969a), Dermocystidium sensu Goldstein (Goldstein and Moriber, 1968), Althornia cronicii (Jones and Alderman, 1973), and Ostracoblate implexa (Alderman and Jones, 1971a)—which defy incorporation into the afore-mentioned groups, have also attracted increasing interest.

Fungi of oceans and coastal waters have been found in sediments, water samples, algae (Phycomycetes), wood and related material (Asco- and Deuteromycetes), animals, and calcareous materials like shells. Most fungi appear to be saprophytes, and only a few species are probably obligate or facultative parasites, e.g. Eurychasmidium tumefaciens (in Ceramium), Ectroegella perforans (in diatoms), Lageni-

*Often referred to as ‘Phycomycetes’ or ‘Lower Fungi’; these terms are used here for convenience.
cultivation of micro-organisms (j. schneider)

dium callinectes (in crab eggs), Lagenidium coscinodiscus, and Althor7zia crozcchii (in oysters).

Knowledge about marine fungi derives from studies in littoral and neritic zones, rather than from deeper oceanic regions. Many Ascomycetes (including yeasts) belong to taxa that are not normally considered part of the marine biota (e.g. species of Aspergillus, Alternaria, Cladosporium, Fusarium).

While a large number of papers has been published on the morphology and taxonomy of marine fungi, few publications are available on their ecology. Effects of the composition of nutrient media and of salinity on Lower Fungi, Ascomycetes, and Deuteromycetes have been studied by several authors: e.g. Scholz (1958), Vishniac (1960), Goldstein and co-authors (1969), Schneider (1969b), Gaertner (1970), Harrison and Jones (1970), Alderman and Jones (1971a), Booth (1971a, b), Jones and co-authors (1971), MacLeod (1971). Gaertner (1968) developed a method to obtain data about the abundance of Lower Fungi in the sea and their geographical and seasonal distribution pattern.

Certain marine fungi endanger constructions in harbours, fisheries equipment, or food sources; they cause, for example, biodeterioration of wood pilings, cordage, and nets, and diseases in oysters and fishes (Alderman and Jones, 1967, 1971b; Jones, 1968; Jones and Le Campion-Alsumard, 1970a, b; Chapter 9; see also Johnson and Sparrow, 1961).

Cultivation of fungi has been dealt with by Emerson (1958), Johnson and Sparrow (1961), Fuller (1964), Klie (1965), Gaertner (1965), Von Arx (1968), Höhne (1968), and Jones (1971); also of interest are papers by Emerson (1959) and Booth (1971c).

(2) Lower Fungi (Phycomycetes): Chytridiomycetes and Oomycetes

It is difficult to isolate and to cultivate aquatic fungi, especially the lower forms. The reasons are easily listed (Emerson, 1959): (i) These forms consist of very small thalli of only one or a few cells. They grow at least in culture—slowly and are more sensitive and ephemeral than, for instance, Ascomycetes and Deuteromycetes. (ii) Aquatic fungi seldom aggregate in 'blooms'; they live dispersed and hence make collection a problem. (iii) Being heterotrophic, they depend on dead or living organic matter: they are saprophytes, parasites, or pathogens, often with specialized nutritional and environmental demands. In pure culture they usually show restricted growth—unlike the common moulds which develop an extensive mycelium, capable of almost indefinite spreading as long as suitable conditions are maintained; vegetative growth terminates after a few generations, life cycles are reduced, and structures are modified making taxonomic determination difficult or impossible.

An experimental ecologist who wants to cultivate Phycomycetes from marine habitats must take all this into consideration right from the beginning, i.e. sampling. No matter what substrate he chooses—water, mud, algae, plankton, fishes, molluscs, drifting materials—he must take the following precautions: (i) process the sample as soon as possible under sterile conditions; (ii) if sample transport is inevitable, take great care to ensure good aeration (it is better, for instance, to transport a few algae thalli per vessel with a small amount of water just covering the plants than to place a huge bulk in a small bag); (iii) keep transport-container
temperature low. At temperatures above 10° C, oxygen deficiency will seriously reduce survival chances of sensitive, rare, or slowly reproducing fungi.

For sampling of water, plankton, or algae normal sampling equipment (ZoBell water sampler, plankton nets, dredges) is used. Transport vessels should be sterilized before use; if this is not possible, they should at least be rinsed thoroughly with water from the sampling location.

Before processing the samples in the laboratory, plankton and algae thalli should be examined microscopically. In some cases, fungi can be detected without enrichment and be isolated directly from the substrate. In most cases, however, it is necessary to increase the number of Phycomycetes in enrichment cultures. Two enrichment methods have proved especially successful: (i) baiting, (ii) plating on solid media, sometimes after filtration or centrifugation of water samples.

In order to delay the development of fast-growing fouling organisms, only small bits of substrate are placed in the many enrichment dishes; solid material (algae) is rinsed with sterile sea water or water of the sampling area to diminish slimy bacterial layers and protozoans. The cultures are scanned for fungi every other day for 2 to 3 weeks or longer and desired organisms are subcultured as soon as possible in fresh medium.

(a) Baiting

Small quantities of mud, algae pieces, or other substrates collected are distributed in Petri dishes or Erlenmeyer flasks (100 to 300 ml) which contain a sterile seawater mixture (sea water of ca 36% S: aqua destillata = 1:1) or a shallow water layer from the sampling location. Baits are then added, e.g. small amounts of pollen of Pinus sp., pieces of crab chitin or grass blades, fish scale, cellophane, or half hemp seeds, etc. It is recommended to use only one sort of bait in one dish.

Enrichment cultures are then incubated at about 15° C in the dark. After 3 days or longer (in some cases it takes 2 weeks until the first fungi appear) Lower Fungi may be seen attached to the bait upon microscopic examination.

Baiting has several advantages: most baits release only small amounts of nutrients into the surrounding water, sufficient to attract the zoospores of Phycomycetes, but too small to significantly enhance bacterial growth. Once a zoospore has settled on a bait and developed a germ tube, from which rhizoids arise, piercing the outer layers of the bait's cells, the fungus has gained some advantage over competing organisms which must stay 'outdoors'. Since most baits float at the water surface, settling fungal spores develop under fairly good oxygen conditions.

(b) Plating

Numerous authors have successfully used solid nutrient media (mostly on agar basis) for enrichment and isolation of aquatic fungi. Vishniac (1955) was the first to introduce a solid medium into marine mycology. Fuller and co-authors (1964) used a simplified version of Vishniac's medium (Table 3-3).

After autoclaving, the medium still being hot, streptomycin sulphate, 0.5 g, and penicillin, 0.5 g, are added. According to Fuller and co-authors (1964), antibiotics are added dry. No contamination was observed which could be attributed to this way of adding antibiotics.
3. CULTIVATION OF MICRO-ORGANISMS (J. SCHNEIDER)

Table 3-3

Modification of Vishniac's medium (After FULLER and co-authors, 1964; reproduced by permission of the New York Botanical Garden)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Gelatin hydrolysate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Liver extract</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Sea water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

GOLDSTEIN and MORIBER (1966) developed a more complex medium—designed for Dermocystidium sp., but suitable also for other marine Phycomycetes, such as Thraustochytrium sp. (Table 3-4). In this connection the publications of GOLDSTEIN and MORIBER (1966) developed a more complex medium—designed for Dermocystidium sp., but suitable also for other marine Phycomycetes, such as Thraustochytrium sp. (Table 3-4). In this connection the publications of GOLDSTEIN and MORIBER (1966) are of special interest. These studies revealed the requirement of vitamin B₁ and B₁₂ by marine Phycomycetes (Thraustochytrium aureum, T. roseum, Schizochytrium aggregatum). Belsky and GOLDSTEIN (1965) compared the effect of different bacteriostatic and fungistatic compounds on non-filamentous marine fungi. The results suggest that greater flexibility in the isolation of lower marine fungi may be reached by using different antibiotics in combination.

BELSKY and GAERTNER (1963) and GAERTNER (1970) are of special interest. These studies revealed the requirement of vitamin B₁ and B₁₂ by marine Phycomycetes (Thraustochytrium aureum, T. roseum, Schizochytrium aggregatum). Belsky and GOLDSTEIN (1965) compared the effect of different bacteriostatic and fungistatic compounds on non-filamentous marine fungi. The results suggest that greater flexibility in the isolation of lower marine fungi may be reached by using different antibiotics in combination.

Table 3-4

Composition of culture medium for Lower Fungi (After GOLDSTEIN and MORIBER, 1966; reproduced by permission of Springer-Verlag)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>70 mg</td>
<td>Na₂EDTA</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>43 mg</td>
<td>B (as H₃BO₃)</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>47 mg</td>
<td>Fe (as FeCl₃·6H₂O)</td>
<td>0.19 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.4 g</td>
<td>Mn (as MnCl₂·4H₂O)</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.8 g</td>
<td>Mo (as Na₂MoO₄·2H₂O)</td>
<td>0.09 mg</td>
</tr>
<tr>
<td>Casein-hydrolysate (enzymatic)</td>
<td>0.2 g</td>
<td>Zn (as ZnCl₂)</td>
<td>0.06 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.4 g</td>
<td>Co (as CoCl₂·6H₂O)</td>
<td>1.0 μg</td>
</tr>
<tr>
<td>Agar</td>
<td>0.1 g</td>
<td>Cu (as CuCl₂·2H₂O)</td>
<td>2.0 μg</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>20.0 μg</td>
<td>Aqua destillata to 100 ml (glass distilled)</td>
<td>7.3</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>0.3 μg</td>
<td>pH (after autoclaving)</td>
<td></td>
</tr>
</tbody>
</table>

and BELSKY (1963) and GAERTNER (1970) are of special interest. These studies revealed the requirement of vitamin B₁ and B₁₂ by marine Phycomycetes (Thraustochytrium aureum, T. roseum, Schizochytrium aggregatum). Belsky and GOLDSTEIN (1965) compared the effect of different bacteriostatic and fungistatic compounds on non-filamentous marine fungi. The results suggest that greater flexibility in the isolation of lower marine fungi may be reached by using different antibiotics in combination.

GAERTNER (1960) used silica gel for cultivating a freshwater phycomycete; this method is applicable for marine fungi as well. MILLER (1967) employed membrane filtration to concentrate propagules of Phycomycetes from sea and fresh waters, although with moderate success in regard to the marine forms (species of Thraustochytrium and Schizochytrium have been isolated).
According to Miller, the main limitation of this method is the long time necessary to filtrate water containing large quantities of particulate matter. This problem could be eliminated to some extent by allowing the suspended matter to settle prior to filtration (the water sample was decanted and filtered). Fuller (1964) successfully applied continuous-flow centrifugation to isolate species of Dermocystidium, Thraustochytrium, Schizochytrium, Lagenidium, Haliphthoros, Atkinsiella, Pythium and Phytophthora from marine-water samples. Johnson and Sparrow (1961) recovered fungi from concentrated decaying marine phytoplankton samples by means of a sedimentation tank.

Several procedures are available to obtain monospecific axenic cultures of marine Phycomycetes. They represent variations of the methods employed by Couch (1939) to isolate freshwater Phycomycetes (see compilation by Sparrow, 1960). Two methods of isolation have been adopted by many authors: (i) Transfer of single sporangia or of zoospores with glass capillaries, into several successive droplets of sea water; (ii) streaking of infested material on solid media and transfer of single colonies to fresh media.

These two basic isolation methods have been modified in numerous ways, e.g.: If enrichment is achieved by baiting, for example with pollen, the first-mentioned method may be employed. For isolation the following glassware and solutions are required: 25 slides (sterilized by flaming; one in each dish); glass capillaries (inner diameter of the tip approx. 50 μm, the ideal diameter being slightly larger than an average sporangium of the fungus to be isolated) sterilized by steam or alcohol; sterilized Petri dishes (φ 6 cm); a Pt-Ir-inoculation loop; a microscope with low-power objective (a working distance of 8 to 10 mm should be available); a solution of penicillin, 0.5 g, and streptomycin sulphate, 0.6 g l⁻¹ sea water (chloramphenicol 0.1–0.2 g l⁻¹ is also recommended); sterile pine pollen and sea water.

Two droplets of sterile sea water are placed on each of the sterilized slides (to prevent desiccation there should be a little water in the Petri dish, and slides placed on short glass rods). On two separate sterile slides 4 to 6 droplets of antibiotics solution are placed, and the slides kept in dishes, as described above, until they are needed. Then a small drop of enrichment culture is distributed on a third slide with light pressure to separate sporangia from the bait. Some sporangia are collected with a capillary under the microscope and transferred to the first antibiotic droplet; further sporangia are likewise placed on the remaining antibiotic droplets. The capillaries should be cleaned with alcohol and washed with sterile sea water after each transfer. In case the sample is heavily contaminated, another transfer in a second drop of antibiotics solution may be useful.

Following this antibiotic treatment, one sporangium is transferred to each sea-water droplet under microscopic control; this will ensure the growth of a monospecific culture of the fungus. Then, a very small amount of sterile pine pollen is dusted over these microcultures and the slides incubated in their dishes at ca 15°C, in the dark. After 4 to 6 days microscopic control reveals if there has been any fungal development and if the microcultures are free of undesired organisms (especially bacteria, yeasts, moulds, protozoans and flagellates). During this examination of the cultures any draught should be avoided in order not to recontaminate already pure cultures. Phase contrast facilitates recognition of zoospore activity (an important indicator of the vitality of the fungus) and any contamination.
Microcultures which show fungal development (e.g. sporangia and/or good zoospore activity) and no infections by undesired organisms should be processed without delay: the droplet of the microculture is transferred by means of a sterile inoculation wire loop or a capillary to approximately 5 ml sterile sea water in a Petri dish (φ 6 cm), and dusted with pollen. Incubation follows as previously outlined. To ascertain that these macrocultures are free of other organisms, subcultures from well-growing macrocultures are obtained in medium without agar, or some material is plated out on this medium solidified with agar. Such procedure will reveal any contamination. GAERTNER (personal communication) prefers very small glass loops to isolate single sporangia or zoospores.

This method may be modified as follows: single sporangia are isolated in drops of sea water. After sporulation, the zoospores are streaked out on a solid medium, so that they are well isolated from each other. JONES (1971) recommends using low nutrient concentrations, which favour zoospore germination, for instance: plain agar (3% agar), Leitner's agar (2% agar, 0.004% peptone).

Solid substrata such as pieces of algae, fish, shellfish, etc. are placed on solid media (see above) in Petri dishes. The dishes should not be overcrowded. Sometimes it is advisable to rinse the substrate pieces with sterile sea water to reduce the adhering slimy cover of bacteria and other organisms.

Residues of centrifugation or filtration can be distributed likewise after redilution in a small amount of supernatant or filtrate (0-2 ml of suspension is sufficient). After a few days of incubation (15°C, darkness) the first fungal growth can be observed, either in form of hyphae or sporangia (single or in clusters) which originate from the dispersal of zoospores of monocentric forms. Subcultivation on fresh media should take place as soon as possible to reduce contamination by other organisms.

(3) Labyrinthula

Several species of Labyrinthula—reportedly saprophytic or parasitic organisms—live on algae, sea grass (Zostera sp.) and other plants. They have been successfully isolated by a number of authors. KUE (1965) describes a detailed method to isolate L. coenocystis. Two or three steps are generally necessary to obtain di- or monospecific cultures: Infected algae are placed on agar plates (peptone, 0.03 g; agar, 0.75 g; sea water, 100 ml). L. coenocystis accumulates, together with accompanying bacteria, around the plant material. From these plates, agar blocks are cut out—preferably from regions which are less infected by bacteria, diatoms, or flagellates—and transplanted on fresh plates, radially inoculated with Aerobacter aerogenes. L. coenocystis gradually escapes undesired organisms jointly carried over by subinoculation. As cells of L. coenocystis grow in regions free of bacteria and related micro-organisms, it is possible to cut out blocks which harbour only L. coenocystis. KUE (1965) transferred these blocks to peptone-sea-water agar plates as above, with 1-0 ml inactivated beef serum added, and inoculated prior to transfer with suspensions of A. aerogenes or Torulopsis famata. From these dispecific cultures monospecific cultures can be obtained by transplanting blocks on plates previously plated with dead yeast cells or cell fractions. L. coenocystis can also grow on synthetic sea-water agar enriched with dead yeast cells.
(4) Ascomycetes, Excluding Yeasts, and Deuteromycetes (Fungi imperfecti)

A comprehensive compilation of cultivation methods for Ascomycetes and Deuteromycetes has been provided by Johnson and Sparrow (1961). More recently Jones (1971) has published an account on this subject.

(a) Enrichment and Isolation

Three methods are generally recommended and often applied to enrich and isolate Ascomycetes and Deuteromycetes: direct examination of collected material, trapping, plating.

Direct examination of freshly collected marshland grasses and other plants, of driftwood and cordage by means of a hand lens often reveals the presence of perithecia of higher fungi. Sometimes the ascocarps are embedded in the material, and only the necks of the fruit bodies, piercing the surface of the substrate, indicate the presence of fungi. Old pilings are often inhabited by these organisms even if impregnated against fouling organisms, and should be included in the list of substrates to be examined, as well as submerged cellulose material, like rootstocks. Kohlmeier (1966) applied this method with much success. Fungal spores have been found in scums and foam on beaches (Kohlmeier, 1967). Anastasiou and Churchland (1969) report fungi from decaying leaves exposed to sea water.

Some authors have obtained higher fungi from marine habitats by trapping (e.g. Johnson and co-authors, 1959; Jones, 1962, 1963). The principle of this method is to expose steam or gas-sterilized wood panels for weeks or months to the sea. Untreated, unimpregnated rough blocks, 20 × 8 × 15 cm, of Pinus sp., Fagus sp., Tilia sp., Maple sp., Ochroma sp. (balsa wood) are suspended in strings—each panel well separated from the other by a plastic washer. A weight should be attached to keep the panels well under water. It is advisable to have a second rope loosely attached in case the main rope breaks. At the test site, hydrographical data (e.g. temperature, salinity, water movement, turbidity, pollution) are recorded and the degree of test-panel colonization by other micro- and macro-organisms determined.

The test panels are brought to the laboratory under sterile conditions and processed: They are scanned (perithecia of Ascomycetes present are picked up with a sterile needle and distributed on a solid medium; see following) and incubated in damp-chambers to enable mycelia development into perithecia. Glass or plastic boxes are lined with blotting paper and sterilized by autoclaving or ethylene-oxide treatment. All panels are carefully cleaned of adhering macro-organisms, slightly blotted with sterile paper towelling and placed in the boxes in such a way that they do not touch each other. Some authors (e.g. Johnson and Sparrow, 1961; Jones, 1971) emphasize that Fungi imperfecti often do not form conidial stages unless the surface water has drained off. On the other hand, the panels should not be allowed to dry down completely; small amounts of sterile sea water must be added to the boxes from time to time. Usually, several weeks of incubation are required until reproductive stages can be observed. Johnson and co-authors (1959) recommend to dry test blocks on which perithecia have formed in order to enhance spore release. They also describe a method of 'forcible ascospore discharge': Agar plates are prepared and a pad of moist sterile filter paper is placed on the inside of
the Petri dish cover. The plate is then inverted and small segments of panels infested with ascocarps placed on the paper so that the ostiola are directed towards the agar surface. Ejected bacteria-free spores adhere to the overlying agar. Developing mycelia can be transplanted to fresh media.

Incubating panels in damp chambers shortens the time required for the preceding exposition of the test material to sea water (JOHNSON and SPARROW, 1961). In general, an exposition of 7 to 14 days is sufficient under damp-chamber conditions.

Plating of infested material (plant culms, wood slivers or cores) directly on agar media often leads to the development of lignicolous fungi; difficulties may arise, however, in obtaining isolates by this method in regard to contamination by other fouling organisms, such as bacteria and protozoans. To avoid contamination, JOHNSON and SPARROW (1961) recommended to dry the test substrates rapidly. This considerably reduces the incidence of bacterial contaminants.

Media for isolation of marine fungi should be low in nutrient content. The following formula is used by many authors (Jones, 1971): Yeast extract, 0.1 g; glucose, 1.0 g; agar, 18 to 20 g; sea water, 1000 ml. Cornmeal agar or cellulose agar also have been recommended (BOOTH, 1971c). Sometimes, addition of wood flour to a simple yeast extract–glucose medium enhances growth of the fungi desired.

Many authors use a diluted, aged sea water to prepare the media. The sea water is filtered to remove detritus, and kept in a cool dark place for several months. It is then diluted with distilled water to ca 25%. To reduce the development of undesired contaminants, antibiotics may be added: 0.1 g chloramphenicol, penicillin or streptomycin sulphate (single or in combination) is sufficient in most cases. Chloramphenicol can be added before autoclaving. The pH of the medium should be above 7.

It is not easy to obtain reproductive stages (ascocarps) of marine lignicolous fungi in culture. MEYERS and REYNOLDS (1959) had some success with species of Lachworthia, Ceriosporopsis, Corollospora, and Torpedospora after adding balsa-wood slips to a 0.1% yeast extract–sea-water broth. Temperature, time and substrate also affect the reproduction of marine Ascomycetes (see also Volume I). KIRK (1969) employed a similar technique. He used a more complicated medium in combination with birch-wood sticks.

Some authors have obtained lignicolous fungi from sea water in a system, in which sea water is pumped continually over test blocks. A simple circulation system which can be sterilized has been described by JOHNSON and GOLDS (1959). Methods for obtaining single-spore isolates from rough cultures—a prerequisite for identification and for physiological and ecological studies—have been reviewed by BOOTH (1971c).

(b) Sustenance

In contrast to materials infested with Ascomycetes or Deuteromycetes (wood, cordage), substrates containing Phycomycetes usually cannot be stored without vitality loss of fungi within a short time (days or weeks). Material suspected to contain marine Phycomycetes should, therefore, be processed without delay.

For long-term sustenance of living pure cultures of Higher Fungi, JOHNSON and
SPARROW (1961) recommended storage on agar slants under mineral oil. The widely used lyophilisation, thus far, has not proved practicable with marine Ascomycetes and Deuteromycetes, since they do not produce abundant masses of spores. Also, no reliable and generally applicable method for sustaining cultures of marine Phycomycetes over long periods of time seems to be available. Species of Thraustochytrium and Schizochytrium, however, can be kept viable on agar slants for 3 to 5 months. The composition of the agar is as follows: peptone, 1.0 g; yeast extract, 2.0 g; agar, 8.0 g; sea-water mixture, 1000 ml (750 ml sea water, 35% S, and 250 ml distilled water). To prepare stock cultures, 5 ml of a good growing culture of the fungus are pipetted on the slants. The tubes are incubated at room temperature, in the dark, until the organisms have developed well on the agar. The cultures are then stored at about 5°C.

Fastidious Phycomycetes of terrestrial and limnetic origin have been stored in liquid nitrogen (HWANG, 1966). For further pertinent information consult FENNEL (1960) and ONIONS (1971).
3. CULTIVATION OF MICRO-ORGANISMS

3.2 FUNGI

3.22 YEASTS

H.-G. Hoppe

(1) Introduction

Vegetative reproduction by budding is a principal quality of yeasts; hence cultivation of both ascosporogenous and asporogenous genera will be treated here. The presence of yeasts in oceans and coastal waters was first established by Fischer (1894) who isolated bacteria and yeasts on an Atlantic Ocean cruise. He was not able, however, to cultivate Lower Fungi; these organisms require complicated culture techniques. Because of the similar size and nutrient requirement, there is little difference between culture and sampling methods of yeasts and saprophytic bacteria (Chapter 3.1). This fact may also be significant for assessing the ecological role of these organisms in the marine environment. Nearly all marine substrates sampled harbour yeasts and bacteria together. Antiyeast activities of certain marine bacteria have been described by Buck and Meyers (1965) and Buck (1967).

Most marine occurring yeasts can be identified on the basis of Lodder’s (1970) ’Taxonomic Study’, which includes mainly terrestrial forms. From this may be concluded that the majority of the yeasts found in oceans and coastal waters are of terrestrial origin. Thus far, only a few species have been isolated exclusively from marine sources. To these belong Metschnikowiella zobellii and M. krissii (van Uden and Castelo-Branco, 1961), Candida marina, Torulopsis torresii and T. maris (van Uden and Zobell, 1962) and C. suecica (Miranda and Norrfrans, 1968), which are well adapted to the marine environment and perhaps autochthonous marine yeasts.

Most yeasts isolated from marine habitats can be cultivated by employing standard methods. In order to obtain ecologically valid information, light, temperature, salinity and pH should be comparable to habitat conditions. On the other hand special requirements of the yeasts in respect of these factors must be regarded. It is not possible to obtain all types of yeast present in a marine water sample by using only one isolation medium. A larger number of different media has been listed by Aaronson (1970) and Booth (1971b). A review of the nutrition and solute uptake by yeasts was made by Suomalainen and Oura (1971).

Ecological studies involving cultivation of yeasts from marine substrates have attracted increasing attention during the last thirty years. Kriss and Novozhilova (1964) and Meyers and co-authors (1967b) studied the yeast flora of the Black Sea. Yeasts in the Indian Ocean received attention from Bhat and Kachwalla (1955) and Fell (1967), those of the Pacific Ocean from van Uden and Castelo-Branco (1963). Yeasts from aquatic regions of Florida were isolated by Fell and co-authors (1960) and Ahearn and co-authors (1968). The yeast flora of the North H.-G. Hoppe
Sea was studied by Meyers and co-authors (1967a), that of the Baltic Sea by Norrkrans (1966a) and Hoppe (1972a, b). Both individual density and variety of species tend to decrease with increasing distance from the shore. Only some selected species are able to grow and reproduce in offshore regions. These organisms may be called obligate marine or facultative marine—to follow the terminology of Van Uden and Fell (1968), according to their dependency on specific marine environmental factors. Whereas there is hardly any difference between yeast species composition in fresh water and estuaries (Ahearn and co-authors, 1968), offshore regions are primarily occupied by the genera Rhodotorula, Candida and Debaryomyces. Seaward distribution of yeasts appears to be limited by additive environmental stress due to such factors as temperature, salinity, pH and low concentration of nutrients.

After 20 years of studying primarily distribution and taxonomy, investigators are now beginning to concentrate on specific ecological, physiological and biochemical problems. Studies on substrate uptake and threshold concentrations of nutrients analogous to those of bacteria (Jannasch, 1970) would be very instructive. Culture methods may help to reveal yeast species of special ecological significance and specific metabolic capacities.

(2) Isolation and Cultivation

(a) Yeasts from Water Samples

The density of yeast cells in marine habitats is low. Hence isolation by plating is successful only in eutrophic inshore areas, such as harbours, estuaries and sewage outlets. For quantitative sampling of yeasts from offshore waters, membrane filters are used. Water is collected by a sterile sampler (Zobell, 1941b; Nitschin, 1962). Depending on the expected yeast density, water volumes from 1 ml to 1000 ml are sucked through a membrane filter of 0.2-0.6 μm-pore size. The filter is then placed on isolation agar, solidified in Petri dishes. In heavily polluted areas, series of dilutions up to $10^{-3}$ may be necessary to obtain countable densities of 10 to 100 colonies ml$^{-1}$. At an incubation temperature of 20°C, the grown macrocolonies are counted after 5 and 7 days, at 12°C to 14°C after 9 days (Meyers and co-authors, 1967a), at 10°C after 7 and 14 days (Hoppe, 1972a). The lower incubation temperatures correspond to the sea-water temperatures prevailing in northern regions; they limit the growth rates of moulds without reducing the number of yeast colonies.

Solid media can be replaced by carton discs and liquid media (Gocke, unpublished). Inoculated membranes are then placed on soaked cartons in Petri dishes. This method is very practical on long ship cruises; it can also be used for isolating bacteria.

In the microcolony method small colonies on the membranes are counted microscopically after short-time incubation of 1 to 3 days and staining (e.g. 1% erythrosine in 5% phenol). In this way, the results are quickly available.

Direct counting of yeasts by bright field, phase contrast or fluorescence microscopy requires considerable experience; but this technique is promising in pure
Yeast cultures. Kriss (1959) compared results from plate counts with those obtained by direct microscopic examination. The presence of yeasts could be detected in 56% of his water samples from the Black Sea when observed microscopically, in agar plate test only in 49%. Such discrepancies in enumeration methods are caused by dead or inactive cells or the attachment of several cells to organic particles which form only one colony on membrane filters. Some yeasts may not grow at all on the culture media thus far employed (van Uden and Fell, 1968).

For isolation and cultivation of yeasts from marine substrates, numerous nutrient media are in use. Except for synthetic media, there are no essential differences in the composition of these media. Therefore, a few examples will suffice:

The isolation medium used in the reviewer's laboratory contains: glucose, 20 g; peptone (Difco), 5 g; meat extract (Lab-Lembco-Powder, Oxoid), 2.3 g; yeast extract, 1 g; and agar-agar, 15 g; dissolved in 11 aged sea water or appropriate seawater-freshwater mixtures. The medium is autoclaved for 20 mins at 2 atm, and acidified after cooling to pH 4.5 with 10% lactic acid. To prevent bacterial growth, different antibiotic mixtures are added to the media before plating: (i) Chlortetracycline HCl, 10 mg %; chloramphenicol, 2 mg %; streptomycin sulphate, 2 mg % (Fell and co-authors, 1960). (ii) Chloramphenicol, 50 mg %; chlortetracycline, 10 mg %; streptomycin, 15 mg % (Myers and co-authors, 1967b). (iii) Chloramphenicol, 0.5% or Gentamycin sulphate (Schering), 0.2 g l⁻¹ (Ahearn and co-authors, 1968). (iv) Dihydrostreptomycin or Binotal (Bayer), 0.25 g l⁻¹ (Hoppe, 1972a). Mould overgrowth can be suppressed by fungistatic agents, such as sodium propionate and diphenyl (Hertz and Levine, 1942). Morris (1968) and Seshadri and Sieburth (1971) questioned the use of acidic media in cultivating yeasts from alkaline sea water, because bacterial growth can be suppressed completely by sufficient antibiotics. Seshadri and Sieburth recommended a supplement of 100 mg l⁻¹ chloramphenicol. For vital staining of colonies they used a supplement of 100 mg l⁻¹ TTC (2,3,5-triphenyltetrazolium chloride at pH 7.0).

A simple medium, appropriate for isolation of yeasts from various marine substrates, has the following composition in % w/v (van Uden and Castelo-Branco, 1963): glucose, 2; peptone (Difco), 1; yeast extract (Difco), 0.5; agar, 2; filtered sea water. The pH of the medium is adjusted to 4.5 with lactic acid. From isolated yeast colonies, pure cultures can be obtained by repeated streaking (same medium) and isolation of single-cell colonies. Preservation by freeze drying has proved best for the sustenance of pure cultures. In this way the capacity for ascospore formation is maintained for a long time (Wickerham, 1951), and laborious long-term laboratory cultivation is no longer necessary. Cultures on agar slants, especially of Rhodotorula species, should be transferred frequently (monthly) to fresh medium. For species with oxidative metabolism the culture tubes should be closed with cellulose stoppers.

(b) Yeasts from Marine Sediments

Sediments are sampled with a sterilized spoon or a sawed-off syringe; in greater depth, with a special bottom sampler. An appropriate sediment volume (1 cm³) is distributed in a portion of sterile sea water and ground by an emulsifier or by shaking with glass beads to detach the organisms from the substrate. A sufficient quantity
(1 ml) of the emulsion is then plated with agar, or filtered through a membrane filter after settlement of coarse particles. Accurate quantitative results cannot be anticipated by this method. If the sediment layer on the filter is rather thick, bacterial colonies may develop despite added antibiotics.

(c) Yeasts from Marine Plants

For isolating yeasts from seaweeds (Seshadri and Sieburth, 1971), up to 5% seaweed (wet weight) is suspended in sterile artificial sea water. Adhering yeast cells are set free by shaking with glass beads for 10 mins, or by blending in precooled (4°C) artificial sea water for 2 to 5 mins. To avoid transfer of inhibitory substances from the seaweeds, the yeasts above the filter are held in suspension by a stirring blade, while the tenfold sample volume of sterile sea water is added. Then the sample is reduced to 5 ml and quantitatively transferred to a second filter for incubation.

Studies on yeasts isolated from marine plankton were carried out by Suehiro and co-authors (1962) and Suehiro and Tomiyasu (1962, 1964). Phytoplankton is collected with a net and directly transferred to sterilized cotton-plugged flasks. After adding chlorotetracycline (50 ppm) and citric acid (200 ppm), the samples are stored at 20°C and surveyed for yeast development at 24-hr intervals. The isolation medium consists of 3% malt extract (Difco) and 1.5% agar prepared with sea water.

(d) Yeasts from Marine Animals

Various isolation techniques for yeasts from internal and external surfaces of invertebrates and fishes have been adapted by Roth and co-authors (1962), Siepmann and Hörnke (1962) and van Uden and Castelblanco (1963). Small animals or aseptically removed pieces of animals are placed or streaked out directly on the agar surface. The whole sample may be incubated in liquid media or adhering yeasts rinsed with sterile sea water. Gut contents of birds and other animals are suspended in sterile sea water and plated with agar after serial dilution. Exact quantitative analysis should not be expected from these methods.

(e) Selective Cultivation

For isolation of the human pathogenic yeast species Candida albicans several methods are used. Stedham and co-authors (1966) recommended a modified Pagano Levin Medium to isolate Candida species selectively from highly contaminated materials. The medium contains: Pagano Levin base (Difco); 2,3,5-triphenyl-tetrazolium chloride (TTC, Difco), 0.1 mg ml⁻¹; cycloheximide, 0.5 mg ml⁻¹; and chloramphenicol, 0.05 mg ml⁻¹.

According to Lodder (1970), yeasts frequently metabolize mono-, di-, and tri-saccharides, polyols and organic acids as well as polysaccharides (starch and inulin). The compounds of the plant cell wall, cellulose and pectin, can be decomposed only by certain species. Cellulolytic activity was substantiated, especially for Trichosporon cutaneum and T. pullulans (Denis, 1972), which may perhaps act as primary invaders. Cellulose breakdown was tested according to the method of Bravery (1968). Pectolytic properties were revealed for the yeast species T. cutaneum, T.
pullulans, Cryptococcus macarans and Candida albidas by DENNIS (1972). The selective medium consists of yeast extract, glucose, peptone, meat extract, and agar (WICKERHAM, 1951), supplemented with 0.5% CaCl₂ and layered with 2% sodium polypectate. T. cutaneum, in particular, was frequently isolated in contact with marine phytoplankton (SUHIBO and TOMIYASU, 1962) and putrified algae.

(3) The Importance of Environmental Factors in Yeast Cultivation

Survival and reproduction of terrestrial yeasts in a marine environment depends on their responses to environmental factors, such as temperature, salinity, pH and concentration of assimilable nutrients. Culture experiments to investigate minimum, optimum, and maximum tolerances of yeasts in regard to these factors have been made by Ross and MORGAN (1962), NORKRANS (1966b, 1968), NORKRANS and KYLIN (1969) and HOPPE (1972a). The growth rate was estimated by turbidity measurements (610 nm), plating, or direct counting after various periods of incubation in sterilized media (filtration).

The incubation temperature greatly affects the development of yeasts in seawater media (see also Volume I: OPPENHEIMER, 1970). Solid media (NORKRANS, 1966b) as well as liquid media (HOPPE, 1972a) have been used to determine the cardinal temperatures of yeast growth. Liquid media seem to yield more detailed results in critical ranges. HOPPE used the multiple temperature-incubation apparatus (OPPENHEIMER and DROST-HANSEN, 1960) for maintaining temperature gradients and estimated growth by turbidity measurements. In a suitable nutrient medium (glucose, 8 g; peptone, 3 g; yeast extract, 2 g; KH₂PO₄, 0.3 g; MgSO₄ · 7H₂O, 0.3 g; 1000 ml water with suitable salinity, pH 4.5), yeasts develop from nearly 0° to 42°C. Yeasts isolated from the Western Baltic Sea grew best between 25° and 30°C (HOPPE, 1972a); such high temperatures are reached only in summertime in shallow coastal waters; the average temperature is 9°C. Most of the yeasts isolated from the Western Baltic Sea are able to grow between 1° and 5°C after a prolonged lag period. Microscopical observation indicates no cell damage at low temperatures, but progressive destruction of cell structures at supra-optimal temperatures. In Rhodotorula strains, pigmentation is reduced considerably at supra-optimal incubation temperatures. Psychrophilic strains of Rhodotorula infirmo-minuta have a minimum temperature for growth of 4°C, an optimum of 14° to 18°C, and a maximum of 26° to 28°C (AHERN and ROTH, 1966). Strains from seawater showed a special requirement for vitamins of the B-complex.

The salinities tolerated vary from 0 to 10% w/v NaCl (Saccharomyces cerevisiae, Cryptococcus neoformans) to 0 to 22% w/v NaCl for Debaryomyces species (ROSS and MORGAN, 1962; see also Volume I: MACLEOD, 1971). Salinity tolerance is much lower than sugar tolerance (KOPPENSTEINER and WINDISCH, 1971); it is based on tolerance to osmotic pressure. Fermentation activity of cell-free extracts ceases at an osmotic pressure of 30 atm in neutral salt solutions, but at 120 atm in sugar media (KOPPENSTEINER, 1969). The cause of different salt tolerance of S. cerevisiae and D. hansenii was studied by NORKRANS and KYLIN (1969). Incorporation experiments with the isotope pairs ²²Na⁻²⁴Na and ⁴²K⁻⁸⁶Rb showed that the relation of K to Na was higher in the cells than in the medium, and higher in D. hansenii than in S. cerevisiae. This result indicates more effective Na extrusion and K uptake in D. hansenii than

ENVIRONMENTAL FACTORS IN YEAST CULTIVATION
in *S. cerevisiae*. In some yeasts (*S. mellis, S. rouxii, S. bisporus* and *Pichia pastoris*), KOPPENSTEINER and WINDISCH (1972) recorded increased compatibility of salt solutions at increasing concentrations of fructose or galactose and lactose. The kind of nitrogen source available affects the growth capacity of yeasts at different salt concentrations (ROSS and MORRIS, 1962; Table 3-5).

### Table 3-5

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>% w/v NaCl in medium</th>
<th>(NH₄)₂SO₄ Peptone Asparagine Urea Fish extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pichia membranaeformis</em> 180</td>
<td>21-0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>22-0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>23-0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>24-0</td>
<td>—</td>
</tr>
<tr>
<td><em>Debaryomyces kloeckeri</em> 354a</td>
<td>21-0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>22-0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>23-0</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>24-0</td>
<td>—</td>
</tr>
<tr>
<td><em>Debaryomyces kloeckeri</em> 132</td>
<td>21-0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>22-0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>23-0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>24-0</td>
<td>—</td>
</tr>
<tr>
<td><em>Rhodotorula glutinis</em> 321p</td>
<td>21-0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>22-0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>23-0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>24-0</td>
<td>—</td>
</tr>
</tbody>
</table>

Supranormal salinities may prolong lag phase and generation time. For example, the lag phase of *Rhodotorula rubra* lasts 28-5 hrs at 8-1% NaCl, but 68 hrs at 16% NaCl. Generation time for *Cryptococcus albidus* was 3 hrs at 0 to 4%, 7 hrs at 8%, and 21 hrs at 12% NaCl (NORKRANS, 1966b). Respiration and fermentation intensities remained nearly the same from 0% to 4% w/v NaCl in yeast strains of marine origin (*Debaryomyces hansenii, Saccharomyces cerevisiae, Cryptococcus albidus, Candida zeylanoides, NORKRANS, 1968*).

Cultivation experiments revealed no specific sodium-chloride requirement for yeasts from marine waters. Yeasts usually exhibit good or best growth in media without sodium chloride.

Hydrogen ion concentration (pH) of the medium is important for successful yeast cultivation. Most yeasts from terrestrial and aquatic habitats are acidophilic. Usually yeasts from the Western Baltic Sea exhibit the following growth characteristics in relation to pH: minimum at pH 2 to 3, optimum at pH 3 to 6-6, maximum at pH 8 to 9. While cells die off in extreme acid media, cell damage was not noticed
at maximal pH values (up to pH 10 tested), with the exception of *Saccharomyces cerevisiae*; this may be significant for the survival of yeasts in sea water (pH 7.5-8.4).

At alkaline pH values, Norkrans (1966b) established a decrease in growth rates and prolongation of the lag phase in several marine yeasts. Increasing precipitation in alkaline culture media disturbs turbidity measurements. Attempts to reduce precipitation by admixture of EDTA failed, because this substance inhibits growth in some yeast species (Norkrans, 1966b).

**4** Cultivation of Yeasts in Natural Sea Water

Yeasts may develop differently in nutrient media and in natural sea water. In sea water low contents of assimilable compounds seem to be primarily responsible for limited growth. Survival, reproduction, generation time and morphology of yeasts in this medium have been studied by Kriss (1961), Norkrans (1966b), Madri (1968), and Hoppe (1972a). Sea water with its natural microflora, or sterile filtered sea water, were inoculated with pure cultures and incubated in batch culture or continuous culture. Yeast development was estimated by plating techniques, on membrane filters, or direct counts in chambers.

Kriss (1961) cultivated *Torulopsis pulcherrima* and *T. candida*, which occur in marine habitats, in sea water from the Black Sea and from the Pacific Ocean. Both species are capable of reproducing in sea water; maximal cell counts (10 to 15-fold of the inoculum) were obtained after 12 to 30 days incubation. In the Black Sea yeasts have been isolated from the anaerobic zone in 1000 to 1500 m depth. According to Kriss (1961), many marine yeasts are capable of denitrification enabling them to live in oxygen-free media.

Norkrans (1966b) tested the survival of yeasts in sea water of 34% NaCl enriched with nutrients. After 120 days incubation survival rate was 10% for *Rhodotorula glutinis*, *R. rubra* and some strains of *Debaryomyces hansenii*; less resistant were *Candida* sp., *Cryptococcus laurentii, C. luteolus* and *Saccharomyces cerevisiae* with 5% surviving cells after 60 days.

When exposed to sea water, *Candida albicans* formed typical mycelium and chlamydospores only in exceptional cases (Madri, 1968). The development of yeasts and various ecological groups of bacteria in a 5-l water sample (16.3% S) was estimated by Hoppe (1972a, Fig. 3-9). After 33 days the number of survivors had decreased to 1.6% for the yeasts, 10% for ‘freshwater’ bacteria, and 22% for marine bacteria. In brackish-water batch culture with progressively decreasing nutrient concentration yeasts seem to be limited more severely than bacteria.

Experiments with pure cultures of *Saccharomyces cerevisiae* (H 116) and *Rhodotorula mucilaginosa* (H 25) were performed in sterile filtered Baltic Sea water (16.4% or 17.3% S) and tap water. *S. cerevisiae* showed at no time an increase in cell counts. In tap water the cell numbers decreased even more quickly than in sea water. At an incubation temperature of 20° C, the rate of survival was 50-7%; at 4° C, 70% (Fig. 3-10). The mean size of the cells decreases from 8.3 × 6.4 μm (nutrient agar) to 6.9 × 4.5 μm (20° C, sea water) to 5.7 × 4.5 μm (4° C, sea water). Sporulation was stronger at 20° than at 4° C. Supplementary glucose caused a significant increase in cell counts correlated with germination of ascospores. Addition of NH₄NO₃ caused only
3. CULTIVATION OF MICRO-ORGANISMS (H.-G. HOPPE)

Fig. 3-9: Development of yeast and bacteria populations in a 5-l brackish-water (16.3‰, S) sample, which was stirred and incubated in the dark at 20°C. ZS: modified yeast extract-peptone-agar according to OPPENHEIMER and ZOBELL, 1952; ZL: ZS prepared with tap water; yeast medium: see p. 349. (After HOPPE, 1972a; modified; reproduced by permission of Institut für Meereskunde, Kiel.)

Fig. 3-10: Development of Saccharomyces cerevisiae (H 116) and Rhodotorula mucilaginosa (H 28, isolated from the sea) in sterile filtered sea water (17.3‰, S), stirred and incubated at 4°C and 20°C. (After HOPPE, 1972a; modified; reproduced by permission of Institut für Meereskunde, Kiel.)
a weak and temporary increase in yeast development. This indicates that the concentration of assimilable carbohydrates is of decisive importance for survival and reproduction of yeasts in sea water.

Rhodotorula mucilaginosa, isolated from sea water, was able to grow in all media tested. At an incubation temperature of 4°C, an 18-times higher cell concentration was yielded than at 20°C (Fig. 3-10). While generation time is usually longer at 4°C, the better survival chances may be ecologically more important; at 20°C, generation time is short, but death rate high.

In a chemostat, Rhodotorula mucilaginosa can be cultivated continuously in sterile filtered sea water. At 25°C, a concentration of 5020 cells l⁻¹ with a generation time of 178 hrs could be maintained; at 4°C, the corresponding values were 23,800 cells l⁻¹ and 263 hrs. This means that the yeasts in question are capable of building up a self-perpetuating population in sea water when competitors are excluded.

(5) Some Aspects of the Use of Yeast Cultivation

In general yeasts are mesophilic and able to develop over a fairly wide range of pH values; hence their isolation and maintenance normally presents few, if any, difficulties (van der Walt, in: Lodder, 1970, p. 36). This statement may be applicable also to yeasts found in marine habitats. Cultivation experiments with yeasts from marine sources have been mainly concerned with ecological considerations.

To all appearances, yeasts may have a bearing on the supply of vitamins and nutrition of marine animals (Chapter 5). Siepmann and Höhnk (1962) observed a strong riboflavin extrusion of Debaryomyces subglobosus during assimilation tests. 33 from 68 strains of this organism were isolated from the interior of fishes. Russian investigators have grown the polychaete Nereis sp. exclusively on yeast cells (cited from Siepmann, 1963). Rodina (1960, in: Siepmann and Höhnk, 1962) demonstrated the importance of yeasts as a source of nutrients for planktonic and benthic animals of lakes and fish ponds. Yeasts can be used as food for fry (Assman, 1957, in: Siepmann and Höhnk, 1962).

Some species of yeast are considered to be pathogenic to man and animals (Gentles and La Touche, 1969). Among these, especially Candida tropicalis, C. pseudotropicalis, C. parapsilosis, Trichosporon cutaneum and Rhodotorula mucilaginosa were also found in coastal and offshore areas. For details consult Chapters 8 and 9. The occurrence of the human pathogenic Candida albicans and the industrially important Saccharomyces cerevisiae can serve as an indicator of pollution in coastal waters.

Substrate uptake is of considerable importance in aquatic ecology. van Kleeff and co-authors (1969b) used a modified autoradiographic method for the detection of radionuclides accumulated in bacterial as well as yeast colonies. They tested 150 classified yeast strains for accumulation of ⁶⁰Co, ⁶⁵Sr, ¹⁰⁶Rn, ¹³⁷I, ¹³⁴Cs and ¹⁴⁴Ce. Candida humicola accumulated almost all cerium presented after 24 hrs incubation, while about 80% cobalt was taken up after 16 days shaking in the medium (Kokke and co-authors, 1969). To study the localization of the radionuclides within the yeast cells, a method described by van Kleeff and co-authors (1969a) may be used. This technique necessitates electronmicroscopic examination.
Yeasts contribute to heterotrophic remineralisation of organic substances in the sea. (MEYERS and AHEARN, 1974). Since most yeasts isolated from marine substrates do not grow optimally in sea water, their remineralization capacity in the sea seems inferior to that in fresh water. While marine bacteria are reported to be predominantly proteolytic, the ecological importance of yeasts may be based on their preference for carbohydrates (with the exception of some species, such as Candida lipolytica and C. tropicalis, which specialize on hydrocarbons; HUG and FIECHTER, 1973). With increasing distance from the shore, the 'biomass' of yeasts decreases more than that of bacteria. In Kiel Bay the relation 'biomass' of yeasts to marine bacteria changes from 1:61 to 1:180 (HOPPE, 1972a).

These facts indicate that yeasts are mainly inshore-inhabiting organisms or marine transients. They are much less tolerant to marine waters than are marine bacteria. The low nutrient concentration of the sea seems to favour oxidatively dissimilating yeast species, resulting from the better energy balance of this metabolic type. Yeast distribution in marine environments depends on microzonation and ecological niches providing sufficient assimilable nutrients.

Literature Cited (Chapter 3)


3. CULTIVATION OF MICRO-ORGANISMS


LITERATURE CITED


3. Cultivation of Micro-organisms


LITERATURE CITED


3. CULTIVATION OF MICRO-ORGANISMS


4. CULTIVATION OF PLANTS

4.1 UNICELLULAR PLANTS

R. UKELES

(1) Introduction

(a) Importance, Purpose and Scope of Unicellular Algae Cultivation

A basic prerequisite for the critical analysis of the relationship between unicellular algae and their environment, abiotic and biotic, is our capability to cultivate these algae under adequate conditions. Progress in modern marine ecology depends heavily upon more exact information on environmental and nutritional requirements. There is great demand for ecologically meaningful information obtained under controlled and reproducible conditions.

Ecological, physiological, biochemical, life history, and phylogenetic investigations of micro-algae benefit from studies on laboratory cultures that often elucidate characteristics not apparent in isolates taken directly from nature (Parke and Adams, 1960; Braarud, 1961; Wilson, 1967; Kornmann, 1970). To conduct these various investigations it is first necessary to develop culture methods for a particular species that will provide dense cell populations, preserve the integrity of the species and yield predictable results. In many cases, axenic cultures maintained under well-defined environmental conditions are most desirable. Although less rigorous standards are satisfactory for some purposes, the interpretation of experimental results is generally enhanced by high standards of purity.

Since the introduction of *Chlorella* species as an experimental tool for investigation of photosynthesis by Warburg (1919)—because it was simple to handle under controlled conditions and gave better reproducibility than higher plants—this alga and others have become favourite organisms for elucidating photosynthetic mechanisms, as well as other problems in biochemistry and cell physiology. There has developed a considerable literature of investigations into the fundamental biochemistry of photosynthesis (Brody and Brody, 1962), pigments (Schiff, 1964), metabolic pathways, and nutritional requirements (Provasoli, 1958b) in which micro-algae are used as experimental tools. With the advent of electron microscope techniques, micro-algae are providing much of the information on membrane structure and cell-organelle development (Pitelka, 1963; Brown and co-authors, 1970).

The large uninucleate unicellular alga *Acetabularia mediterranea* (Fig. 4-1a), isolated from the littoral zone of tropical and subtropical environments, is an ideal object for investigations of nuclear-cytoplasmic interrelationships (Hammerling, 1931; Hammerling and co-authors, 1959). Particularly useful for studies of the relationship between cytoplasm and nuclear division is stalk division leading to
dichotonic and trichotonic cells. This is only sometimes found in nature but can be induced in the laboratory (Fig. 4-1b; Bonotto and Bonnlns-Van Gelder, 1969). *A. mediterranea* is also well suited for studies on RNA-synthesis, interspecies grafting, and ionizing-radiation effects on the morphogenetic process (Pueux-DAO, 1962; Pueux-DAO and co-authors, 1972; Bonotto and Kerckmann, 1972). Marine algae are also useful for studies on ionic relations and membrane transport at the cellular level (Gutknecht and Dainty, 1968).

![Fig. 4-1: Acetabularia mediterranea. (a) Reproductive cap (9 mm diameter); (b) branched cells, each branch differentiating a reproductive cap (cell length 35 mm). (Unpublished photographs taken by, and reproduced by permission of S. Bonotto.)](image)

The potential of unicellular algae as a food supplement for man in the daily diet (Osnitskaya and Goryunova, 1962) or as a food for such esoteric pursuits as submarine travel or space exploration has been evaluated in numerous publications (Casey and Lubitz, 1963; Casey and co-authors, 1963). Bonott (1964) proposed that man's physiological needs for O₂, water, food, and sanitation disposal could be met by a closed system involving a continuously managed culture of micro-algae. Thus far, freshwater species of Chlorella have received most of the attention, but some investigators (e.g. Eddy, 1956) have already recognized the importance of considering other species and that isolates from salt and brackish-water environments may offer some special advantages. Recently, a concept of industrial photosynthesis has become popular that describes a multi-dimensional process of wastewater treatments, food production, and water reclamation. The major emphasis in such a system is on the algal photosynthetic conversion of solar energy into a high protein food while simultaneously reclaiming water and disposing of waste (Muttoni and co-authors, 1965; Schmitt, 1965; Ryther and co-authors, 1972). Marine and brackish-water algal species can then be utilized directly as a food for rearing crustaceans and molluscs or the algal cultures processed and used to supplement food of other animals (Fig. 4-2; Chapter 5).
As pressures for additional food sources increase, the controlled industrial cultivation of many unicellular marine algae on a large scale is a likely consequence (HUTNER, 1964). Methods already developed for the rearing of bivalves depend on the large-scale culture of particular species of unicellular marine algae as a food source in the early stages of bivalve development (LOOSANOFF and DAVIS, 1963; DAVIS, 1969; UKELES, 1971; Chapter 5). Among prospects for the industrial use of micro-algae is a proposal that species of *Chlorella* could be cultivated as a source of amino acids and added to cereal diets (HUNDLEY and co-authors, 1956). It is believed that algae are nutritionally equivalent to yeast, being higher in protein, fat and vitamin content than other vegetable sources, with the exception of some seed products, and with a potentially higher yield per unit space than conventional crops (FISHER, 1955; LUBITZ, 1963).

Industrial marine micro-algal cultivation also has a potential for the production of special substances: the gel-forming carrageens, of which *Porphyridium cruentum* is an excellent source (GOLUEKE and OSWALD, 1962); or pharmaceuticals (HUTNER, 1964) as provitamin A which can be obtained from *Dunaliella salina*. Sometimes beta-carotene pigment is present in *D. salina* in concentrations that are 10 times higher than in most green algae and leaves. A method of isolating the carotene was developed and a type of beta-carotene preparation made available that suggests positive results in animal husbandry and medicine (RABOTNOVA and MILEKO, 1966). No doubt, products of other synthetic capacities in marine micro-algae could be put to practical use, possibly the production of thiamine by *D. tertiolecta* and *Skeletonema costatum*, or B₁₂ by *Coccolithus huxleyi*, or biotin by *D. tertiolecta*, *Phaeodactylum tricornutum* and *S. costatum* (CARLUCCI and BOWES, 1970).

For some time it has been a hope that certain species of algae would be valuable assay organisms for examining water quality. With the growing interest in marine pollution (Volume V), micro-algal assay organisms are being proposed for evaluating...
toxicants or eutrophic conditions in freshwater, brackish-water and marine environments. ALEEM (1970) bioassayed sea water from the English Channel at Plymouth and from the Celtic Sea with uni-algal cultures of the pelagic diatoms Chaetoceros dydimus, Phaeodactylum tricornutum and a 'reddish flagellate A'. In an extension of the study of the problem of 'good' and 'bad' sea water that dates back to the classical studies of ALLEN and NELSON (1910), ALEEM's results agreed with WILSON's (1951) regarding the grading of different sea water as good or bad for rearing larvae. Bioassay experiments were also undertaken by JOHNSTON (1963b) using Skeletonema costatum and Peridinium trochoideum; a rapid monitoring of water quality was developed using a Chlorella species (CULLMORE and McCANN, 1972). Assay experiments with Chlorella ovulifl S. costatum and P. tricornutum also yielded information on the quality of water in an Oslofjord with various polluted discharges (SKULBRO, 1970). SMAYDA (1970) noted marked differences in water quality of surface waters collected from different stations in Phosphorescence Bay, Puerto Rico, based on the growth response of the diatoms Bacteriastrum hyalinum, Cyclotella nana, S. costatum and Thalassiosira rotula. Vitamin content in sea water was assayed by RYTHER and GUILLARD (1962), BELSER (1963) and GOLD (1967) utilizing unicellular marine algae. The industrial application of marine microalgae to assay of vitamins, and cytotoxic pharmacological agents was also proposed (AARONSON and co-authors, 1964), using what GAVIN (1956) defined as 'analytical microbiology'—a branch of microbiology in which micro-organisms are used for the qualitative determination of certain chemicals.

Whatever the ultimate importance and purpose of unicellular algae cultures, there are certain principles that must be adhered to. These may be modified or amplified depending on the facilities available, the scale of cultivation, the particular use of the culture and strain requirements. However, the basic approach to cultivation is similar in all cases. A physical and chemical environment must be provided that will induce a particular species of micro-algae to increase in cell numbers with no change in characteristics unless intended by the experimenter.

(b) Availability of Unicellular Algae Species for Cultivation

DROOP (1961a) observed the significant fact that the majority of unicellular marine algae species in culture are neritic, littoral or supralittoral in origin. Species usually found in estuaries are known as laboratory 'weeds' growing in laboratory culture without great difficulty, e.g., Phaeodactylum tricornutum, Skeletonema costatum, Dunaliella tertiolecta, Chlorella sp. and Nannochloris sp. It is not known why so many phytoplankters will grow well in a relatively low nutrient sea water, as the ocean, and will not grow in such water or in enriched sea water in the laboratory. WOOD (1968) could get truly oceanic species to grow in the laboratory from time to time but could not obtain reproducible results. DROOP (1961a) suggested that the reason for the difficulty of culturing planktonic species does not lie in the capacity of ordinary laboratory media to satisfy a nutritionally exacting species, but that these species have developed a narrow tolerance to physical or physicochemical conditions as a result of becoming adapted to the constancy of natural oceanic habitats. These habitats cannot always be duplicated in the laboratory. Some conditions in a culture medium are very different from those in nature, such
as enclosure within a small space, need to sterilize solutions, artificial pH buffers and conditions of nutrient and light saturation. However, concern about the artificiality of the culture environment may often be overemphasized. Many species are available today in cultures that were at one time considered not amenable to the unduly restrictive environment of laboratory culture. According to Ackman and co-authors (1970), any bloom must result from an imbalance of the factors that normally restrict growth and reproduction of a pre-existing parent stock. There would be no reason to believe that the analytical data from a particular natural bloom sample would be more typical of a species than those obtained from artificial cultures in which certain growth components were eliminated or provided in excess.

Organisms isolated from the natural marine habitat are obtained from plant fragments or other debris in the water, from naturally occurring blooms or from plankton tows. Wood (1968) discussed the inefficiency of plankton tows, particularly in regard to loss of small organisms as μ-flagellates and very small diatoms. Each of the other collection methods, such as filtration, continuous centrifugation or closing samplers, offers some improvements but presents its own difficulties, particularly for collection of the more delicate species.

Another source of marine algae for culture are symbiotic forms, zooxanthellae of marine invertebrates. With new separation techniques, antibiotics and defined media, axenic culture of symbionts is now possible. The history and present status of these techniques have been described in numerous publications (McLaughlin and Zahl, 1957, 1959, 1962; Zahl and McLaughlin, 1957; Freudenthal, 1962; Lee and Zucker, 1969).

Established culture collections offer a variety of species with a known culture history for experimental investigation. The 5 major collections in the world are: (i) Culture Centre of Algae and Protozoa, 36 Storey's Way, Cambridge CB3 0DT, England; (ii) Sammlung von Algenkulturen, Pflanzenphysiologischen Instituts der Universität Göttingen, Nikolausbergerweg 18, Göttingen, West Germany; (iii) Sbirka Kultur Autotrofnich Organismù ČSAV, Viničá, 5, Praha, 2, Czechoslovakia; (iv) Culture Collection of Algae, Institute of Applied Microbiology, University of Tokyo, Japan; (v) Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Indiana 47401, USA.

While most collections have a preponderance of freshwater species and few marine isolates, many smaller collections exist that concentrate on marine species. Tropical and subtropical cultures are maintained in the laboratory of W. H. Thomas, Scripps Institution of Oceanography, La Jolla, California (USA); a variety of marine and brackish-water forms are in the Oceanographic Institution, Woods Hole, Massachusetts and also at the National Marine Fisheries Service Laboratory, Milford, Connecticut (USA). A few marine species are available from the American Type Culture Collection, Washington, D.C. Published lists of culture collections contain information on the marine isolates available, fees and maintenance media for stocks (Provasoli, 1958a; Starr, 1960, 1964, 1971a, b; Baslerová and Dvorskáková, 1962; Koch, 1964).

According to Wood (1968) the word 'marine' is usually taken to include oceanic environments, waters and sediments of the continental slopes, estuaries, and some lakes that are as salty as the sea. It is sometimes difficult to decide where the freshwater environment ends and the marine begins. On the whole, marine and fresh-
water phytoflagellates show similar requirements, except for differences in salinity. In fact, salinity requirements can be very different for species that are taxonomically closely related. For example, the Euglenophyta are most abundant in fresh water, but brackish-water and salt-water forms are also known (Volume I: GESSNER and SCHRAMM, 1971). Species of other genera (Chlorella, Stichococcus) also range from marine to freshwater habitats, which are apparently inexact about salinity (GEORGE, 1957). Much of algal culture methodology defies any distinction between marine and freshwater culture techniques. This chapter concentrates on culture methods applied to generally accepted marine and brackish-water species, although references to freshwater species will occasionally be necessary. Discussion will be limited to the culture of those organisms which are unicellular in the adult stage thereby excluding spores of multicellular algae. The blue-green and phagotrophic species of marine micro-algae are covered in other chapters.

The terms employed are used as given by STRICKLAND (1966). Phytoplankton refers to plant organisms suspended in the water with very limited mobility which cannot or do not maintain their distribution against local water movements. Microplankton, nanoplankton, and ultraplankton describe size distributions in the range of 50 to 600 µm, 10 to 50 µm and 0-6 to 10 µm, respectively.

(2) Laboratory Culture

(a) Technological and Procedural Aspects

Culture Enclosures

Cleaning of glassware is an important aspect in algal culture (Chapter 7); failure to cultivate a unicellular alga or to obtain reproducible results may sometimes be attributed to improper glassware treatment. New glassware may be contaminated with spores from packing material or a residual film of alkali (remove by soaking in 1% HCl). All organic and metal contaminants must be carefully removed. After cleaning, the glassware should have a neutral reaction and be free from residual anti-microbial or stimulating agents. Cleaned glassware should be carefully drained or oven-dried and stored to avoid dust.

Cru (1942) recommended preliminary rinsing in hot tap water, soaking for 2 days in strong potassium dichromate sulphuric acid solution, followed by heating at 70° C for 1 hr, and successive rinses with hot water, distilled water and Pyrex glass-distilled water. Kain and Fogg (1958a) also cleaned glassware by soaking in chromic acid followed by 30 rinses in tap water and 3 in distilled water. Other workers believe that the use of cleaning solution with chromate should be avoided since chromic ions tend to absorb on the glass and then slowly leach out into the culture medium (HERVEY, 1949). Lavin (1934) found that 10 rinses did not completely remove the dichromate from glass, and Richards (1936) reported that a dichromate-cleaned pipette yielded 0.1 to 0.2 ppm after the 10th rinse. Lewin (1959) suggested concentrated sulphuric acid, saturated with commercial-grade sodium nitrate, for washing. When detergents are used special care must be taken
to wash them out since they are sometimes inhibitory (UKELDIS, 1965a). Caution in the use of high detergent concentrations has also been recommended by Bernhard and Zattera (1970) although they found that detergents have little or no effect on three phytoplankters in concentrations which probably remain on glassware. Lewin (1959) regarded multiple changes of distilled water unnecessary, but other scientists employ elaborate rinsing procedures. For example, Cummins and co-authors (1966) rinsed 5 times in distilled water plus 5 times in double-distilled water after washing glassware in a 95% ethyl alcohol sodium hydroxide mixture. Hayward (1968b), in a study on metal requirements, washed glassware with detergent, rinsed with Pyrex double-distilled water, steamed for 30 mins with constantly boiling HCl, rinsed again with double-distilled water and then autoclaved glassware filled with double-distilled water capped with beakers, as suggested by Waring and Werkman (1943). A small amount of chelating agent added to the rinse water is often recommended. After frequent use, culture vessels may become coated with material that is resistant to normal washing procedures; in this case Quayashi and Spencer (1971) recommended that glassware be boiled in soap and sodium hydroxide solution before the usual washing procedure. Provasoli and Gold (1962) baked glassware for 2 hrs at 200°C to remove adhering organic matter.

Conventional culture methods for marine unicellular algae call for the inoculation of solid or liquid media dispensed into various types of culture containers. Culture container sizes range from a hanging drop to the deep tank described by Strickland and co-authors (1968). Containers for micro-algae can be adapted from those available for other types of microbiological culture techniques: e.g., culture-wet film, hanging drop, slide immersion, agar blocks, or flat capillary techniques (Hartman, 1968; Norris and Ribbons, 1969). The more conventional culture containers are limited to various size test tubes, flasks or carboys. Microbiological cultivation techniques require an abundance of glassware and other equipment described in a comprehensive review by Hutner and co-authors (1958).

Screw-capped test tubes (19 X 150 mm or 19 X 120 mm) or 125-ml flasks are recommended for stock cultures. While vessel size is determined by preferences and particular needs, a good surface-to-volume ratio must be insured to avoid CO₂ limitations if CO₂ is not delivered to the culture from an outside source. Culture-medium volumes recommended for Erlenmeyer flasks are 40 to 60 ml for 125-ml flasks, 60 to 100 ml for 250-ml flasks and 150 to 250 ml for 500-ml flasks. Roux flasks are also sometimes used as culture containers (Eddy, 1956; Birdsey and Lynch, 1962) and are particularly recommended for culturing species of *Acetabularia* (Shephard, 1970). Where larger volumes of culture medium are needed, a 2800-ml wide-neck Fernbach flask with a cotton plug containing a glass siphon for periodic harvesting is very satisfactory. If it is necessary to shake cultures for keeping cells in suspension, a modified Fernbach flask can be used. The main feature of this new type of flask is a conical base around which 2 l of water can circulate with the flask on a reciprocal shaker (Fig. 4-3; Walsby, 1967). Numerous other ingenious culture enclosures have been described to meet special requirements. One such example is the pipette container constructed from a volumetric pipette. The culture compartment is contained in the broad part of the pipette, the lower part is bent into a U through which aeration is supplied (Jorgensen and Steemann Nielsen, 1961).
Culture enclosures should be made of good quality borosilicate glass with a low thermal expansion, although for certain types of nutritional investigations (e.g., mineral requirements) this type of glass is not satisfactory and vycor, polycarbonate or coated glass is called for. Autoclaving nutrient media in Pyrex can leach boron, as well as silicon, from the glass (McBRIDE and co-authors, 1971). For investigations of silicate uptake it is no longer necessary to resort to coating glass interiors with pure paraffin as in the past (PRINGSHEIM, 1946b) since specially manufactured polycarbonate culture vessels that are transparent, non-toxic, autoclavable and do not leach silicon into the medium are now available (BUSBY and LEWIN, 1967). During an investigation of iron requirements, GOLDBERG (1952) found that 80 to 90% of the iron added as ferric citrate was adsorbed within 1 day on the wall of the culture bottles. To eliminate this adsorption, the bottles were treated with 'Desicote', a silicon polymer (supplied by Beckman Instruments, USA).

Closures on culture containers must prevent contamination and permit good gas exchange; each particular closure should be examined for its effect. Closures may be non-absorbent cotton-wool plugs, metal or polypropylene caps. Some authors state that with the latter type of closure contamination is no greater than with
cotton wool (Elliott and Georgala, 1969) but other workers do not concur (Sutter, 1969). Lewin (1959) obtained good results with inverted glass vials on rimless culture tubes as closures. Although traditional cotton plugs are convenient and inexpensive, their use may cause problems. The possibility exists of introducing unwanted nutrients present in the cotton wool (Sherwood and Singer, 1944; Robbins and Schmitt, 1946); in addition, moisture accumulating in the cotton may facilitate bacterial contamination through water channels in the plug. Cotton plugs should be loosely covered with paper cups or caps of wax paper to reduce evaporation and to prevent soiling with dust. Screw-capped tubes and flasks offer a solution to the problems caused by cotton plugs, but two cautions should be mentioned: (i) Drying of agar slants and evaporation of liquid media must be kept at a minimum, but caps cannot be so tight that they do not permit gas exchange. Screw caps should be left loose during autoclaving, allowed to cool in the closed autoclave and tightened immediately when removed; (ii) Caps must be of some inert material that does not introduce unwanted nutrients or toxic materials. Often, the resins used to seal cap liners are toxic. In the reviewer’s laboratory, cap liners are thoroughly removed by soaking in acetone, scraping out the liner material, washing with detergent and rinsing in distilled water.

A type of culture flask common in tissue culture work, but not often applied to the cultivation of micro-algae, is the perfusion chamber. By adopting the liquid transport principle of paper chromatography, a perfusion chamber of a new type was developed and used to cultivate unicellular algae (Fig. 4-4; Sem, 1966). For producing dense populations of cells in a medium free from complex constituents or toxic products, dialysis cultures are suitable. Some variables in dialysis culture and different types of membranes were investigated by Gerhardt and Gallup (1963); the subject of dialysis culture of micro-organisms as a whole was reviewed by Schultz and Gerhardt (1969). In a recent report on some exploratory studies of the value and potential of dialysis techniques for phytoplankton studies Jensen and co-authors (1972, p. 241) state:

'It seems quite astonishing that the many useful possibilities offered by the dialysis technique have not been further exploited for the cultivation of autotrophic organisms.'

For some experiments Jensen and co-authors used Sartorius membrane filters of various porosities in a modified dialysis culture flask based on the apparatus of Gerhardt and Gallup (1963); for other experiments, 1/2" (1.6 cm) dialysis tubing, or regenerated cellulose in the form of a bag (Fig. 4-5). Dialysis culture of marine algae can be set up in the field or laboratory and for many species yields dense populations when cultivated in non-enriched sea water, demonstrating the efficiency of removing toxic wastes from the culture (see also p. 218).

Many culture containers are suitable only for the production of large volumes of algal culture; some of these are discussed in the section on mass culture in this review. In general, size and shape vary: vertical glass columns (Myers and Clark, 1944; Reisner and Thompson, 1956), Pyrex carboys (Davis and Ukeles, 1961; Ukeles, 1971, 1973), polycarbonate and fibre-glass tanks (Ukeles, 1965b, 1973), wooden vats (Loosanoff, 1951), concrete tanks (Ansell and co-authors, 1964) and a coated steel 'deep tank' (Strickland and co-authors, 1969) have been used.
The results obtained may be seriously affected by using materials with toxic components during sampling, storage or experimentation (Chapter 7). Dyer and Richardson (1962) tested numerous materials for their effects on chlorophytes and found that plastics, in general, were non-inhibitory except in a few cases. They
emphasized that one cannot rely on a particular material because one formulation was found non-toxic; each individual formulation must be tested for toxicity. Of the metals tested, aluminium alloys gave the best results but these varied greatly with copper-containing alloys. Stainless steels were generally good although two types were occasionally inhibitory; toxicity was also noted by Cummins and co-authors (1966). Even if stainless steel has no discernible effects on algal cultures, adverse effects on other species feeding on algae exposed to the metal can become apparent. This was found to occur even after a short exposure of algae to blenders or filters with stainless steel components (Davis and Ukeles, unpublished). An impressive list of the effects of chemicals released from 70 different materials on

6 marine phytoplankters (Bernhard and Zattera, 1970) revealed that there are no suitable non-toxic laboratory gloves and only surgeons' gloves can be used in washing and handling laboratory glassware. Many widely used rubber and plasticized polyvinyl chlorides are highly toxic; but teflon, polyethylene, tygon, polypropylene and polycarbonate are considered safe. For further details consult Chapter 7.

Isolation

Unicellular algae are isolated from crude samples brought into the laboratory (e.g., from plankton tows, tidal pools, submerged slides or other objects coated with nutrient agar or gelatin, body surfaces of marine species or decalcified shell
fragments of barnacles and molluscs as shown by Prud'homme van Reine and Van den Hoek, 1966). If the samples contain dilute algae suspensions, cells must be concentrated by centrifugation or other methods although some cells may not survive the process; where applicable, the gentle concentrating device of Dodson and Thomas (1964) is very useful. Concentrating phytoplankton samples on Millipore filters, as described by Clark and Sigler (1963), is sometimes convenient. After filtering a sample the filter is removed from the support, either cupped in the hand or put in a special holder, and the organisms washed from the filter into a dish of sea water with a compressed air atomizer.

The next step after collecting the crude sample is to obtain uni-algal clone cultures (vegetative progeny of one individual) and then bacteria-free cultures if possible (this may depend on defining the minimal and optimal conditions for growth and reproduction). Basically, all methods of obtaining clonal cultures depend upon isolation of single cells. Whenever pure cultures are necessary, the culture should originate from a single cell (Lewin, 1969); introduction of more than one cell may invalidate the concept of a clonal culture and increase the chances of contamination with another strain.

Growth of algae under a particular set of environmental conditions in nature does not necessarily mean that these are optimum conditions; however, trying to emulate this environment is a good starting point in attempting isolations from nature. Hardy species can be streaked on agar and colonies isolated by normal bacteriological techniques. Small unicellular algae with firm cell walls or pellicles grow especially well on agar; however, Butcher (1951) reported an abundance of abnormal forms obtained from agar slants. Delicate species may not survive agar-plate treatment that tends to flatten and even disrupt naked cells, but some species grow with persistent efforts of the experimenter, e.g., the chrysomonads Monochnosis lutheri and Isochrysis galbana, generally considered unable to grow on agar, are now being routinely cultured on agar slants in the reviewer's laboratory (Fig. 4-6). A wire loop or glass spreader is used in preference to a needle for streaking or spreading a diluted algal suspension on agar. Agar Petri-dish plates or test-tube

Fig. 4-6: Stock cultures of unicellular marine algae growing on enriched natural sea water agar media. (Original.)
slants are made up as a mineral-enriched sea-water medium with 1.5 to 2.0% agar, and incubated for several days or weeks until colonies begin to appear. These are then examined microscopically, and selected isolated colonies aseptically transferred with a sterile loop or needle into tubes of liquid media. The process is repeated to ensure that isolation of a single strain has been accomplished. DROOP (1954) found that test-tube slants of 1% agar are preferable to Petri dishes since drying out of the medium is delayed; however, considerably more skill is required for isolations. Some algae that can withstand temperatures of 40°C for a few minutes will grow in agar overlays on pour plates. In the reviewer's laboratory, Dunaliella euchlora, Chlorella autotrophica, Platymonas sp. and Phaeodactylum tricornutum grow throughout the depth of a thin agar overlay. Colonies of Prymnesium parvum were obtained from single cells in soft agar overlays on a solid medium in an atmosphere of high humidity by PADAN and co-authors (1967). LEWIN (1969) described a technique for isolating cells enmeshed in debris in which a small drop or clump of the mixture is placed on a thin film of agar and teased out with fine needles under the binocular microscope so that the selected cell is drawn away from other material. As liquid is absorbed into the agar, the block on which the cell stands is cut out or drawn into a fine capillary and transferred to a tube of liquid medium. Another suggestion is that cells of pennate diatoms be allowed to creep over the surface of an agar plate under unidirectional lighting which may separate the diatoms from debris and other organisms.

For isolating single cells over 5 μm, an interesting device was developed by EDMONDS (1972). It consists of a cat hair (vibrasse) of 50 μm or less in diameter, the distal end of which is held in a 26 to 27 gauge hypodermic needle. A drop of the suspension is spread on a slide and the desired cell is pushed with the loop to the edge of the drop. When free of other cells the loop is placed over the cell to be isolated, lifted out and transferred into a culture tube. KNIGHT-JONES (1951) considered the serial dilution of sea-water samples in test tubes of culture medium the most useful way of isolating representatives of single algal species. This method was used by ALLEN and NELSON (1910) and has been employed with great success by GROSS (1937), PARKE (1949) and BUTCHER (1951) for obtaining uni-algal cultures of many species. Although this dilution technique is useful, isolation by micropipette washing, as described by PRINGSHEIM (1946b), is considered the preferred method. The advantage of this latter method is that the alga isolated is not selected for by the medium and the ratio of bacteria to algae is greatly reduced at each manipulation although the method has limited applicability to species less than about 10 μm. A microcapillary pipette with a bore several times the cell's diameter is used to transfer unicellular algae from a droplet into a watch glass containing sea water placed inside a Petri dish; this procedure is repeated until transfer into new watch glasses with sterile sea water results in the isolation of one species from the others, and followed finally by transfer to a tube with medium. Cells should not be drawn up or expelled violently and should be subjected to a minimum of handling. A device described by BOLEYN (1967) employs a C clamp and rubber bulb for conveniently controlling the capillarity. This washing technique for isolation of phytoplankters from natural mixed populations has been described in detail by PRINGSHEIM (1946b, 1951) and by other authors (DROOP, 1954; LEWIN, 1959).
Droop (1954) suggested use of phototactic responses (Volume II: Seitz, 1975) for purifying micro-flagellates, since many species exhibit a strong negative taxis when transferred from the wild to a culture medium. In a drop of material put at the window side of a watch glass the flagellates will congregate on the side away from the light after 5 to 10 mins. This procedure can be repeated and, finally, the algal aggregation transferred en masse to a culture tube with medium. This method was used for isolation of Prymnesium parvum, Monochrysis lutheri, Hemiselmis sp. and several Chlorophyceae, all below 10 μm in size. For purposes of concentrating positively phototactic species a flask was designed by Meeuse (1963) that consists of a test tube-like projection attached to the side of a Florence flask. Positively phototactic flagellates concentrate in the tube when exposed to illumination. Superfluous liquid can be decanted without disturbing the flagellates, thus isolating them from debris and non-motile species. Cultures of Acetabularia species can be purified by making use of the phototactic behaviour of the gametes (Keck, 1964).

Johnstone (1969) reviewed methods of isolating single-celled organisms, mostly bacteria; some of the ingenious methods described can probably be applied to unicellular algae. Colourless algae can sometimes be isolated by taking advantage of their mode of nutrition. Although isolation of micro-organisms by selecting an enrichment medium is an established practice in bacteriology, it has seldom been applied to unicellular algae. This method can be used only if the nutritional requirements of the organism to be isolated are known. Rahat and Dob (1967) utilized the enrichment method of isolation in Prymnesium parvum, based on the observation that ethionine could serve as the sole source of nitrogen for this flagellate (Rahat and Reich, 1963), but is toxic for other algae (Johnson and co-authors, 1967). In serial subculture with ethionine the number of P. parvum increased and the culture was uni-algal by the 5th transfer.

**Purification**

Some investigators maintain that work with non-axenic cultures reflects a more ‘realistic’ approach to studies of organisms in relation to their natural environment than axenic cultures, and that non-axenic cultures provide more characteristic information on the water mass in micro-algal assay studies (Aleem, 1970). This point of view is supported by results such as Johnston’s (1963b), who obtained little or no growth of pure cultures of Skeletonema costatum in over 200 water samples from the North Sea enriched with nitrogen, phosphorus and silicon with or without trace metals. Classical taxonomists also have been critical at times of the study of cultured species because of the so-called ‘artificiality’ of the cultivation environment (Fritsch, 1935). However, the significance of pure cultures to experimental work was recognized by microbiologists as far back as the late 19th century when the first pure cultures were isolated. Pure cultures of marine unicellular algae are necessary for definitive studies on nutritional requirements, specific aspects of organism-environment relations, physiology, and biochemistry. In non-axenic cultures the results obtained are in danger of being obscured or modified by the presence of other organisms. When bacteria-free cultures are not available the assumption is sometimes made that growth and reproduction would be the same.
with or without a small bacterial population (Goldberg and co-authors, 1951; Kain and Fogg, 1958b; Craig, 1969); the incorrectness of this assumption has been demonstrated in numerous publications. McLachlan and Yentsch (1959) showed that growth and reproduction of Dunaliella echloror were stimulated by an organic nitrogen source derived from the hydrolysis of organic matter by bacteria, and if bacterial growth occurred before inoculation, some inhibitor was released that affected algal growth. Berland and co-authors (1972) reported that certain bacteria could be toxic for marine algae, e.g., Pseudomonas aeruginosa strongly inhibited growth of Tetraselmis striata. In general, the relationship between bacteria and phytoplankton is not well understood. Enhanced growth of certain marine algae with bacteria has been attributed to the release of organic growth-promoting substances (Provasoli, 1958c; Guillard and Cassie, 1963; Guillard, 1968). Results are further complicated by the antibiotic substances produced by some algae (Steemann Nielsen, 1955; Duff and co-authors, 1966; Bruce and co-authors, 1967). At one time a ‘harmonious relationship’ between bacteria and algae was postulated only because of the frequent correlation between diatom and bacterial populations in the pelagic environment (Waksman and co-authors, 1933, 1937). Soli (1963) suggested a relationship between the presence of bacteria in cultures and the formation of auxospores, based on the observation that bacterized cultures of Chaetoceros dydimus formed endospores in 8 days but cultures without bacteria after a much longer period. Although algal species often depend nutritionally on bacterial excretions, it is possible that physico-chemical effects, such as buffering of the medium, influence on heavy-metal solubility or lowering of the oxidation-reduction potentials, are of greater importance in bacterial-algal interrelationships.

Purification of algal species from contaminating organisms can take place only if a culture medium is available that will satisfy all nutritional requirements and if the species undergoing purification does not harbour an endosymbiotic bacterial flora of the kind described for Gymnodinium splendens and Cryptothecodinium cohnii (Gold and Pollingher, 1971). Recent algal subcultures or isolates with a minimum amount of organic material and a low bacterial population should be used in initial efforts at purification. The advantage of a recent isolate is emphasized by the experiments of Droop and Elson (1966) that suggest healthy pelagic diatoms are virtually free from bacteria. Enriched, sterile, natural sea-water media rather than synthetic media should be used for initial subculturing in the purification process. Since the enrichments that are perhaps needed by the alga may be complex, it is sometimes necessary to establish a two-membered culture, i.e., the isolated alga cultured with a known bacterial population.

A conventional technique for obtaining pure cultures is to streak mixed cultures on an agar plate prepared with an enriched sea-water medium, with or without antibiotics. Well-isolated algal colonies are cut out of the agar with a flamed needle, transferred to sterile liquid media, and mixed to disperse cells; the entire process is repeated several times. Pure cultures are eventually obtained, but the procedure is deficient for some species since not all algae will form isolated colonies on agar.

Chu (1946b) and others obtained bacteria-free cultures by laboriously washing cells. Purification by repeated micro-pipette washings was reported for Gyrodinium californicum, Gyrodinium resplendens and Gymnodinium splendens (Provasoli
and McLaughlin, 1963). All apparatus and media used in washing are, of course, sterilized before use. An individual picked out under a dissecting microscope is transferred with a capillary pipette into a sterile watch glass containing a few ml of medium, with or without antibiotics, and then is similarly transferred to other dishes of sterile sea water or sterile media. The washing process should be carried out in an isolating chamber or transfer hood previously treated by ultra-violet irradiation or washed with a disinfectant, being careful to avoid volatile antiseptics whose vapours may be a potential source of toxicity. Sweeney (1951) washed Gymnodinium splendens cells in a lucite transfer chamber previously sprayed with 70% alcohol. She found that transferring a single cell through successive drops of sterile nutrient solution was not practical since the evaporation from a single drop of sea water was rapid enough to change the salinity and cause disintegration of the cell. Lewin (1959) recommended that droplets be supported on an isotonic agar surface to prevent evaporation. With such modification this author considered washing by the capillary method the preferable technique for purifying large flagellates. If an epiphytic bacterial flora occurs on the naked flagellates or if there is a mucilaginous coat on the alga where bacteria are embedded, it is not likely that these can be removed by persistent washing. In these cases the use of antibiotics or other bactericidal agents is indicated.

Chu (1946b) suggested that bacterial growth could be reduced by inoculation onto silica gel which supports diatoms but not bacterial growth. The gel was prepared according to Pringsheim’s (1926) method by mixing equal volumes of diluted HCl and aqueous silicon, diluted to the required specific gravity, allowed to set in a Petri dish, washed with sea water, dried and sterilized. Colonies of diatoms that developed after inoculation were isolated and subcultured on nutrient agar. To obtain pure cultures, Allen (1914) employed the ‘differential poisoning’ method. A chemical, such as CuSO₄, is added to a number of culture flasks in a series of diminishing strengths for various exposure times with the hope that at some point the bacteria will be killed without affecting the algae. ZoBell and Long (1938) also used various bactericidal compounds among which acriflavine was found to be bacteriostatic in concentrations that did not affect algae. Along a similar direction, Sohl (1963) recommended purification of tough diatoms, e.g., Chaetoceros dydimus, by a few minutes exposure to 0.1% alcoholic iodine solution and subsequent washing and centrifuging in sterile medium. Some chlorophytes have been purified by shaking with a mixture of phenol and detergent (McDaniel and co-authors, 1962).

Allen (1914) described a method of electrolysis for obtaining bacteria-free cultures. Charcoal-treated and filtered sea water is subjected to an electric current of 1.5 to 1.7 amp for 3 mins causing a considerable formation of hypochlorous acid. The culture to be purified is added to the electrolyzed water and then subcultured after various exposure times. Coler and Gunner (1969) designed an apparatus for the separation of algae and bacteria utilizing the difference in electrophoretic mobility of both groups and the greater susceptibility of bacteria over algae to the lethal effects of an electric field. Another unorthodox method of purification described by Wurtz (1964) utilizes ultra-violet light. A dilute suspension of algae is placed in a quartz window chamber and irradiated for 20 to 30 mins with 2750 nm ultra-violet light from a quartz-jacketed mercury vapour lamp. Generally,
the algae are more resistant to ultra-violet irradiation than the bacteria. Brown and Bischoff (1962) reported success in obtaining axenic cultures by subjecting cells to repeated rinsing in liquid medium interspersed by occasional ultra-sonification combined with detergents. Wiedeman and co-authors (1964) extended the

Table 4-1
Antibiotics used for purification of unicellular-alga cultures
(Compiled from the sources indicated)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Species</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin (CaCl₂), 500 units ml⁻¹ + penicillin (Na)², 500 units ml⁻¹</td>
<td>Nitzschia closterium</td>
<td>Spencer (1952)</td>
</tr>
<tr>
<td>Penicillin G, 100 mg l⁻¹ (1645 units mg⁻¹) + dihydrostreptomycin sulphate, 50 mg l⁻¹; in culture for 18-36 hrs</td>
<td>Detonula confervacea, Cyclotella nana 3X</td>
<td>Guillemard and Ryther (1962)</td>
</tr>
<tr>
<td>K penicillin G, 500 units + chloramphenicol, 5 µg; neomycin, 25 µg + polymixin B, 15 units + dihydrostreptomycin, 500 µg + tetracycline, 25 µg + canicidin, 15 units; in culture for 15 days; concentration per ml</td>
<td>Gyrodinium cohni</td>
<td>Provasoni and Gold (1952)</td>
</tr>
<tr>
<td>Penicillin, 200 units + chloramphenicol, 1 unit + neomycin, 1 unit + polymixin B, 1 unit ml⁻¹</td>
<td>Gyrodinium resplendens</td>
<td>Provasoni and McLaughlin (1963)</td>
</tr>
<tr>
<td>Polymixin B, 50 µg ml⁻¹ + neomycin, 50 µg ml⁻¹</td>
<td></td>
<td>Soli (1963)</td>
</tr>
<tr>
<td>Penicillin G, 100,000 units + streptomycin, 100 µg; solution added as 0.05 ml 100 ml⁻¹ medium; in culture for 48 hrs</td>
<td>Acetabularia sp.</td>
<td>Keck (1964)</td>
</tr>
<tr>
<td>Streptomycin SO₄, 200 µg ml⁻¹ + benzyl penicillin SO₄, 1000 µg ml⁻¹ + chloramphenicol, 25 µg ml⁻¹</td>
<td>Diatoms</td>
<td>Droop (1967)</td>
</tr>
<tr>
<td>Benzyl penicillin SO₄, 1000 µg ml⁻¹ + streptomycin sulphate, 200 µg ml⁻¹ + chloramphenicol, 10 µg ml⁻¹ + neomycin, 50 µg ml⁻¹</td>
<td>Diatoms</td>
<td>Droop (1967)</td>
</tr>
<tr>
<td>Carbenicillin, 1 mg ml⁻¹; penicillin G, 1 mg ml⁻¹; vancomycin, 1 mg ml⁻¹; kanamycin, 100 µg ml⁻¹; each agent added to medium for 10 days</td>
<td>Acetabularia mediterranea</td>
<td>Shepard (1970)</td>
</tr>
</tbody>
</table>

Brown and Bischoff technique by following the treatment with a method of plating cells that utilizes compressed air to spray an inoculum of cells onto sterile agar plates.

Antibiotics alone or combined with detergents are often used to purify contaminated cultures, as a direct treatment or in conjunction with some of the other purifica-
ion techniques. SPENCER (1952) experimented with penicillin and streptomycin in obtaining axenic cultures of *Nitzschia closterium* and two chlorophytes. Antibiotic mixtures, effective for purifying diatoms, have been tested by PROVASOLI and co-authors (1951), PROVASOLI and PINTNER (1953), MCLAUGHLIN and ZAHL (1957), GUILLARD and RYTHER (1962), and PINTNER and PROVASOLI (1963). The technique described by DROOP (1967) has been successfully employed for purification of a variety of organisms, including pelagic flagellates and sublittoral diatoms. Based on the work of PROVASOLI and co-authors (1951) and OPPENHEIMER (1955), the general principle is to use a high concentration of a wide spectrum antibiotic mix during short exposure. The method calls for a series of cultures with a varying ratio of cell numbers to antibiotic concentration, plus a drop of the sterility test medium in each tube to induce bacterial division. After 24 hrs each culture is transferred to antibiotic-free medium. Sterility tests are carried out on all subcultures after incubating for several weeks. Usually, some tubes show bacterial growth and some viable bacteria-free algae.

Although standardized antibiotic mixes have been used in many laboratories to purify algal cultures (Table 4-1), sensitivities to antibiotics vary with the different bacterial flora contaminating each culture. Evidence of the wide spectrum of antibiotic sensitivities displayed by bacterial populations that contaminate algal cultures is shown in Table 4-2. In the reviewer's laboratory, one procedure for purification is to culture the bacterial population contaminating a particular algal species in an organically enriched sea-water medium; an agar pour plate is made with this culture and antibiotic discs applied on the plate. After a suitable incubation period the antibiotic sensitivity for each contaminating population is evaluated. The particular antibiotic mix is utilized in each of the purification techniques, as washing, plating or serial dilution. DROOP (1964) points out that many isolations with antibiotics could also have been done with other methods avoiding the possibility of producing physiological changes in the purified species. Antibiotics, however, have been widely employed to purify strains of unicellular algae, bypassing some of the other tedious procedures.

Sterility-testing media commonly employed are varieties of highly enriched sea-water media (Table 4-3). Some investigators recommend incubating stock cultures in 1% agar with 0.1% peptone and 1% glucose to reveal slow-growing contaminants (Kuhl and LORENZEN, 1964). However, growth on agar of some species (e.g., *Monochrysis lutheri*, *Isochrysis galbana*) may be inhibited by the presence of this added enrichment (UKELES, unpublished). DROOP and ELSON (1966) suggested a liver infusion or soil-extract sea-water medium. GUILLARD and RYTHER (1962) used a sterility-test medium of ½-strength nutrient broth and nutrient agar made up with 20% distilled water and 80% sea water. The STP medium of PROVASOLI and co-authors (1957) is allowed to incubate at algal growth temperature for 3 weeks to reveal contaminants. In the reviewer's laboratory both fluid thioglycollate medium (prepared with sea water) and an enriched sea-water agar medium are used routinely for sterility tests; tests are incubated at both 20° and 32° C. DROOP (1954) recommends that 4 sterility tests be made routinely on the following media to ensure sterility: (i) freshwater liquid; (ii) freshwater agar; (iii) salt-water liquid; (iv) salt-water agar in a medium containing yeast, beef, soil extract and glucose. It is unlikely that even fastidious forms would not be revealed by these tests.
Table 4-2
Antibiotic sensitivity spectrum of bacterial contaminants in cultures of unicellular algae (Original)

<table>
<thead>
<tr>
<th>Species</th>
<th>Triple sulphur 50 µg</th>
<th>Ampicillin 10 µg</th>
<th>Penicillin G 10 units</th>
<th>Dihydrostreptomycin 15 µg</th>
<th>Erythromycin 30 µg</th>
<th>Novobiocin 30 µg</th>
<th>Kanamycin 30 µg</th>
<th>Polymyxin B 300 units</th>
<th>Chlorotetra cycline 30 µg</th>
<th>Oxytetracycline 30 µg</th>
<th>Tetra cycline 30 µg</th>
<th>Chloramphenicol 30 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletonema costatum</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Chaetoceros sp.</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Diatom sp. (E.)</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>Prorocentrum parvum</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dictyosperma sp.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Cryptomonas sp.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Rhodomonas sp.</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Prorocentrum micans</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>Stichococcus G</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chlamydomonas sp. (≠ 11)</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chlamydomonas sp. (≠ 113)</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>Chlorophyceae sp. (≠ N)</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

Inhibition zones: 0 none, + 1-2 mm, ++ 2-3 mm, +++ 3-4 mm, ++++ over 4 mm.
CULTIVATION OF PLANTS (R. UKELES)

Table 4-3

Sterility test media for marine bacteria

<table>
<thead>
<tr>
<th>Component</th>
<th>STP</th>
<th>ST₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea water</td>
<td>80  ml</td>
<td>70  ml</td>
</tr>
<tr>
<td>Water</td>
<td>15  ml</td>
<td>25  ml</td>
</tr>
<tr>
<td>Soil extract</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>20-0 mg</td>
<td>5-0 mg</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1-0 mg</td>
<td>—</td>
</tr>
<tr>
<td>Na₂ glycerophosphate, 5H₂O</td>
<td>—</td>
<td>1-0 mg</td>
</tr>
<tr>
<td>Hy-Case (Sheffield Chemical)</td>
<td>—</td>
<td>2-0 mg</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>—</td>
<td>1-0 mg</td>
</tr>
<tr>
<td>Yeast autolysate (Nutritional Biochemicals)</td>
<td>20-0 mg</td>
<td>—</td>
</tr>
<tr>
<td>Liver oxid L-25 (Oxo)</td>
<td>—</td>
<td>2-0 mg</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0-005 µg</td>
<td>0-015 µg</td>
</tr>
<tr>
<td>Thiamine, HCl</td>
<td>0-02 mg</td>
<td>0-02 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0-01 mg</td>
<td>0-01 mg</td>
</tr>
<tr>
<td>Putrescine, 2HCl</td>
<td>4-0 µg</td>
<td>4-0 µg</td>
</tr>
<tr>
<td>Calcium pantothenenate</td>
<td>0-01 mg</td>
<td>0-01 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0-5 µg</td>
<td>0-5 µg</td>
</tr>
<tr>
<td>Pyridoxine, 2HCl</td>
<td>4-0 µg</td>
<td>4-0 µg</td>
</tr>
<tr>
<td>Pyridoxamine, 2HCl</td>
<td>2-0 µg</td>
<td>2-0 µg</td>
</tr>
<tr>
<td>D.L-Alanine</td>
<td>10-0 mg</td>
<td>2-0 mg</td>
</tr>
<tr>
<td>Trypticase (BBL)</td>
<td>20-0 mg</td>
<td>—</td>
</tr>
<tr>
<td>Glycine</td>
<td>10-0 mg</td>
<td>2-0 mg</td>
</tr>
<tr>
<td>Glycyglycine</td>
<td>—</td>
<td>4-00 mg</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>—</td>
<td>2-0 mg</td>
</tr>
<tr>
<td>Sodium acetate, 3H₂O</td>
<td>—</td>
<td>4-0 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>—</td>
<td>4-0 mg</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>—</td>
<td>4-0 mg</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>1-0 µg</td>
<td>1-0 µg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0-05 µg</td>
<td>0-08 µg</td>
</tr>
<tr>
<td>Choline H₂ citrate</td>
<td>0-05 mg</td>
<td>0-05 mg</td>
</tr>
<tr>
<td>Inositol</td>
<td>0-1 mg</td>
<td>0-1 mg</td>
</tr>
</tbody>
</table>

Media Preparation, Sterilization, Subculturing

The common practice in preparation of culture media is to combine appropriate amounts of the individual medium components from concentrated stock solutions. Glass-distilled water or, at least, de-ionized water from an ion-exchange column is used for preparing stock solutions; light-sensitive solutions are stored in amber coloured reagent bottles. HUTNER and co-authors (1958) recommended the use of 'dry mixes' for basal media to avoid the necessity of storing solutions under refrigeration and to make the repetitive compounding of complex formulations more efficient. Dry mixes keep well, except for thiamine, tyrosine and phenylalanine; hygroscopic ingredients should be avoided. An alternative to either of the two methods mentioned is to combine nutrient solutions needed for a particular volume of medium as a sterile solution in a sealed ampule; this is then added aseptically to previously sterilized sea water or distilled water.

Scepticism must be exercised in regard to the purity of the solutions used for culture media in nutritional studies. The purity of commercially available reagents
and the amount of contaminants that enters solutions during preparation may be considerable. Distilled water is suspect of carrying trace nutrients. WANGERSKY (1965) found carbon compounds in distilled water that survived triple distillation. Fingerprints can transfer enough nutrients to support life in unlikely places and raise blank values in assay procedures. Microbes have been known to grow in almost any solution and will even multiply in distilled water contained in glass bottles. Fluids awaiting analysis and stock solutions may be protected against microbial action by the addition of a preservative that is removed on autoclaving by a steam-distillation effect. The preservative developed by HUTNER and BJERKNES (1948) consists of a mixture of 1 part O-fluorotoluene, 1 part 1,2-dichloroethane and 2 parts n-butyl chloride. The fluorotoluene (boiling point 114°C) was replaced later by chlorobenzene (boiling point 132°C). The preservative is dispensed from a dropping bottle into all organic nutrient solutions, as well as solutions of inorganic salts.

<table>
<thead>
<tr>
<th>Component</th>
<th>STP</th>
<th>ST₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymine</td>
<td>0.08 mg</td>
<td>0.08 mg</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>0.026 mg</td>
<td>0.026 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.25 µg</td>
<td>0.25 µg</td>
</tr>
<tr>
<td>Folinic acid</td>
<td>0.02 µg</td>
<td>0.02 µg</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0 mg</td>
<td>—</td>
</tr>
<tr>
<td>NaH glutamate</td>
<td>50.0 mg</td>
<td>—</td>
</tr>
<tr>
<td>pH</td>
<td>7.5-7.6</td>
<td>7.9</td>
</tr>
</tbody>
</table>

**Burdholder (1963)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Enriched sea water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptase</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Soytone</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Yeast</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Marine mud extract*</td>
<td>100 ml</td>
</tr>
<tr>
<td>Sea water</td>
<td>900 ml</td>
</tr>
</tbody>
</table>

**Spencer (1952)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Peptone sea water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-peptone</td>
<td>0.5%</td>
</tr>
<tr>
<td>Ferric phosphate (dissolved in 75% sea water)</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

**Spencer (1952)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Casein sea-water agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-peptone</td>
<td>0.05%</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.1%</td>
</tr>
<tr>
<td>Soluble casein</td>
<td>0.05%</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>0.05%</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.02%</td>
</tr>
<tr>
<td>Agar (dissolved in 75% sea water)</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

* Autoclave 1 kg of wet marine mud together with 1 l sea water and filter; preserve under toluene and store in refrigerator.
For axenic cultures, complete sterilization of media and culture vessels must be carried out. Methods of sterilization for general purposes are limited to chemical, steam under pressure, dry heat, membrane filtration, irradiation and electric discharge. The choice of sterilization method depends on the material to be sterilized, the equipment available and the standards to be met. Sterilization of glassware and pipettes may be accomplished in a sterilizing oven; the pipettes are placed into canisters (Pyrex are preferred to metal) and kept for 3 to 4 hrs at sterilizing temperature (190°-194°C). Autoclaving avoids the accumulation of decomposition products of cotton from dry heat, although condensation from the autoclave presents a contamination risk. Hutner and co-authors (1958) recommended that pipettes for aseptic operations be cotton plugged and autoclaved in individual borosilicate tubes, plugged at both ends, and the damp pipettes be allowed to dry by standing overnight in the autoclave. Large culture vessels, flasks, test tubes and other miscellaneous apparatus are usually steam sterilized. Ultra-violet irradiation has occasionally been applied to the sterilization of media. Irradiation was also used for sterilization of a deep open tank although its importance for an open non-bacteria-free culture system was questionable. After the tank walls were scrubbed, rinsed and the accessories wiped with isopropyl alcohol, all fixtures were irradiated immediately before filling the tank by slowly raising and lowering a 1200-W Hanovia Englehardt mercury arc tube (model 189 A). The efficiency of the treatment was confirmed by bacterial tests which left no doubt that the all-exposed surfaces were sterilized (Strickland and co-authors, 1969). Chemical sterilization of open cultures, tanks and sea water has been practised since the early days of mass culture. Allen and Nelson (1910) sterilized aquarium-tank water with ozone. They found that ozonated oxygen-treated sea water gave distinctly better cultures than untreated water. Chlorine sterilization of tanks was carried out by Raymont and Adams (1958). NaOCl was added to a tank, left for at least 48 hrs, and the excess neutralized with Na₂S₂O₃.

All culture media must be regarded as heat labile to some extent. Chemical changes are bound to occur at the temperatures and pressures employed in autoclaving that are considered damaging to spores, i.e., 115° to 121°C for 15 to 20 mins; 118° to 121°C for 30 mins kills resistant spore forms and drives off volatile preservatives. Some investigators use shorter autoclaving times, i.e., 15 lbs for 5 mins, but these should not be considered safe for absolute sterility (Hutner and co-authors, 1958). On the other hand, opinions differ on the minimum permissible time and pressure of autoclaving. In Droop’s laboratory all solutions are autoclaved as lightly as possible. Contamination did not occur in media autoclaved in pressure cookers with the pressure brought up to 15 lb in⁻² and left there for only 1 min (Provasoli and co-authors, 1957). Armstrong and co-authors (1966) suggested that ultra-violet-irradiated sea water may be usable for cultures with no further treatment than brief autoclaving.

There is some concern and disagreement as to the importance of precipitates that appear in autoclaved sterilized media. Hayward (1968c) correlated the lack of Phaeodactylum tricornutum growth with media that developed an excessive precipitate after autoclaving. An excessive precipitate may entrap organisms and leaves the medium somewhat undefined in composition; on the other hand, it can provide a slowly soluble source of nutrients. In the reviewer’s laboratory an en-
TECHNOLOGICAL AND PROCEDURAL ASPECTS

riched sea-water medium that produces a moderate amount of precipitate on autoclaving is used routinely without encountering any difficulty; Takano (1964) reports similar experience.

Filtration, as a procedure for sterilization of culture media, is widespread and necessary when heat-labile components present a sterilization problem. However, caution is in order when dealing with the wide array of ultra-fine filters that are now commercially available. Porter and co-authors (1967) tested four types of hydro-sol filters, two re-usable (diatomaceous and fritted glass) and two disposable (asbestos and membrane filters). Only one type of membrane filter in its manufacturer's own holder consistently gave sterile results. If a high degree of certainty is required for sterile heat-labile filtrates, it is suggested that the liquid be passed through two or more filters in a previously tested and proven system. Seitz filters should not be used for preparing media as several components of this apparatus are toxic to marine phytoplankters (Bernhard and Zattera, 1970; Chapter 7). Sterile disposable bacteriological filter units made of plastic have toxic impurities which inhibit the growth of the protozoan Leishmania tarentolae (Simpson, 1966); four different lots of the filters exhibited this phenomenon. Similar observations were made on artificial sea-water media filtered through this unit in which the media became toxic to unicellular marine algae (Ukeles, unpublished).

To avoid contamination at the inoculating bench it is desirable to mount an ultra-violet lamp in a well-ventilated (but not drafty) inoculating area. Good housekeeping should be maintained and floors waxed often to trap dust. Commercially available inoculating hoods are inexpensive and can be valuable in maintaining sterile conditions during inoculations. Glass pipettes are preferred for inoculations (1 ml graduated to 1/100; 2, 5 and 10 ml graduated to 1/10), but sterilizable hypodermic syringes, capillary pipettes or platinum loops may also be used.

Droop (1961b) showed that the final yield of cells in Skeletonema costatum varied with the time interval between autoclaving and inoculating the medium and was influenced by the amount of sodium sulphide added at the time of inoculation. The re-entry of oxygen or carbon dioxide after autoclaving may be responsible. The inoculating schedule is determined, in part, by convenience, by need, by conditions of incubation, and by culture requirements. In any event, a relatively consistent schedule should be employed and stocks maintained on a variety of media to ensure against loss of strains. Axenic stock cultures on agar slants require the least frequent subculturing, enriched natural sea water more frequent, and artificial sea-water medium the most frequent transfers. A schedule of about 8, 6 and 4 weeks, respectively, is satisfactory; bacterized cultures should be transferred relatively often. Subcultures should be checked for contaminants and inspected for mutations that may alter characteristics of the original strain.

Discussing the influence of the size of the inoculum on the future growth of the culture, Wurtz (1964) stated that the amount of inoculum does not influence the growth of the culture or the final number of cells obtained, but does vary the rate of multiplication throughout the growth period. There seems to be a complex relationship between cell numbers and physico-chemical conditions of the medium that triggers cell-division cycles. Attempts by Pringsheim (1942) to prepare clone cultures by the isolation of single cells failed to show extensive multiplication al-
though 10 or more cells did. However, by greatly enriching the medium (putrefaction culture method), almost every uni-flagellate culture was successful although the lag period was extremely long. If inocula are small, there may be a delay in the
onset of exponential population growth and sometimes a decline in numbers before active growth proceeds (KAIN and FOCO, 1958b; BUNT, 1968). Some results suggest that an apparent growth lag may have been caused by viability loss of a proportion of the cells in the inoculum; this may be offset to some extent by supplementing culture media with appropriate chemical substances (BUNT, 1968). It is generally
believed that a lag phase does not occur in algal cultures if cells from a recent transfer are used for subculture under the same conditions as previously.

Agitation of cultures is a procedure on which there is little general agreement. The necessity for agitation depends on factors such as size of culture container, population density, medium composition and the strain cultured (see also Volume I, Chapter 5). Adhesion of cells on glass surfaces may occlude light and reduce rates of growth and reproduction. The mechanism of adhesion appears to be an electrostatic interaction between cells and glass surfaces (Nordin and co-authors, 1967) which can be minimized by some agitation and regulating mineral composition in the medium. Aloszus (1961) described an apparatus, called 'shaking table', that provides agitation and intense illumination at constant temperature for a large number of cultures. Birdsey and Lynch (1962) developed a table that holds 16 culture flasks. Some investigators, however, have found that agitation is detrimental to growth: Eppley and co-authors (1967) reported no growth of the diatom Didymosphenia geminata with constant agitation. Agitation of 80 strokes min⁻¹ prevented growth of Gyrodinium colinitum, while 20 to 40 strokes min⁻¹ were no better than non-agitated tubes (Provasoli and Gold, 1962).

The most usual agitation procedure is aeration of culture media which gives a gas stream, as well as gentle water movement. Compressed air, enriched with CO₂, is passed through filters and delivered to cultures through sintered glass or aerators (Kain and Fogg, 1968a; Davis and Ukeles, 1961; Hayward, 1965; Ukeles, 1965b, 1971). The need for supplementary aeration is questionable if a good surface-to-volume ratio is maintained in the culture vessel. In the reviewer's laboratory test-tube or flask (125–2800 ml) cultures are not aerated, but aeration systems are employed in 20-l culture carboys. Open tank cultures are also provided with agitation via a compressed air stream, in addition to periodic hand mixing with a paddle. Some examples of laboratory culture facilities are shown in Figs 4-7 and 4-8.

Specialized Types of Culture

Mass cultures

Mass cultures represent, in essence, scale-ups of conventional, small-scale cultivation procedures. The term 'mass culture' has been applied to marine species in culture volumes of 2.5 l (McLaughlin and co-authors, 1960), 15 l (Ketchum and Redfield, 1938; Davis and Ukeles, 1961), 80 l, 280 l and 1000 l (Ukeles, 1965b, 1971) and 2400 l (Raymont and Adams, 1958). Considering the problem of mass cultivation, Myers and co-authors (1951, p. 539) write:

'If pushed to a stage of practical development, the mass culture of algae will become an engineering problem. Before any such stage is reached, however, the problem is essentially biological.'

The biological problem is to determine the conditions necessary to obtain maximum levels of population density, rates of growth and reproduction and maximum efficiency of utilization of light and nutrients; these aims are similar to those of small-scale laboratory cultures.
In a general way, mass cultures can be considered as closed systems or open systems (see also Chapter 2). Either one can be maintained in an indoor laboratory environment under artificial illumination or outdoors using solar energy. The closed system designed by Wisely and Purday (1961) provides up to 400 l per unit for micro-algal cultures used in rearing marine invertebrate larvae (Chapter 5). Each unit consists of four 44-gal (166.5 l) galvanized, open-head fuel drums coated internally with white fibre glass and externally with polyurethane paint, filled with pasteurized enriched sea water and aerated (Fig. 4-9). After inoculation a fluorescent
tube, totally enclosed in a pasteurized waterproof tubular jacket, is lowered and held in the culture drum by a lid flange and the pressure of the culture medium. When a suitable population density is reached, most of the culture is pumped to an elevated upper reservoir for subsequent use; the remainder serves as inoculum to start a new culture. The drums are cleaned and pasteurized by raising the temperature with an immersion heater to 70°C for 4 hrs while the water is aerated vigorously to ensure mixing. A soil-extract medium was used to support the growth of Isochrysis galbana, Dunaliella tertiolecta and Phaeodactylum tricornutum in this system.

Another closed system of mass cultures of marine algae in 5-gal (18.9-l) carboys was developed for the same purpose, i.e., cultivation of species to be used as food for marine invertebrates. The culture apparatus consisted of sixteen 5-gal (18.9-l) Pyrex carboys or twenty 9-l Pyrex serum bottles, immersed 3 to 4 inches (7.6 to 10.1 cm) in a cold-water bath (19°C). Illumination was provided by four 40-W cool-white fluorescent tubes mounted about 1 to 2 feet (30-60 cm) behind the growth chambers and three 40-W lamps above the cultures. A mixture of CO₂ and air was provided to each culture in sterile inorganic enriched sea-water media. Cold sterilization of media was accomplished by filtering sea water through cotton orlon filters to remove large particles and then passing the sea water with a nutrient enrichment through a bacterial ceramic filter attached to each culture carboy (Fig. 4-10; Davis and Ukeles, 1961). The growth chamber, together with the filter and tubing connectors, is autoclaved assembled. Variations of this system are now used where carboys are incubated in a cold room and illumination provided by a bank of vertical fluorescent lamps (Figs 4-11 and 4-12). The medium consists of inorganic-enriched natural sea water that was autoclaved in 4- or 8-l bottles. Part of the culture is harvested periodically and the harvested volume replaced with sterile medium constituting, in effect, a semi-continuous culture. The construction and operation of this mass culture system have been described in detail (Ukeles, 1973). This system was designed and maintained to deliver axenic unicellular algae in large quantities. Frequent sterility tests must be carried out to ensure against failure of mechanical components.

Mass-culture in identical carboys, rather than in much larger volumes as is sometimes advocated, has several advantages: it can be inexpensive; it allows the culture of numerous species simultaneously; axenic cultures can be sustained and cultures that are not functioning or not needed can be removed without losing the entire culture volume.

A closed mass culture system similar to that described above was developed for culture volumes of 80 to 100 l in Lexan® (polycarbonate)* plastic tanks (Fig. 4-13). In this system all parts were steam sterilized but media filter sterilized. Sea water, prefilled through a series of cotton orlon filters of decreasing size (15 μm, 10 μm, 5 μm), then through a 0.45-μm pleated membrane cartridge filter and finally through a 0.22-μm Millipore filter, was collected in a 20-l Pyrex carboy (Fig. 4-14). This sterile sea water was dispensed via a system of tubes and connectors to 4 sterile tanks. Inocula and nutrient concentrates were added aseptically through an orifice in the tank cover.

*Produced from General Electric Lexan® resin.
An open system of mass culture consisting of 1000 l in a 2000-l fibre-glass tank was developed as a method of providing a constant supply of unicellular algal food of known species composition to adult invertebrate herbivores. The fibre-glass tanks are located in a temperature-controlled room (20°C) with 4 overhead fluorescent lamps (Vita-Lite) over each tank (Fig. 4-15). The growth medium consists of an artificial sea salt plus enrichments dissolved in well water (Table 4-12) since it is less expensive and simpler to assemble than a completely synthetic sea-water medium and avoids the introduction of other species that would be present in natural sea water. High-density cultures from 5-gal (18.9 l) Pyrex carboys are used to inoculate tanks at about 10 l 100 l⁻¹ of media. Tank cultures are harvested continuously at a rate of 350 ml min⁻¹ by a gravity feed. After the entire 1000 l are consumed the tank is washed and the culture started again.
Although the possibility of supplementing human food supplies with algae has been thoroughly reviewed (Burlew, 1953; Tamiya, 1957, 1960; Oswald and Golueke, 1968a), little attention has been given to the selection of a suitable species. A great quantity of the experimental work and pilot-plant effort has been devoted to species of *Chlorella* which possess a rapid growth rate and attain high cell densities. Eddy (1956) tested a limited number of unicellular algae for their suitability as alternatives to *Chlorella* for mass culture; *Dunaliella bioculata* was shown to achieve favourable yields, as compared with *Chlorella* sp. Effley (1963) evaluated the usefulness of flagellated Volvocales for mass culture. Three strains displayed growth rates above average (*Dunaliella tertiolecta*, *D. primolecta* and *Tetraselmis tetrahele*).

![Mass cultures of unicellular algae](image)

**Fig. 4-11**: Mass cultures of unicellular algae. Culture carboys containing axenic cultures in enriched natural sea-water media. (Original.)

and a salinity range allowing selection of a strain suitable for mass culture with almost any salt content. In the reviewer's laboratory numerous species have responded well in mass culture; some examples are *Monochrysis lutheri*, *Isochrysis galbana*, *Diceratium inornata*, *Phaeodactylum tricornutum*, *Chlorella autotrophica*, *Dunaliella tertiolecta*, *Tetraselmis* sp., *Porphyridium cruentum*, *Cryptomonas* sp. and *Chlorella stigmatophora*.

**Synchronous cultures**

The technique of synchronization refers to the experimentally induced co-ordination of individual cycles in a population of unicellular organisms that has a random age distribution. Synchronization is necessary to obtain large numbers of cells, that are at the same life-cycle stage, for investigation of developmental and
physiological events that vary during different stages of the cell cycle. Many reviews are available on synchronization in general; some are restricted to synchronization of algae (HOOGENHOUT, 1962, 1963; PIRSON and LORENZEN, 1966; TAMIYA, 1966).
Fig. 4-13: Mass culture of unicellular marine algae in sterilized polycarbonate tanks each containing 80–100 l of culture fluid. (Original.)

Fig. 4-14: Filtration system for cold sterilization of natural sea water. a: 15-μm polypropylene filter; b: 1-μm polypropylene filter; c: 0.45-μm pleated membrane cartridge filter; d: 0.22-μm Millipore filter. (Original.)
Hoogenhout (1963) divides the methods for obtaining synchrony into two types: (i) mechanical selection of the starting material, e.g., by differential filtration, sedimentation velocity, fractional centrifugation, individual collection of dividing cells, or spore collection; (ii) induction, e.g., via an extended period of depletion in an environmental entity, followed by a synchronizing regime, such as shifts from starvation to enrichment, temperature cycling (shifts or shocks), sudden dilution of crowded cultures, or alternating light and dark periods. In photosynthetic species, nearly all synchronization has been achieved by periodic illumination. It is probable that many populations growing in nature are synchronous to some extent from natural light–dark periods. In some species, only one change in the illumination pattern can induce synchrony; more often, regularly repeated changes are necessary. Once synchronization is obtained, it will fade away after a few generations unless reinforced. If regularly re-synchronized, cultures will remain synchronous as long as conditions for growth and reproduction do not deteriorate.

Cell division in cultures of Nitzschia palea could be synchronized after cells were kept in the dark for 10 days and then illuminated in continuous light, but synchrony died away. However, with a 6:6-hr light–dark rhythm, repetitive synchronous cell division began every second light period and was completed in the ensuing dark period (von Denffer, 1960). In order to synchronize cell division in Cylindrotheca fusiformis, population growth under constant illumination was followed by a 24-hr dark period, then a more intense illumination and the addition of cysteine L-methionine, and sodium lactate (Levin and co-authors, 1966). A programme of

Fig. 4-15: Open-system fibre-glass tanks containing 1000 l culture fluid. (Original.)
12:12-hr light and dark was used by Jørgensen (1966) to obtain synchronous cultures of *Skeletonema costatum*; cell division started in the middle of the light period similar to that of other diatoms where periodicity of cell division was induced by light-dark changes. Also, in cultures of the marine plankton diatoms *Ditylum brightwellii* and *N. turgidula*—synchronized by different combinations of light intensity and photoperiod—cell division generally took place in the light. The degree of synchrony was highest under short photoperiods of bright light. *D. brightwellii* was strongly inhibited by continuous light but *N. turgidula* was not (Paasche, 1968a).

One theory for the sensitivity to continuous light of some species is based on observations with *Cyclotella cryptica*. This diatom converts carbohydrates to protein in the dark (Werners, 1966). If this conversion were completely inhibited by strong light, a major part of the organic matter would be unavailable unless subjected to darkness (Paasche, 1968b).

*Porphyridium aerugineum* was synchronized by a 24:24-hr light-dark regime; following an initial 48-hr period of darkness the cells started to divide about 6 hrs before each light period (Hoogenhout, 1963). Cultures of the chrysomonad *Coccolithus huxleyi* were synchronized by four different combinations of light intensity and day length. In all four cases cell division was restricted to 6 out of 24 hrs. Synchronized cell division took place in the dark, but was not directly related to the onset or termination of illumination (Paasche, 1967). In cultures of dinoflagellates, the period of cell division varies with the species (Hastings and Sweeney, 1964). Synchronization of *Dunaliella tertiolecta* was reported by Wegmann and Metzner, using a combined light-dark and high-low temperature treatment.

Continuous cultures

To avoid or reduce the uncontrollable changes of environment and cell population, inherent to batch cultures, many investigations are now being carried out in continuous cultures. Whenever large quantities of cell material are required at frequent intervals, continuous cultures provide a greater output and a more consistently uniform cell population than batch cultures.

Continuous cultures may be operated on the principle of a chemostat or turbidostat (Chapter 2). A chemostat involves continuous dilution with fresh medium of a fixed-volume culture, in which one of the nutrients acts as a limiting factor. After equilibrium is established with the rate of dilution, population growth rate adjusts to a maximum level determined by the rate of supply of the limiting nutrient or light source. A turbidostat is an apparatus in which the population is sustained at a constant turbidity level by automatically adding fresh medium to the growth chamber (dilution) when the cell concentration exceeds a predetermined value. Continuous culture systems need not be complex and expensive; some inexpensive and relatively simple types of apparatus have been described in the literature.

A turbidostat was first designed for work with algae by Myers and Clark (1944), to supply uniform Chlorella cells as experimental material over a period of time. This work was followed by many other systems of continuous algal cultures (Pipes and Koutsoyannis, 1962; Maddux and Jones, 1964; Eppley and Dyer,
TECHNOLOGICAL AND PROCEDURAL ASPECTS

1965; Droop, 1966; Howell and co-authors, 1967; Carpenter, 1968; Cook, 1968; Hare and Schmidt, 1968; Taub and Dollar, 1968; Fuhs, 1969; Ukeles, 1973). The growth chamber used by Maddux and Jones (1964) consists of a 1-l flask, magnetically stirred and incubated in a water bath. The medium is supplied from a constant head reservoir (8-l Pyrex bottle). When the population density increases above a predetermined setting, the open solenoid permits additional medium to enter until the turbidity is reduced (Fig. 4-16). Using this apparatus, a set of symmetrical relationships was found between light, temperature and nutrient concentration in which the interaction of any two factors was modified by the third (see also Chapter 2).

Carpenter (1968) devised a simple chemostat that does not use action pumps or elaborate gravity flow systems and also can be sterilized as a unit. The culture container is a three-neck distilling flask in which the volume of the medium is regulated by the height of the overflow tube. An electric pump generates a gas...
mixture that displaces the culture medium from the reservoir to the culture vessel
(Fig. 4-17). This apparatus was used in several nutrient-limited experiments of
axenic cultures of two plankton diatoms. Caperon (1968) obtained data from
continuous culture experiments for *Isochrysis galbana* using nitrate as limiting nutrient. He concluded that the concentration of some form of internal rather than environmental nitrogen controls the population growth rate when nitrogen is limiting. An inexpensive 250-ml chemostat was developed by Droop (1966, 1968, 1969, 1970) for studying the kinetics of vitamin B$_{12}$ limitation in the chrysophyte *Monochrysis lutheri* (Fig. 4-18; see also p. 214). An exhaustive mathematical interpretation of results led to the conclusion that the specific growth rate in the chemostat depends on the nutrient concentration within the cells.

![Continuous culture system](image)

Fig. 4-19: Continuous culture system. (After Dunstan and Menzel, 1971; modified; reproduced by permission of American Society of Limnology and Oceanography.)

Dunstan and Menzel (1971) investigated the effect of a sewage effluent on phytoplankton contained in a large-scale heterogeneous continuous culture. The phytoplankton cultures were grown in 15-l Plexiglas containers with circulating water jackets for temperature control. The cultures were agitated by a magnetic stirring bar and the containers scraped with a polypropylene spatula. The continuous flow system was controlled with micro pumps which provided fresh media and a siphon gravity system for outflow (Fig. 4-19). Sea water diluted with secondary-treated sewage effluent provided an excellent enrichment for the maintenance
of mixed natural populations of marine phytoplankton. A 15-l continuous culture system, in which medium was provided by micro pumps and a siphon gravity system for outflow, was described as a method of providing large quantities of algal

Fig. 4-20: Continuous culture system. 1: 20 mm aseptic filling bell on media intake port; 2: effluent cotton air filters; 3: effluent gas outlet; 4: inoculating port; 5: media filling line; 6: needle valve for gas pressure regulation; 7: influent gas tube with stone air breaker and cotton filters; 8: voltage regulator; 9: 70-mm aseptic filling bell; 10: to aeration and CO₂ system. (After UKELES, 1973: modified; reproduced by permission of Cambridge University Press.)

foods for invertebrate larvae (Fig. 4-20). In this system all parts of the apparatus were sterilized and axenic cultures maintained of several species of chlorophytes and chrysophytes (UKELES, 1973).
Nutritional studies on micro-algae have been largely directed toward clarification of the role of various chemical agents in natural productivity or in regulating yields of laboratory cultures. The success in algal culture methodology has closely followed acquisition of information on nutritional requirements. Some aspects of algal nutrition will be briefly reviewed as information essential to the understanding of culture methods.

Minerals

Growth and normal biochemistry of micro-algae, as in other organisms, require the availability of a varying number of mineral elements, probably 15 to 20, with the possibility that others may still be added to the list. Elemental nutrient requirements are often considered in two groups—the macronutrients, used directly or indirectly for cellular building blocks (C, H, O, N, P, S, K, Mg), and the micronutrients, needed in a lower concentration as catalysts or for unique functions as structural material or osmotic regulators (Fe, Mn, Cu, Zn, Mo, V, B, Cl, Co, Ca, Si, Na). The compounds used to supply the major minerals and the concentration ranges in which they are generally required are shown in Table 4-4. In reviewing the subject of mineral nutrition in algae, O'KELLEY (1968) commented that the inherent heterogeneity of the algae makes it difficult to answer the questions of which elements are essential to algae. The following mineral elements were listed by this author as being required by one or more algae: N, P, K, Mg, Ca, S, Fe, Cu, Mn, Mo, Na, Co, V, Si. Of these N, P, Mg, S, Fe, Cu, Mn, and Mo were considered to be required by all algae; but to some extent S, K, and Ca could be replaced.

To demonstrate the essential need of an alga for minerals it is necessary to purify the medium to such an extent that the alga stops growing, and normal growth is resumed only when the element is supplied. The following procedures for investigating metal requirements are recommended (HALL, 1965): (i) establish a clone or 'pure line' that will grow on any medium that supports growth; (ii) establish an axenic clone in medium that will support growth after autoclaving which may contain sugars, vitamins, salts, fatty acids and protein digests; (iii) replace natural materials by known chemicals of measured quantity; (iv) determine minerals that are indispensable for serial transfer. The absence of essential trace elements may limit growth of unicellular algae by limiting growth rate, total population size or both (SPENCER, 1957).

BOWEN and co-authors (1965) depended on demonstrating optimal growth rates to establish essentiality of boron; but failure of growth after the omission of a metal is often difficult to interpret. Even if all the required metals are present, a failure to grow may be the result of the essential metal ion becoming unavailable by binding to a component of the medium. A non-essential metal may be preventing an essential one from forming a needed complex. If stimulation occurs as the result of adding a metal, interpretation is still not clear since the added metal could simply be displacing a required metal from a complex. Other complications are the
presence of contaminants in so-called pure chemicals, the occurrence of a sparing action, and the physico-chemical form in which the element is present.

The early concept of a defined medium for marine species was that a preparation, as close as possible to the composition of natural sea water, was the ideal culture medium. With the development of recent culture methods, population growth of unicellular algae has been shown to occur in culture solutions whose total ionic content and chemical composition depart from that of natural sea water. Because of poor buffering and precipitation of salts after autoclaving the trend has been to lower the overall composition of salts (PROVASOLI and co-authors, 1967). This trend in the decrease of mineral composition in culture medium (DROOP, 1961b) is shown in Table 4-5 (see also Chapter 2). The high tolerance to variations in ratios of concentrations of Na, K, Ca and Mg suggested to HAYWARD (1970) that

Table 4-4

Compounds used to supply major and minor elements required by unicellular algae (After S. AARONSON, 1970, Experimental Microbial Ecology; reproduced by permission of Academic Press)

<table>
<thead>
<tr>
<th>Element</th>
<th>Compounds</th>
<th>Concentration range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>CO$_2$, CO$_3^{2-}$, organic molecules</td>
<td>g</td>
</tr>
<tr>
<td>O</td>
<td>O$_2$, H$_2$O, organic molecules</td>
<td>g</td>
</tr>
<tr>
<td>H</td>
<td>H$_2$O, organic molecules</td>
<td>g</td>
</tr>
<tr>
<td>N</td>
<td>N$_2$, NH$_4^+$, NO$_3^-$, NO$_2^-$, amino acids, purines, pyrimidines</td>
<td>g</td>
</tr>
<tr>
<td>Na</td>
<td>Several inorganic salts, i.e., NaCl, Na$_2$SO$_4$, Na$_3$PO$_4$</td>
<td>g</td>
</tr>
<tr>
<td>K</td>
<td>Several inorganic salts, i.e., KCl, K$_2$SO$_4$, K$_3$PO$_4$</td>
<td>g</td>
</tr>
<tr>
<td>Ca</td>
<td>Several inorganic salts, i.e., CaCO$_3$, Ca (as chloride)</td>
<td>g</td>
</tr>
<tr>
<td>F</td>
<td>Several inorganic salts, Na or K phosphates, Na$_2$ glycerophosphate, 5H$_2$O</td>
<td>g</td>
</tr>
<tr>
<td>S</td>
<td>Several inorganic salts, MgSO$_4$, 7H$_2$O, amino acids</td>
<td>g</td>
</tr>
<tr>
<td>Mg</td>
<td>Several inorganic salts, CO$_3^{2-}$, SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>mg</td>
</tr>
<tr>
<td>Fe</td>
<td>FeCl$_3$, Fe(NH$_4$)$_2$SO$_4$, ferric citrate</td>
<td>mg</td>
</tr>
<tr>
<td>Zn</td>
<td>SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>mg</td>
</tr>
<tr>
<td>Mn</td>
<td>SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>mg</td>
</tr>
<tr>
<td>Cu</td>
<td>SO$_3^{2-}$ or Cl$^-$ salts</td>
<td>µg</td>
</tr>
<tr>
<td>Co</td>
<td>Vitamin B$_{12}$, SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>µg</td>
</tr>
<tr>
<td>B</td>
<td>B$_2$O$_3$</td>
<td>mg</td>
</tr>
<tr>
<td>Mo</td>
<td>Na or NH$_4$ molybdate salts</td>
<td>µg</td>
</tr>
<tr>
<td>V</td>
<td>Na$_3$VO$_4$.16H$_2$O</td>
<td>µg</td>
</tr>
<tr>
<td>Sr</td>
<td>SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>µg</td>
</tr>
<tr>
<td>Al</td>
<td>SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>µg</td>
</tr>
<tr>
<td>Rb</td>
<td>SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>µg</td>
</tr>
<tr>
<td>Li</td>
<td>SO$_4^{2-}$ or Cl$^-$ salts</td>
<td>µg</td>
</tr>
<tr>
<td>Cl</td>
<td>as Na$^+$, K$^+$, Ca$^{2+}$ or NH$_4^+$ salts</td>
<td>g</td>
</tr>
<tr>
<td>I</td>
<td>as Na$^+$, K$^+$, Ca$^{2+}$ or NH$_4^+$ salts</td>
<td>µg</td>
</tr>
<tr>
<td>Br</td>
<td>as Na$^+$, K$^+$, Ca$^{2+}$ or NH$_4^+$ salts</td>
<td>mg</td>
</tr>
<tr>
<td>Si</td>
<td>Na$_2$SiO$_3$.9H$_2$O</td>
<td>mg</td>
</tr>
</tbody>
</table>

*Per 100 ml of medium.
algaes are able to maintain an internal ionic composition despite a wide variation in
the external composition of the medium. Evidence was presented to show that
cellular concentrations varied during different phases in batch culture; divalent
cations increased to a maximum toward the stationary phase, monovalent cations
increased during the exponential phase and then decreased almost immediately.
A comparison of the results obtained with cells grown in full strength and \( \frac{1}{2} \) strength
artificial sea-water medium (ASP) showed that the cellular composition changed
very little with the composition of the medium, but cell numbers increased faster
in more dilute medium. Hayward (1970) suggested that energy available to the cell
may be expended in ionic control to maintain an internal environment against a
high concentration gradient, but in dilute medium this energy becomes available
for growth (see also Volume I, Chapter 4).

Hutner and co-authors (1950) introduced the use of chelating compounds into
the culture medium to avoid the heavy precipitation of metals. The addition of a
chelating agent as ethylenediaminetetraacetic acid (EDTA) to sea water sets up

table 4-5

<table>
<thead>
<tr>
<th>Na</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>Cl</th>
<th>SO_4</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.5</td>
<td>0.379</td>
<td>0.411</td>
<td>1.28</td>
<td>19.0</td>
<td>2.64</td>
<td>Dittmar's analysis (1884)</td>
</tr>
<tr>
<td>11.1</td>
<td>0.405</td>
<td>0.432</td>
<td>1.34</td>
<td>29.1</td>
<td>3.24</td>
<td>Allen (1914)</td>
</tr>
<tr>
<td>11.8</td>
<td>0.522</td>
<td>0.292</td>
<td>1.53</td>
<td>22.0</td>
<td>2.65</td>
<td>Pringsheim (1946b)</td>
</tr>
<tr>
<td>9.40</td>
<td>0.334</td>
<td>0.183</td>
<td>1.14</td>
<td>10.7</td>
<td>2.32</td>
<td>Leving (1946)</td>
</tr>
<tr>
<td>7.05</td>
<td>0.313</td>
<td>0.100</td>
<td>0.44</td>
<td>10.4</td>
<td>1.93</td>
<td>Provasoli and co-authors (1957)</td>
</tr>
<tr>
<td>5.90</td>
<td>0.260</td>
<td>0.117</td>
<td>0.30</td>
<td>10.2</td>
<td>0.28</td>
<td>ASP_2</td>
</tr>
</tbody>
</table>

Equilibria in which cations compete for available EDTA bonds, but enough ions
are released through mass action to satisfy the requirements of the growing cells.
The generally accepted fact is that EDTA is not readily metabolized (Spencer,
1957). Since many required elements may be toxic in high concentrations, chelators
permit the growth of an organism in a medium containing metal concentrations
that may otherwise be harmful. Chelators other than EDTA are shown in Table 4-6.
Many different types of media have been designed with variation in the major
mineral components; but little general agreement has resulted on the benefits to be
derived from each variation, partially due to the inherent complications in the inter-
pretation of data. One example is the low requirements for trace metals shown for
the chrysomonads (Pintner and Provasoli, 1963) which does not imply that
these minerals are not needed, but that the requirements are satisfied by impurities
in other salts and distilled water. Considering the composition of growth medium
for micro-algae, it is probably well to recollect a statement made by Pringsheim
and quoted by Ketchum (1954, p. 58):

'Most algaes are not affected by minute changes in the composition of the medium,
otherwise they could not live under natural conditions. Changes
effect the algae themselves are often more decisive than the differences between various media.'

Nitrogen. There is no evidence that inorganic nitrogen can be fixed as a nitrogen source in micro-algae other than in Myxophyceae (Ketchum, 1954). The inorganic nitrogen sources commonly utilized are ammonium salts, nitrate and possibly nitrite. Inorganic nitrogen sources play an important part in regulating phytoplankton growth; hence a considerable literature exists on their utilization, particularly ammonia and nitrate. Wattenberg (1929) was one of the first investigators to suggest that forms of nitrogen other than nitrate, then assumed to be the essential one, be investigated as a possible nitrogen source. Schreiber (1927) had shown that more Carteria cells can be produced with ammonia than from equivalent amounts of nitrate. Species of Chlamydomonas and Chlorella were also cultured with ammonium salts as a nitrogen source (Braarud and Foyn, 1931), and Cooper's (1933) investigations in the English Channel indicated that ammonia may be directly utilized from sea water by Biddulphia mobiliensis. ZoBell (1935) reported an immediate increase in cell populations in ammonia not observed in other nitrogen sources; ammonia is preferentially absorbed. It is toxic at high concentrations and the optimum concentration of nitrate for Nitzschia closterium is over 100 times higher than for ammonia. Although the centric diatom Biddulphia aurita can utilize each of the 3 inorganic nitrogen sources, ammonia nitrogen is removed twice as fast as nitrate or nitrite (Lui and Roels, 1972). This preference also exists for Ditylum brightwellii when supplied with both forms of nitrogen; addition of ammonia immediately lowers nitrate or nitrite uptake. If nitrate and nitrite are present in equal concentrations, both ions are assimilated simultaneously (Eppley and Rogers, 1970). Guillard (1963) reported that the diatoms Skeletonema sp., Cyclotella nana, C. cospi, Coscinodiscus asteromphalus and N. seriata grew in ammonia or ammonia + nitrate but not as successfully as nitrate alone. Ammonia

---

Table 4-6


<table>
<thead>
<tr>
<th>Compound</th>
<th>pH for best use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>6.0-7.5</td>
</tr>
<tr>
<td>Nitrilotriacetic acid</td>
<td>Below 6.0</td>
</tr>
<tr>
<td>Citric acid</td>
<td>Below 7.0</td>
</tr>
<tr>
<td>DL-Malic</td>
<td>6.0-7.0</td>
</tr>
<tr>
<td>Ethylenediamine(0-hydroxyphenyl) acetic acid</td>
<td>Below 6.0</td>
</tr>
<tr>
<td>1,2-Diaminocyclohexane tetraacetic acid</td>
<td>6.0-7.5</td>
</tr>
<tr>
<td>Diethylene-triamine pentaacetic acid</td>
<td>6.0-7.5</td>
</tr>
<tr>
<td>Ethylene glycol bis(aminoethyl ether) tetraacetic acid</td>
<td>7.0-7.5</td>
</tr>
<tr>
<td>Ethylene glycol bis(aminoethyl ether)</td>
<td>7.0-7.5</td>
</tr>
</tbody>
</table>
induces an initial lag in *Cylindrotheca closterium* var. *californica*, but if three forms of nitrogen (nitrate, ammonia and urea) are offered in combination, ammonia is used first followed by urea and then nitrate (Grant and co-authors, 1967).

Dinoflagellates thrive on nitrate in low concentrations (0.001 mg l$^{-1}$) (Barker, 1935; Provasoli and McLoughlin, 1963), but the addition of more than 0.01 mg l$^{-1}$ ammonium chloride inhibits *Prorocentrum micans* (Barker, 1935). The five species of chrysomonads studied by Pintner and Provasoli (1963) differ in their tolerance to ammonium sulphate; *Pavlova gyranus* and *Hymenomonas* sp. withstand 20 mg%, other species utilize this nitrogen source at 10 mg%, but it is toxic to *Coccothrix huxleyi* at 1 mg%. In contrast to this tolerance is the sensitivity of *Prymnesium parvum* to ammonium salts in alkaline medium while being a good nitrogen source in the neutral or acid range at low concentrations (less than 0.5 mg%; McLoughlin, 1968).

Physiological studies with *Dunaliella tertiolecta* showed that this alga assimilates nitrate faster in the light than in the dark and grows better with ammonia than with nitrate at all light intensities (Grant, 1967, 1968; Grant and Turner, 1969). Paasche (1971) found that cells utilizing ammonia have a greater concentration of ribulose diphosphate carboxylase; he suggested that increased growth with ammonia may be partly a consequence of the general increase in net protein synthesis resulting in greater content of photosynthetic enzymes. In spite of reports of increased growth with ammonia, Lewin (1953) recommended avoiding ammonium salts for the following reasons: (i) in alkaline media even a few parts per million may be inhibitory or toxic to sensitive forms; (ii) ammonia distils off when media of high pH are autoclaved, the nitrogen thereby being reduced to an unknown level; (iii) uptake of ammonium ions is always more rapid than that of nitrate with the result that the pH drops; in a weakly buffered solution it may reduce the pH to a toxic level.

Marine flagellates and other green algae were studied for their ability to utilize organic nitrogen (Schrøiber, 1927; Braarud and Foy, 1931; Droop, 1955, 1957, 1959; Gibor, 1956; McLoughlin, 1958). No organic nitrogen source seemed to be suitable for all species, but there were indications that uric acid and urea could generally be utilized. In one study of 40 uni-algal clones representing 36 species about half had a fair capacity to utilize urea (McCarthy, 1971). From experiments with natural populations of diatoms Harvey (1940) concluded that amino acids are not utilized significantly unless first decomposed by bacteria, but that uric acid and urea are generally acceptable. Two species, however, were later found to require amino nitrogen: the cryptomonad *Hemiselmis virescens*, which grew best on glycine (Droop, 1957), and the phagotrophic dinoflagellate *Oxyrrhis marina*, which used valine, proline or alanine (Droop, 1959). Harvey’s conclusion that simple amino acids do not serve as a source of nitrogen for diatoms was confirmed, with two exceptions, by Guillard (1969). In a study of 15 clones of centric and one pennate diatom only two species, Melosira sp. and Coscinodiscus asteromphalus, utilized glutamate and glutamine as readily as nitrate. *Cyclotella caspia* and *Cyclorella nana* isolated from polluted waters can utilize uric acid, but of 9 clones isolated from the Sargasso Sea only one, Skeletonema sp., utilizes urea and uric acid. *Melosira nummuloides* has the capacity to take up amino acids with restricted utilization of some as nitrogen sources (Hellebust and Guillard, 1967). One isolate of *Phaeo-
Dactylium tricornutum was reported to utilize a variety of organic nitrogen sources for growth, including amino acids and nicotinamide (Hayward, 1965).

In a study of the heterotrophic capacities of the chrysomonads Pintner and Provasoli (1963) observed that many organic nitrogen sources can be utilized. Only Syracosphaera sp. utilizes low levels of urea (0.01–1 mg%), as well as nitrate; but Hymenomonas sp. and Pavlova gyraea utilize urea at higher concentrations. The chrysomonad Prymnesium parvum utilizes organic nitrogen sources as creatine, asparagine, arginine, alanine, histidine, methionine and acetyl-urea, but guanidine and acetamine are not incorporated (McLaughlin, 1958). The amino acids methionine and ethionine serve as nitrogen sources for P. parvum (Rat and Rexor, 1963) and even at high concentrations do not exert the growth inhibiting effect described for the freshwater chrysomonad Ochromonas malhamensis (Johnson and co-authors, 1957).

Some amino acids are utilized by dinoflagellates as nitrogen sources: arginine and asparagine by Gyrodinium uvacenum, G. californicum, G. resplendens and Amphidinium carteri and A. rhynchocephalus; glycine by A. carteri, A. rhynchocephalus, G. californicum and Gymnodinium splendens clones; glutamic acid and alanine by A. carteri, A. rhynchocephalus and G. splendens; methionine by both species of Amphidinium (Provasoli and McLaughlin, 1963). Rytcher (1954) observed that in estuaries contaminated with waste from duck farms the dominant phytoplankters Nannochloris and Stichococcus utilize a wide variety of nitrogen sources as nitrate, nitrite, ammonia, urea, uric acid, cystine, asparagine and glycine; but urea utilization produces only 57% of the growth attained with nitrate. When low concentrations of glycine or arginine are supplied to a marine Platymonas sp. uptake rates are sufficient to meet the nitrogen requirements, and nitrate and ammonia do not interfere with amino-acid uptake (North and Stephens, 1971). According to Birdesey and Lynch (1962), the red alga Porphyridium cruentum utilizes nitrate and ammonium chloride, but none of the organic nitrogen sources tested. Jones and co-authors (1963) also observed that P. cruentum grows equally well in nitrate and ammonia but that L-amino acids do not support growth of the alga, with the exception of L-asparagine. In general, species isolated from estuarine and neritic environments use urea or uric acid, but the inability to utilize these nitrogen sources may be widespread in oceanic populations. Carpenter and co-authors (1972) caution against the hasty extrapolation of laboratory studies on urea decomposition to field conditions. These investigators observed that the rate of decomposition of urea by Stephanopogon costata (Skeletonema costatum), as found in the laboratory, is 3.5 to 3.7% of the in situ rate measurements.

Phosphorus and sulphur. Inorganic phosphorus is a good source of phosphorus for all phytoplankton, generally varying little with the form in which it is supplied. Pyrophosphate (H₂P₂O₇) is not utilized as effectively as orthophosphate (H₄PO₄) by Skeletonema costatum, Nitzschia closterium or Phaeocystis pouchetii, and the CaMg salt of phytic acid can support S. costatum and N. closterium as well or better than orthophosphate (Cru, 1946a). Hayward (1968c) described similar experiments comparing effects of orthophosphate (Na₃H₂PO₄. 2H₂O; Na₂HPO₄; KH₂PO₄; K₂HPO₄), pyrophosphate (Na₂P₂O₇. 10H₂O; K₂P₂O₇), potassium glycophosphate and sodium inositol hexaphosphate (sodium phytate) at 2 mg l⁻¹ phosphorus. Several isolates of Phaeodactylium tricornutum grow well with the 4
forms of orthophosphate, less well with pyrophosphate and utilize the organic forms of phosphate, as well as the orthophosphate, but sodium hypophosphite (NaH$_2$PO$_3$·H$_2$O) is toxic to all isolates.

Organic phosphates—glycerophosphate, adenylic acid, guanylic acid and cytidylic acid—are utilized by several chrysomonads and species of the dinoflagellates Gyrodinium and Amphidinium in addition to the inorganic source (Pintner and Provasoli, 1963; Provasoli and McLaughlin, 1963). Dunaliella tertiolecta and Olisthodiscus sp. are unable to utilize glycerophosphate or do so only to a limited extent (McLachlan, 1964). The utilization of organic monophosphates is difficult to evaluate because many marine diatoms and chrysophytes have alkaline phosphatases that act at the cell surface to hydrolyze glucose-6-phosphate (G-6-P), adenosine-monophosphate or alpha-glycerophosphate. The ability of 13 marine unicellular algae to grow on G-6-P could be related to the amount of surface phosphatase produced (Kuenzler, 1965; Kuenzler and Perras, 1965).

Concentrations of phosphorus that limit exponential growth rates are difficult to determine, partly due to the introduction of unknown factors from the formation of calcium and iron phosphate precipitates. Nevertheless, Ketchum (1939) showed that wide variations in phosphate concentrations had no effect on the growth constant of Nitzschia sp., and for Asterionella japonica remained the same within 0·01 to 0·31 µM phosphorus (Kain and Fogg, 1958a). Apparently, some cells can accumulate large stores of phosphorus that are used when there is a depletion of external phosphorus in the medium (Kuenzler and Ketchum, 1962). On the other hand, oceanic diatoms, such as Chaetoceros gracilis, become growth limited by low concentrations of phosphate in the medium (Thomas and Dodson, 1968).

Sulphur in artificial sea water is usually supplied as MgSO$_4$·7H$_2$O at a concentration approximating that of natural sea water (2·76 g l$^{-1}$ at 35‰ S; Harvey, 1957). Ranges of 1·0 to 3·1 g sulphate l$^{-1}$ have been employed in media used by Hutner (1948), Provasoli and co-authors (1957), Takano (1964) and Taylor (1964). Droop's media S 22 and S 32 contain less, 0·29 g l$^{-1}$. Experiments on levels of sulphate in artificial sea water demonstrated that 0·0047 g l$^{-1}$ is sufficient to give a maximum yield for many species but results in a depressed growth rate, 0·14 g l$^{-1}$ being needed to maintain the growth rate at a maximum (McLachlan, 1960). Jones (1962) reported that methionine, L-cysteine, L-cystine, glutathionine and sulphide are not utilized by Porphyridium cruentum as a source of sulphur, but MgSO$_4$, Na$_2$SO$_4$ or Na$_2$S$_2$O$_3$ in the range of 5·4 to 27 mM result in good population growth. Such a high sulphate consumption was also reported by Lewin and Busby (1967), which is to be expected since this alga accumulates an extracellular acidic polysaccharide-protein complex with 10% bound sulphate.

Silicon. Hydrated amorphous silicon is a constituent of the diatom cell wall and is, therefore, essential in relatively large quantities to most species of diatoms, with the exception of Phaeodactylum tricornutum (Lewin and Guillard, 1963), and also to chrysophytes with silicious skeletons. The mineral is normally supplied as metasilicate (Na$_2$SiO$_3$·9H$_2$O) which hydrolyzes to give orthosilic-acid forming silicic ions in skeletons. The final culture yield of many diatoms is proportional to the amount of silicon, although growth rates can be independent of silicate down to very low concentrations since sufficient amounts can be dissolved from culture vessels in alkaline media. Silicon utilization is influenced by other factors, such as
the concentration of phosphate, the duration and intensity of illumination, and temperature (Hughes and Lund, 1962). Divalent sulphur and amino acids seem to play a role in silicate utilization since the ability to utilize silicate that is lost in washed diatom cells is partially restored by the addition of sulphate or other reduced sulphur compounds (Lewin, 1954; Lewin and Chen, 1968). The rate limiting concentration of silicon appears to vary with the species of diatoms but is probably less than 50 to 100 mg l\(^{-1}\) (Strickland, 1965). The physiology and biochemistry of silicon deposits in diatoms have been reviewed by Lewin (1955, 1966b) and Lewin and Guilland (1963).

Na, K, Mg, Ca. Growth in the laboratory will take place in salt concentrations much lower than in sea water, provided various ionic balances are maintained.

Table 4.7

<table>
<thead>
<tr>
<th>Species</th>
<th>Calcium concentration</th>
<th>NaCl</th>
<th>400 mM</th>
<th>100 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg : Ca →</td>
<td></td>
<td>7-5 mM</td>
<td>2-5 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5:1</td>
<td>10:1</td>
<td>40:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100:1</td>
<td>160:1</td>
<td></td>
</tr>
<tr>
<td>Monochrysis lutheri</td>
<td>95</td>
<td>92</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>Syracosphaera carterae</td>
<td>99</td>
<td>97</td>
<td>95</td>
<td>71</td>
</tr>
<tr>
<td>Olisthodiscus sp.</td>
<td>101</td>
<td>97</td>
<td>75</td>
<td>22</td>
</tr>
<tr>
<td>Thalassiosira fluviatilis</td>
<td>98</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>Cyclotella cryptica</td>
<td>100</td>
<td>96</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>102</td>
<td>96</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td>Amphidinium carteri</td>
<td>98</td>
<td>93</td>
<td>77</td>
<td>45</td>
</tr>
<tr>
<td>Porphyridium sp.</td>
<td>100</td>
<td>101</td>
<td>97</td>
<td>103</td>
</tr>
<tr>
<td>Cryptomonad (3C)</td>
<td>106</td>
<td>104</td>
<td>100</td>
<td>82</td>
</tr>
</tbody>
</table>

Metabolically inert low-molecular weight cationic, anionic, and zwitterionic electrolytes or osmotically active non-electrolytes, such as pentaerythritol, can spare only 90% of the requirement for sodium in the brine alga Dunaliella salina (Hutner and co-authors, 1957b). The concentration of sodium chloride affected certain biochemical changes in D. tertiolecta, which appears to have an absolute requirement for sodium (McLachlan, 1960) that is not evident in growth studies alone (Craigie and McLachlan, 1984). Of 9 phytoplankters studied only growth of Skeletonema costatum was reduced at 200 mM NaCl, as compared with 400 mM NaCl in an artificial sea-water medium. At two lower concentrations, 50 mM and 100 mM, Amphidinium carteri and Olisthodiscus sp., in addition to S. costatum, were inhibited, whereas growth of other species was little affected. Sodium could not substitute for potassium in any of the 9 species (McLachlan, 1964). Olisthodiscus sp., A. carteri and S. costatum were sensitive to potassium concentrations reduced to 1-0 mM.
and 0.5 mM, as compared with 10 mM, but *Monochrysis lutheri*, *Syracosphera carterii* and *Porphyridium* sp. could tolerate a reduction to 0.5 mM with no harmful effect. Calcium is needed in very high concentrations by coccolith-bearing chrysomonads, as the formation of the coccoliths depends on high calcium concentrations (ISENBERG and co-authors, 1964). Growth of numerous species was not affected by varying the Mg/Ca ratio from 5.3:1 to 400:1, but with lower NaCl concentrations *Cyclorella cryptica* and *S. costatum* appeared to be more sensitive to reduced calcium (Table 4-7; McLACHLAN, 1964); growth of *Porphyridium* sp., *A. carteri*, *Olisthodiscus* sp. and *S. costatum* was adversely affected when the magnesium concentration was reduced below 40 mM. In culture, brackish-water and marine algae appear to tolerate a wide range of Mg/Ca ratios and total amounts of calcium and magnesium that are much less than found in natural sea water (PROVASOLI, 1965b; see also Volume I, Chapter 4).

**Trace Metals.** Trace metals may sometimes appear non-essential in culture medium due to their availability from trace metal contamination in chemicals and glassware (PINTNER and PROVASOLI, 1963). According to ALEEM (1970), addition of copper, as well as soil extract, improves the quality of 'bad' sea water. BERNHARD and ZATTERA (1970) cited evidence that small additions of copper and zinc to sea water can easily exceed the upper tolerance concentrations for *Phaeodactylum tricornutum* and *Coccolithus huxleyi*; the normal concentration of copper in sea water is only about 15 times lower than the maximum test concentration tolerated and the corresponding value for zinc is about 30. The upper tolerance concentration for chromium was about 150,000 times the concentration in sea water; for cobalt, 20,000 times.

Inshore water collected near Plymouth, even after enrichment with nitrate, phosphate and iron, did not support continuous growth of a chrysomonad, a cryptomonad, a chlamydomonad and a *Chlorella* unless manganese was added (HARVEY, 1947). Chlorine, in addition to manganese, is another catalytic element known to be specifically concerned with O₂ production during photosynthesis; but the role of chlorine is not clear and chlorine was not shown to be required for algae studied by McLACHLAN and CRAIGIE (1967). Bromine could replace chlorine in the medium, and bromine concentrations as high as those found in the Dead Sea (0.05 M) had no adverse effect on 9 phytoplankters belonging to different taxa. Molybdenum is essential for green plants; it plays a role as a cofactor in nitrogen metabolism and influences respiration and photosynthesis; information on the specific role of molybdenum in these processes is not available (WYESSNER, 1962). A requirement for cobalt exists in algae capable of synthesizing vitamin B₁₂.

EYSTBR (1968) expressed doubt that boron is essential for algae; he suggested that boron requirement is related to the amount of calcium utilized and increases with it. Boron did not stimulate 4 *Chlorella* species; levels up to 50 ppm were tolerated without reduction in growth rate (BOWEN and co-authors, 1965). In a culture medium for diatoms the silicon–boron ratio was important, suggesting an interaction between these elements (LEWIN, 1965, 1966c). Analyses of boron requirements are complicated due to boron contamination from autoclaved borosilicate glassware; such contamination can now be avoided with polycarbonate culture vessels (see also Chapter 7). LEWIN (1966a) demonstrated that the marine diatom
Cylindrotheca fusiformis will multiply in an artificial sea-water medium in polycarbonate vessels only when borate is added. All compounds active in supplying boron to this diatom had at least two H groups attached to the boron atom, such as boric acid, phenylboronic acid and its derivatives. The conclusions on boron requirements in this diatom were confirmed by Nealles (1967). In other studies, boron was shown to be required by 12 species of marine pennate diatoms, 4 centric diatoms, Amphidinium carteri, Pyramimonas sp. and Platymonas sp.; but no requirement could be demonstrated for the chlorophyte Dunaliella tertiolecta or the coccolithophorids Cricosphaera carterae and Coccolithus huxleyi (Lewin, 1966c).

That inorganic iron could stimulate phytoplankton growth was first demonstrated by Gran (1933) with Skeletonema sp. The function of iron is obviously associated with numerous iron-containing enzymes (peroxidases, catalases, cytochromes) and with pigment production. One of the difficulties in the study of iron nutrition is the comparatively high iron content present as contaminants in commercial chemicals (Hayward, 1968b; Davies, 1970). Dissolved iron may change to colloidal ferric hydroxide with age and autoclaving will produce chelated complexes with EDTA or citrate (Eyster, 1968). In the absence of chelators, the pH of the culture media results in almost complete precipitation of iron. Droop (1961a, b) has commented on the importance of redox potentials of marine media in relation to the relative concentrations of ferrous and ferric ions.

The amount of iron that can exist in true solution in sea water at pH 8 in equilibrium with ferric hydroxide is very small: 10^{-4} M (Cooper, 1937a). Harvey (1937a, b) described the three forms of iron present in sea water as (i) iron in true solution in equilibrium with Fe(OH)^{2+}, ferric or ferrous ions; (ii) ferric hydroxide and phosphate colloidal micelles or large aggregates; (iii) iron in organic combination. Whereas it may be possible that some unicellular algae can mobilize their essential trace elements from very low ionic concentrations, the work of Harvey (1937b) and Goldberg (1952) suggests that there are species which can utilize the particulate or colloidal form of iron. Asterionella japonica utilizes particulate and/or colloidal iron, but iron complexed with citrate or humic acid cannot be incorporated (Goldberg, 1952). Although the need for iron has been well documented, many aspects of its role in culture media are not obvious. Phaeodactylum tricornutum became increasingly efficient in utilizing small amounts of iron by a gradual adaptation to iron-limiting conditions (Hayward, 1968b). Studies with 15 species of phytoplankters showed that, on the whole, trace-metal contents of algae increase with increasing concentrations in the medium; differences could not be related to taxonomic groups (Riley and Roth, 1971).

**Carbon**

Photosynthetic algae are generally photo-autotrophic, synthesizing organic carbon from CO₂. Although molecular CO₂ is considered the primary source of CO₂ for photosynthetic species, there is still uncertainty regarding the form in which the carbon is assimilated (Volume I: VidaV, 1972). Some studies indicate that CO₂ is not the preferred form of carbon for marine algae. Cultures of Platymonas sp., Nitzschia closterium and Porphyridium cruentum utilized a carbamino-complex of alanine in preference to inorganic CO₂ and some aquatic plants, particularly those in a marine environment, utilized bicarbonate for photosynthesis (Strickland,
CHEMICAL ASPECTS

1965). This is to be expected in oceans and coastal waters since the kinetics of the conversion of carbon dioxide to bicarbonate in an alkaline medium (Volume I: KALLE, 1972) are such that bicarbonate cannot limit growth providing that CO₂ is present. In sea water at its normal pH of 8.2 the bicarbonate ion concentration is about 100 times greater than molecular CO₂. HOOD and PARK (1962) cited evidence for utilization of bicarbonate ions by marine species of Chlorella, Platymonas sp. and N. closterium in contrast to Chlorella pyrenoidosa or species of Skeletonema and Cyclotella that exclusively utilize molecular CO₂ (DEGENS and co-authors, 1968). According to STEEMANN NIELSEN (1966), naked clones of the coccolithophorids utilize only CO₂ in photosynthesis but for coccolith formation the bicarbonate ion is used, calcium carbonate being precipitated in the coccoliths. One study seems to indicate that growth in artificial media without bicarbonate is limited by carbon, with the exception of a cryptomonad and Dunaliella tertiolecta; both grow equally well in media with or without added bicarbonate (MCLACHLAN, 1959, 1964).

Where carbon dioxide needs to be added to culture media, the gas can be taken from high-pressure cylinders containing the appropriate CO₂/air mixture or from a continuously monitoring gas-mixing device in which the two gases from an air compressor and CO₂ tank are combined under constant pressure. WARBURG's (1919) practice of aerating cultures with a 5:95 ratio of CO₂ to air has often been adopted, but many investigators find that lower concentration ranges of 1 to 2% CO₂ are more satisfactory. It is helpful to monitor the pH changes in mass cultures for maintaining appropriate CO₂ concentrations.

Carbon nutrition in algae has often been claimed to be derived from photosynthetic utilization of CO₂. However, there is evidence that algae with carbon heterotrophic capacities (i.e., assimilation of reduced carbon) exist in the marine environment. Healthy phytoplankton populations have been found below the photic zone or under ice-covered arctic regions. A heterotrophic mode of nutrition has been postulated to account for these observations (BERNARD, 1963). Heterotrophy may also be of advantage in areas of high organic content, such as littoral zones and sewage oxidation ponds, and indeed a high incidence of auxotrophy is reported in isolates from these areas (see also Volume II: PAHDELAX, 1976; SCHLEGEL, 1975).

In the terminology adopted by LWOFF (1951), DROOP and McGILL (1966) and DROOP (1974) the major types of carbon nutrition are described as photo-organotrophic, where carbon is photosynthetically assimilated, and chemo-organotrophic (chemotrophic) where carbon is oxidatively assimilated bypassing the photosynthetic cycle. One of the puzzling aspects of carbon utilization is stimulation by a carbon source in the light, but non-utilization of carbon in the dark. A stimulation of growth over and above the autotrophic growth, dependent on photosynthesis, is known as photoheterotrophy or mixotrophy. Detailed information on the subject of photoheterotrophy in micro-algae may be found in reviews by LEWIN and LEWIN (1960), DANFORTH (1962), THOMAS (1968), and DROOP (1974). An example of mixotrophy in diatoms is Cocconeis sp.; this species reveals growth stimulation with lactate, pyruvate, acetate, succinate or citrate in the light but not in the dark; however, lactate is absorbed in the dark (BUNT, 1969). The light and dark metabolism involving acetate and lactate, the proteins synthesized, and the metabolic inhibitors in this benthic diatom have been investigated by COOKSEY (1972).
Hayward's (1968a) experiments with tracers showed that labeled glucose, mannitol, acetate and lactate did not penetrate Phaeodactylum tricornutum, although a short-term stimulation of respiration occurred that may have been a result of trace-metal contamination in the organic carbon sources.

Heterotrophic capacities are scattered throughout the major taxonomic algae groups; aside from vitamin requirements (auxotrophy) they were considered rare among the pigmented species until, in recent years, more examples have been reported. Absence of heterotrophy is difficult to demonstrate with any degree of certainty. No matter how extensive an investigation is pursued, the possibility remains that some particular carbon source was not tested or that the most suitable combination of other factors (e.g., temperature, salinity, pH, trace-metal mix) had not been investigated. An example is glycerol utilization in Pyramnesium parvum. Light was considered obligatory for the chrysomonad P. parvum until Rarat and Jahn (1965) and Rarat and Spira (1967) showed that growth in the light was enhanced by unusually high concentrations of glycerol and that this compound was specific for dark growth. Population growth of P. parvum and of Chroomonas salina in glycerol was also observed by Cheng and Antia (1970). According to Cheng and Antia, glycerol in the light enhanced growth rate and peak population density of 16 species: members of the Chrysophyceae, Cryptophyceae, one diatom, one rhodophyte and one chlorophyte. Some cells revealed cytological abnormalities, such as cell enlargement and starch accumulation.

The colourless dinoflagellate Gyrodinium colhii utilizes glucose and glycerol as a carbon source, and minimally some fatty acids (Provasoli and Gold, 1962). The pigmented dinoflagellate Gymnodinium breve did not respond to a long list of organic compounds, including fatty acids, TCA intermediates, alcohols and carbohydrates; therefore, it does seem to be restricted to the use of CO₂ in the light (Aldrich, 1962). More than half of 44 pure cultures of marine littoral diatoms (43 pennate, 1 centric) are able to grow heterotrophically preferring glucose as the substitute; 8 can use glucose or lactate, 1 glucose or acetate, 1 glucose, acetate or lactate, and 2 species use only lactate (Lewin and Lewin, 1960). Apochlorotic species, of course, utilize organic carbon sources. Among diatoms isolated from marine muds, 3 species preferred lactate or succinate and 2 species, glucose or glutamate (Lewin and Lewin, 1967). Nitzchia pulrida, another colourless diatom, was cultivated on protein hydrolysate, but growth could not be supported by glucose or acetate alone (Pringsheim, 1951). Cylindrotheca fusiformis can use the following organic compounds as a carbon source: lactate, succinate, fumarate, malate, tryptone, casamino acids or yeast extract but not glucose (Lewin and Hellebust, 1970). Why the yeast extract or complex nitrogen sources served as a substrate is not clear, but contamination with small amounts of organic acids is possible. Of 16 marine centric diatoms, only Cyclotella cryptica (clone O-3A) was capable of heterotrophic growth on glucose but not on lactate. Evidence was obtained for a glucose transport system induced in the dark and inactivated in the light (Hellebust, 1971).

Slight stimulation of population growth in the light by small amounts of lactate and pyruvate was demonstrated with Coccolithus huxleyi, Hymenomonas sp. and Syracosphaera sp. (Pintner and Provasoli, 1963). Efforts to grow other coccolithophorids in the dark were generally not successful (Pintner and Provasoli,
CHEMICAL ASPECTS

1963; Droop and McGill, 1966; Sloan and Strickland, 1966); but one strain of Hymenomonas sp. seemed capable of very slow growth in the dark in the presence of lactate and amino acids, and a large number of different carbon sources were stimulatory in the light (Isenberg and co-authors, 1965). Carbon sources stimulating population growth in the light are pyruvic and lactic acids in Hymenomonas sp.; acetate and glycerol in Pavlova gyrans; lactate and glycerol in Syracosphaera sp. C. huxleyi was slightly stimulated by acetate, pyruvate, glutamate, acetyl glutamate, sucrrose and propylene glycol. Although lactate increased growth rate of Hymenomonas sp. 6 to 8 times in the light, it did not support growth in the dark (Pintner and Provasoli, 1963).

Among the chlorophytes all Chlorococcum strains are obligate phototrophs as are many Chlorella strains (Shihara and Krauss, 1965). Dunaliella viridis is stimulated by glucose and Platymonas sp. by acetate; but dark growth is not supported (Gibor, 1966). According to Kwon and Grant (1971), the failure of D. tertiolecta to use glucose is due to membrane impermeability and not to lack of appropriate enzymes. Since glycollic acid has often been noted as a metabolic product in supralittoral algae, limiting permeability cannot account for the observation that glycollate does not support growth in the dark or enhance growth in the light of the 39 strains tested by Droop and McGill (1966).

Vitamins

The use of mineral media in the isolation of marine micro-algae constitutes bias in favour of the isolation of autotrophs; but with the introduction of soil extract and its eventual replacement by trace metals and vitamins, numerous examples of auxotrophy appeared in isolates. Differences in vitamin requirements of marine micro-algae cannot be correlated with the presence or absence of chlorophyll, taxonomic position or ecological niche. Autotrophs are found in sewage lagoons and auxotrophs in oligotrophic waters (Provasoli and Carlucci, 1974). There are examples of pigmented, as well as non-pigmented species that are strict autotrophs, synthesizing all the vitamins, e.g., the colourless freshwater flagellates Polytoma wella and P. obtusum, and the pigmented marine species Stichochrysis immobis (Pintner and Provasoli, 1963). No difference in growth rate was detected in pigmented Phaeodactylum tricornutum with or without added thiamine, biotin and vitamin B₁₂, either singly or in combination, for 5 isolates in a synthetic sea-water medium (Hayward, 1968b). Vitamin requirements in algae have been reviewed by Provasoli (1958b, c, 1963a), Droop (1962b), Thomas (1968) and Provasoli and Carlucci (1974).

In marine unicellular algae, loss of ability to synthesize vitamins appears to be restricted to vitamin B₁₂, thiamine and, sometimes, biotin. A higher incidence of auxotrophy occurs in chrysomonads and dinoflagellates than in chlorophytes, with other groups falling somewhere in between. A survey of 28 species of chrysomonads (fresh and salt water) revealed a thiamine requirement for all but 3 species (Provasoli and Carlucci, 1973). Of the coccolithophorids Coccolithus huxleyi needs only thiamine, whereas various strains of Cricosphaera sp. require either thiamine or B₁₂ or both (Pintner and Provasoli, 1963). The absence of consistency of vitamin requirements in taxonomically related groups is apparent. While there is no thiamine requirement in Hymenomonas carterae (Provasoli and Pintner, 1963),
418 4. CULTIVATION OF PLANTS (R. UKELES)

the related *Hymenomonas* (*Syracosphaera*) elongata does have such a requirement (Droop, 1958b).

Among the dinoflagellates some species, e.g., *Gymnodinium breve*, need thiamine (Aldrich, 1962), while *G. splendens* does not (Sweeney, 1954). In one survey, 6 out of 28 species of dinoflagellates showed a requirement for thiamine (Provostoli and Carlucci, 1974). The cryptomonads *Cryptomonas ovalis* and *Cyanophora paradoza* need no thiamine (Provostoli and Pintner, 1953), while *Hemiselmis virescens* (Droop, 1958b), *Chromononas salina* (Antia and co-authors, 1969), *Rhodomonas ovalis* (Iwasaki and co-authors, 1969) and *R. lens* (Provostoli, 1958b) need this vitamin. Thiamine is not considered a general requirement among the chlorophytes although there is a requirement in *Brachiomonas submarina* (Droop, 1961d), *Pyramimonas inconstans* (Provostoli, 1958b) and *Acetabularia mediterranea* (Shephard, 1970). Of the marine littoral diatoms isolated by Lewin and Lewin (1960), 14% required thiamine only, 25% cobalamin only, 7% both, and 54% none.

Biotin is definitely required by only a few dinoflagellates and chrysomonads; possibly, the number of isolates is not sufficient to evaluate the extent of this requirement. Biotin requirement has been demonstrated in the dinoflagellates *Ochromonas marina* (Droop, 1959), *Prorocentrum micans* (Kain and Fogg, 1960), *Amphidinium carteri*, *A. rhyzochelaum* (McLaughlin and Provostoli, 1957), and *Gyrodinium cohni* (Provostoli and Gold, 1962). One cryptomonad, *Rhodomonas ovalis* (Iwasaki and co-authors, 1969), has a biotin requirement, but such a need is not known in the diatoms or marine chrysomonads (Provostoli and Carlucci, 1973).

The requirement for vitamin B₁₂ appears to be more widespread than that for biotin or thiamine. The survey of vitamin requirements prepared by Thomas (1968) shows that vitamin B₁₂ is required by all the dinoflagellates with the exception of *Gyrodinium cohni* (Provostoli and Gold, 1962) and possibly *Gymnodinium simplex*. Thirteen of 15 planktonic diatoms required B₁₂, the 2 exceptions being *Melosira nummuloides* and *Dentonula confervacea* (Guillard and Cassie, 1963). Growth of the supralittoral chrysomonad *Monochrysis lutheri* varied directly as the B₁₂ concentration between 0-1 and 100 μg ml⁻¹, each μg supporting 0-85 x 10⁶ cells (Droop, 1961c). In the chlorophytes requirements for B₁₂ appear more frequently than for biotin or thiamine. Even closely related species may have different vitamin requirements; while *Stichococcus cylindricus* has no requirement for B₁₂ (Provostoli, 1958b), *Stichococcus sp.*, isolated from a marine environment, requires small amounts of the vitamin (R. A. Lewin, 1954).

Parameters of vitamin B₁₂ uptake in *Monochrysis lutheri*, as studied in a chemostat (Chapter 2), revealed certain inconsistencies (Droop, 1966). These were later accounted for by the presence of an inhibitor that binds B₁₂ in supernatants of *M. lutheri*, *Isochrysis galbana* and *Phaeodactylum triahormulatum* blocking B₁₂ uptake in old cultures (Droop, 1968). Most of the vitamin B₁₂ taken up in the first third of the log phase is a rapid luxury consumption; the second phase is slow with steadily declining uptake rates. As a result, the specific growth rate depends on the 'cell vitamin quota' and the effects of vitamin B₁₂ binders, rather than directly on concentration of vitamin in the medium (Droop, 1970).

Vitamin requirements of unicellular algae have been used in assays for assessing the vitamin content of natural waters; examples are *Amphidinium carteri* for
biotin assays and *Monochrysis lutheri* for thiamine assays with the $^{14}$C incorporation technique (CARLUCCI and SILBERNAOEL, 1966, 1967). *Gyrodinium colinii* was also used to assay thiamine (PROVASOLI and GOLD, 1962). Rates of $^{14}$C incorporation after exposure of *Cyclotella nana* to $B_{12}$ are proportional to the vitamin concentration in the medium (GOLD, 1964). *C. nana* has also been used in a direct $B_{12}$ assay based on differences in cell density (RYTHEH and GUILLARD, 1962). Other species were used in direct assay by BELSER (1963). A complication in evaluating assays is that unicellular algae also respond to vitamin analogs. The specificity of the algae towards naturally occurring and synthetic variants of vitamin $B_{12}$ is determined by the nature of the nucleotide that is attached to the cobalt containing a porphyrin-like nucleus. The $B_{12}$-requiring organisms fall into 3 groups: (i) mammalian-like with a benzimidazole type of base in the nucleotide, (ii) *Lactobacillus licheniformis* (or *Euglena* sp.)-type with an adenine-like base, and (iii) *Escherichia coli*-like with a nucleotide-free nucleus. A description of the naturally occurring analogs and the response of assay organisms has been provided by SMITH (1965). A summary tabulated by PROVASOLI and CARLUCCI (1974) reports 36 of 72 species analysed to have a mammalian specificity, 15 a *Lactobacillus* specificity and 23 an *E. coli*-type specificity. GUILLARD (1968) examined the responses of 21 species of marine diatoms to analogs of vitamin $B_{12}$ at ecologically significant levels; he observed that the responses are not all or none but vary continuously, and, therefore, considered the assignment of clones to conventional specificity patterns arbitrary. Much of the information on $B_{12}$ specificity of algae has been summarized by PROVASOLI (1963a), GUILLARD (1968) and PROVASOLI and CARLUCCI (1974).

Variability in vitamin requirements of isolates of the same species was first noted by LEWIN and LEWIN (1960). Some isolates of *Amphora coffeaeformis* were autotrophic, some needed $B_{12}$ and some needed thiamine. GUILLARD (1968) isolated 3 clones of *Coccolithus huxleyi* only 2 of which needed thiamine as a growth factor, and 1 needed $B_{12}$. Complications in determining vitamin requirements also arise from unexpected appearances of vitamins and questionable stability in solution. ROBBINS and co-authors (1953) demonstrated that distilled water on storage may develop appreciable quantities of vitamin $B_{12}$; this explains some reports that $B_{12}$-requiring organisms have been cultivated without the vitamin supplement. Variability in thiamine response may sometimes be due to non-biological destruction of thiamine, as demonstrated by GOLD and co-authors (1968). At 37°C and alkaline pH, total destruction of thiamine takes place in 24 hrs; at 60°C, in 1 hr; hence no growth of *Gyrodinium colinii*, which does not utilize the thiamine moieties. Protective agents for thiamine in sea water at 37°C are organic compounds that chelate trace metals, such as amino acids, citric acid or EDTA (GOLD, 1968). Thiamine solutions were protected on autoclaving if cysteine was present and somewhat less so with glutamic acid or thiourea (MIHAILESCU and SAVOPOL, 1966). Thiamine heated for 1 hr at 60°C was protected in the presence of several organic substances including amino acids (WADA and SUZUKI, 1965). Thiamine added to sea water at its natural pH and autoclaved was not destroyed if the pH of the sea water was reduced to 3.0 to 4.0 before autoclaving or if thiamine-enriched sea water was added to the nutrient concentrate at pH 6.4 to 6.8 (GOLD and co-authors, 1966).

Concentrations of $B_{12}$ in filter-sterilized sea water do not change over a 5-month
storage period when kept at -20°C (Carlucci and Silbernagel, 1966). In a later study, Carlucci and co-authors (1969) noted that ecologically significant concentrations of B₁₂ in filter-sterilized sea water, stored in the dark at 5°C, 18°C, 28°C or 37°C, do not change over a 9-week period although some breakdown of B₁₂ occurs at 37°C. However, under similar conditions, biotin activity generally increased, probably due to decomposition to a more active product. Diffused light tends to inactivate B₁₂ in sea water stored in glass bottles (Kashiwada and co-authors, 1959), and vitamins in sea water are not stable under solar radiation (Carlucci and co-authors, 1969). These authors emphasized the significance of the responses of unicellular algae to vitamin analogs and breakdown products which may be as important as responses to the entire vitamin complex.

The effects of auxins on unicellular algae have not been clearly demonstrated although some positive results on multiplication have been reported. Stimulation of population growth of axenic cultures of Exuviaella sp. was observed with kinetin or gibberellic acid (Iwasaki, 1971). Growth promotion of marine phytoplankton in mixed culture by 6 specific gibberellins was not significant; in fact, slight inhibition was more common (Johnston, 1963a). Gibberellic acid stimulates growth of Gymnodinium breve and shortens the lag period; but high concentrations are inhibitory (Paster and Abbott, 1970). Phaeodactylum tricornutum and G. splendens respond to kinetin with a slight increase in yield, but the chlorophyte Nannochloris oculata is not appreciably affected (Bentley-Mowat and Reid, 1989). The work of Thimann and Beth (1959) showed that gibberellic acid and kinesin enhance the growth of Acetabularia mediterranea and kinesin improves cap formation in this species (Spencer, 1968).

Culture Media

Biphasic culture

The biphasic culture method was a favourite technique in the early phases of microbiology. The nutrients, some of which are complex and undefined, become slowly soluble in another phase. This provides a culture system closely parallel to that found in nature, favours the long survival of cultures because of slow diffusion of nutrients and makes possible the culture of species whose nutritive requirements are undefined.

An old and simple method of biphasic culture (Just, 1928) employs mud and grass or seaweeds placed in a container with an equal amount of sea water added. The jars are covered and placed in subdued light; after a period of decay, an abundant growth of diatoms occurs. The diatoms are removed by filtering through plankton bolting silk and then inoculated in prepared media. Another simple biphasic technique (Pringsheim, 1946a) allows the cultivation of many unicellular algae that could not be cultured previously. A culture vessel containing 3 to 4 cm of dry, sifted garden soil, with or without the addition of nutritive substances, and sea water with the desired salinity added to within 5 cm from the top of the tube is heated for several hours (sterility is not intended). Belcher (1968) cultured Pyramimonas reticulata in biphasic culture and observed that the addition of an organic enrichment, e.g., pea, depressed the growth rate of this flagellate.
CHU's (1946b) biphasic culture method was derived from the earlier works of JACOBSEN (1910) and PRINGSHEIM (1921). Flourishing cultures are obtained by the following biphasic procedure: natural mud is dried, pulverized, wrapped in a layer of linen or filter paper about 2.5 to 4 cm (1 to 1.5 in.) in length, put into the bottom of the test tube, which is plugged with cotton wool, and sterilized at 120°C for 30 mins. Sterilized natural or artificial sea water is poured into the tube, about 2.5 to 4 cm (1 to 1.5 in.) over the mud. An alternative is to sterilize the whole preparation at once at 15-lb pressure for 30 mins or by steaming for 3 to 4 hrs; if bacteria-free cultures are not required, the latter sometimes results in better growth. A crushed grain of wheat or barley sometimes stimulates growth; addition of calcium carbonate is suggested with acid mud.

Another type of biphasic culture (PROVASOLI, 1968) is often used by bacteriologists. A 3% agar medium with nutrients added is poured into tubes to form a 5 to 8 cm (2 to 3 in.) layer, autoclaved and solidified. This is overlayed aseptically with 4 to 5 cm sterile sea water. The slow diffusion of nutrients from the agar layer favours long survival of the cultures.

Media prepared with a liquid and solid phase are traditionally considered biphasic; but perhaps it is not too far-fetched to include also cultures in which the successful growth of one species depends upon the presence of another as also constituting a type of biphasic culture. DROOP (1968) gave an example of such a culture in which Monochrysis lutheri could be grown on a vitamin B₁₂-free medium in the presence of Nannochloris oculata. CARLUCCI and BOWES (1970) observed other examples involving 2 phytoplankters in culture where a source of nutrient is slowly released by one species to the benefit of another.

Soil-extract enrichment of sea water

ALLEN (1914) observed that luxuriant growth of Thalassiosira gravida occurs in artificial sea water only if a small amount of natural sea water or of Ulva latissima extract is added to the medium. This was the first example of applying an organic enrichment. Such enrichments were subsequently applied in many ways to media with both an artificial sea-water and natural sea-water base. PEACE and DRUMMOND (1924) reported results similar to those obtained by ALLEN for Ditylum brightwellii and Nitzschia closterium (Phaeodactylum tricornutum).

The use of soil extract, as an enrichment to stimulate growth of unicellular algae difficult to maintain in inorganic media, was first recorded by PRINGSHEIM (1912) and subsequently used by many other investigators (e.g., FOYN, 1934a; LWOFF and LEDERER, 1935; GROSS, 1937; HARVEY, 1939). FOYN's (1934a) Erdschreiber medium—composed of SCHREIBER's (1927) mineral-enriched sea water with the addition of PRINGSHEIM's soil extract—has probably been the most extensively used marine medium, resulting in the successful culture of many species of flagellates, diatoms and dinoflagellates (consult PROVASOLI and co-authors, 1957, for references to specific species, see also Chapter 5.1). Some examples are given in Table 4-8.

BARKER (1935) was probably the first investigator to cultivate the autotrophic dinoflagellates Proorocentrum micans, P. gracilis, Exuviaella sp., Peridinium sp., Gonyaulax sp. and Ceratium sp. for more than a year in an enriched sea-water medium; he made the observation that soil extract is essential for any repeated sub-
culture over a long period of time. Sweeney (1951) isolated and cultivated dinoflagellates in a similar medium made up of aged sea water, nitrate, phosphate, iron and soil extract.

Prinosheim's (1936) investigations showed the active principle in soil extract to be an acid and alkali stable substance, insoluble in alcohol or ether, absorbed by charcoal and destroyed by hydrogen peroxide; but the mystery of its action was not elucidated. Levrino's (1945) experiments with the centric diatoms Chaetoceros decipiens, Skeletonema costatum and Melosira borreri, and the pennate Nitzschia

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO_3</td>
<td>10 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO_3</td>
<td>10 mg</td>
<td>20 mg</td>
<td>20 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH_4Cl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na_2HPO_4 * 12H_2O</td>
<td>2 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_2HPO_4</td>
<td></td>
<td></td>
<td>3.5 mg</td>
<td>3.5 mg</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td></td>
<td></td>
<td>0.05 mg</td>
<td></td>
</tr>
<tr>
<td>Ferric citrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe (as Cl^-)</td>
<td></td>
<td>0.01 mg</td>
<td>0.1 mg</td>
<td>0.097 mg</td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnCl_2</td>
<td></td>
<td>0.0075 mg</td>
<td>0.0075 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn (as Cl^-)</td>
<td></td>
<td></td>
<td></td>
<td>0.02 mg</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>MgCl_2 * 6H_2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO_3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Na_2SiO_3 * 9H_2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na_2S_2O_3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Na_2S * 9H_2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td></td>
<td></td>
<td>1.0 mg</td>
<td></td>
</tr>
<tr>
<td>B_12</td>
<td></td>
<td></td>
<td></td>
<td>0.1 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Thiamine * HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05 mg</td>
</tr>
<tr>
<td>NaH glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin Mix 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (1 : 20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (OXY L25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypticase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacto-tryptone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast autolysate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil extract</td>
<td>5 ml</td>
<td>2 ml</td>
<td>4 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Sea water</td>
<td>100 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aged sea water</td>
<td>100 ml</td>
<td>100 ml</td>
<td>100 ml</td>
<td>75 ml</td>
<td>95 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td></td>
<td></td>
<td>25 ml</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Table 4-8
Natural sea-water media with soil-extract enrichment
closterium to find replacements for the growth-promoting effects of soil extract led him to conclude that the effects are probably due to activities of soil micro-organisms that form growth-promoting substances similar to algal extracts. Following the 1949 report of Hutner and co-authors that B₁₂ is required by Euglena gracilis for growth, several other micro-algae were found to require this external source of vitamin.

Observations with the marine dinoflagellate Gymnodinium splendens suggested B₁₂ to be the factor in soil required for growth (Sweeney, 1964). In this dinoflagellate, amino acids and yeast extract could not replace the soil extract; yeast extract is poor in B₁₂ (Peeler and co-authors, 1951), while soil extract is rich in

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ASW III</td>
<td>E₃</td>
<td>E₁₃</td>
<td>E₆</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0-01 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0-04 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>50 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>50 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0-1 ml</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1-0 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>25 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>25 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>25 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>25 mg</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2-0 ml</td>
<td>0-5 ml</td>
<td>0-25 ml</td>
<td>0-25 ml</td>
</tr>
<tr>
<td>100 ml</td>
<td>50 ml</td>
<td>75 ml</td>
<td>50 ml</td>
</tr>
<tr>
<td>---</td>
<td>50 ml</td>
<td>25 ml</td>
<td>50 ml</td>
</tr>
</tbody>
</table>
B₁₂ (Robbins and co-authors, 1950). Sweeney (1951) reported that soil extract was not active when freshly prepared, became active after several weeks of storage in the sterile condition and lost activity after 2 to 3 months of storage although preservation by deep-freezing was possible. No consistent change in the vitamin B₁₂ activity of any soil extract with age could be detected; therefore, a change in some other factor must account for the aging effect in the soil extract.

In some cases the stimulating effect of soil extract could not be replaced. Prakash (1967) could not grow several species of Gonyaulax on artificial sea water, including a vitamin mix, but growth was supported by soil-extract enriched sea water. The humic component of soil extract acting as a chelator may decrease toxicity or increase availability of trace metals. Prakash and Rashid (1968) cited evidence that humic substances in small amounts are stimulatory to marine dinoflagellates. Fractions of the humic substances derived from soil organic matter are classified into the following groups based on molecular configurations and solubility characteristics: humins, ulmins, humic, fulvic and hymatomelonic acids. Growth responses to enrichments of artificial sea water with humic, fulvic and hymatomelonic acids, as measured by cell yields and generation times, showed that humic acid gives about twice as much growth as fulvic acid, but with hymatomelonic-acid enrichment dinoflagellates remain viable for only 2 days, with no detectable growth response. In a study on the marine diatom Skeletonema costatum Droop (1962a) found that growth was enhanced with the humic acid fraction far more than with the fulvic acid fraction. Prakash and Rashid (1968) speculated that the stimulation of marine algal cells in cellular metabolic processes occurs in some as yet unknown ways while Droop (1962a) suggested that stimulation arises from benefits derived from the weak chelating bonds offered by the soil fractions. Although soil extract may serve as a source of nutrient trace metals and vitamins, oceanic plankton does not tolerate soil humates in concentrations above 10 to 20 mg l⁻¹ (Droop, 1969).

The method of soil-extract preparation varies among investigators. One method of preparing weak extracts is to add boiled water to soil, extract for some time, filter and sterilize. Another method is to autoclave 1 kg of soil with 1 l of double-distilled water for 5 to 10 mins (may be allowed to settle overnight), filter repeatedly until clear, and sterilize. The method preferred by Droop (1969) is to autoclave 1 l of soil with 1 to 2 l of water and 3 g of NaOH. Extraction is taken to be almost complete when the pH remains above 10 overnight. The supernatant is decanted, filtered clear and freeze-dried.

The enriched medium ASW III (Provazol and co-authors, 1957) was devised to improve on the previous enrichment media (Erdschreiber and Levin's media) with the addition of manganese, iron, glutamic acid, glycine, liver extract and vitamin B mix. The vitamin mix and liver extract were added for a source of known and unknown vitamins and growth factors. The manganese was added to be available for such species as Dunaliella tertiolecta with a high requirement for this metal. The richness of the medium also makes it useful as a sterility test medium. The STP enrichment medium (Provazol and co-authors, 1957) can be used for aseptic additions in small amounts (0.5 to 2.0 ml 10 ml⁻¹) to mineral media to satisfy potentially complex growth requirements of some marine species and, also, as a sterility test medium (Table 4-3).
Media devised by Droop (1954) in the E series are useful for isolating in bacteria-free culture a number of littoral and supralittoral pelagic species. Medium E3 was the base formulation that became further enriched with various organic extracts and peptones in E6 to E13. These media are also useful for sterility testing or for aseptic additions to sterile sea water or to a mineral basal medium.

Inorganic and organic enrichment of natural sea water

Natural sea water as a basal culture medium has numerous advantages over a chemically defined sea-water medium, and with the addition of an identified supplement of nutrients it is also superior to culture media with complex enrichments, as soil extract or organic digests. Where a marine medium is needed in large quantities, enriched natural sea water is less expensive and simpler to assemble than artificial formulations. If natural sea water is enriched with defined compounds, it offers the benefit of a culture medium with a relatively standardized composition for ecological investigations, life-history studies, for providing food cultures and for cytological studies. It is the preferred type of marine culture medium for culturing recent isolates.

Sea water itself, however, has a somewhat poor reputation, often yielding inconsistent experimental results in the laboratory from unknown origin (Johnston, 1963b, 1964) and giving rise to such aphorisms as 'good' and 'bad' water. Problems in achieving consistent results can be partly overcome by using sea water collected offshore where there is little phytoplankton activity and then aged in the laboratory (Sweeney, 1951; Spencer, 1954). Evidence obtained by Aleem (1970) agrees with observations of Spencer (1954) that aging of sea water improves its quality, possibly due to the absorption of toxic metals on the container, but the procedure is not always beneficial. In one case it was reported that the water became infertile for growth of Ditylum brightwellii after storage for 9 months, due to the loss of available manganese (Harvey, 1939). Mineral deficiencies were avoided by Sweeney (1951) through a process in which sea water was aged in darkness for at least 2 months, enriched with mineral nutrient solutions, autoclaved and allowed to stand at least 2 weeks before use. Aging of sea water removes organic traces through bacterial action and prepares a 'standard sea water' for experimental use. The possibility remains that unknown organic factors are still present. One of the positive effects of aged sea water may be a result of bacterial growth which is followed by cell lysis, thus adding growth factors to the water (see also Chapters 2 and 3).

For nutritional studies and vitamin assays, organics must be removed from the natural sea water. Charcoal treatment of sea water (Chapter 2) has been used for this purpose and as an alternative to aging. Gold (1964) prepared sea water for B12 assay by adding activated charcoal (Merck powdered N.F. grade 2 g l⁻¹) to sea water and agitating for 30 mins. The charcoal was filtered off by passing through 2 layers of glass fibre filter paper (Reeve Angel, 934, AH or Millipore HA) and treated water then enriched with known nutrients. Guillard (1968) reported that shaking for 5 to 15 mins with washed, activated charcoal was not adequate, but 

\[ \frac{3}{4} \text{ hr was sufficient to remove vitamin B}_{12} \]. A considerable reduction of organic
matter in sea water can be effected by irradiating with high energy ultra-violet light (ARMSTRONG and co-authors, 1966). HAMILTON and CARLUCCI (1966) investigated the toxicity of irradiated water on Monochrysis lutheri and Skeletonema costatum and concluded that this water could be used in preference to aged or charcoal-treated natural sea water.

Sea water has formed the basis of many media for cultivating marine algae, starting with the simple formulations based on Miquel's solution to the more complex mixes of more recent times. ALLEN and NELSON (1910) enriched natural sea water using as a starting point the Miquel (1892) formula that was used for the growth of freshwater species but eliminating the salts found in natural sea water. In Solution A the active ingredient is the nitrate, Solution B lowers the pH and results in the formation of a precipitate which seems to be essential for good growth, possibly due to the co-precipitation of toxic materials or a reservoir of slowly soluble nutrients. ALLEN and NELSON (1910) were able to culture many species of Rhodophyceae, Mixophyceae and Chlorophyceae on this combination of Solution A and Solution B, as enrichments of natural sea water. Media developed on the basis of Solutions A and B are shown in Table 4-9. The deficiencies of these media are that they form precipitates giving inconsistent results and are generally restricted to the growth of bacterized cultures. With the introduction of metal mixes and vitamin supplements, other media formulations based on natural sea water were developed that minimized precipitates and could be used for growth of many species in axenic cultures. Some of these are shown in Table 4-10.

Media with a high organic enrichment of sea water are only suitable for bacteria-free cultures. Inclusion of organics with a poorly defined composition will often bring about abundant growth of estuarine species, particularly those from polluted areas. Such organic enrichments may also support the growth of species with unknown growth-factor requirements, thus possibly avoiding the loss of strains that could not survive on a more well-defined medium if these factors were omitted. Nevertheless, too high a concentration of organics can inhibit some species and will encourage the growth of bacterial contaminants. Organics are included for specific requirements where necessary and can be used to isolate particular species, e.g., ethionine for Prymnesium parvum (RAHAT and DOR, 1967). Examples of seawater medium enriched with organic compounds are shown in Table 4-11.

Another type of enrichment culture is derived from the concept of utilizing treated and untreated sewage as nutrients for algal culture. GOLUEKE and OSWALD (1962) studied mass cultures of Porphyridium cruentum in a medium consisting of sea water, sewage enriched with urea, chelated iron and some salts. The mixture of sewage and sea water was concentrated to 1/2 volume, filtered through a Seitz apparatus, made up to a specific gravity of 1.03 and received additional supplements. In the sewage were present some growth-promoting factors so that growth was stimulated, as compared to the synthetic sea-water medium of Chu (1949) enriched with ammonium nitrate and B\textsubscript{12} or the natural sea water enriched with urea or ammonium nitrate. In other investigations, ANSELL and co-authors (1963a, b) experimented with mass cultures of Phaeodactylum tricornutum employing sewage effluent as a nutrient source. Sewage effluent was added to give a standard concentration of phosphorus. In some cases the salinity was adjusted by addition of a
salt mixture. Purified sewage effluent with or without salinity adjustment was as
good as inorganic fertilizer for the growth of *P. tricornutum*.

Table 4-9

Sea-water inorganic enrichments of the Miquel type (After Provasoli and co-
authors, 1957; reproduced by permission of L. Provasoli)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
<td>10 g</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>5 g</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1 g</td>
<td>—</td>
<td>1 g</td>
<td>—</td>
<td>1 g</td>
</tr>
<tr>
<td>KNO₃</td>
<td>2 g</td>
<td>20:2 g</td>
<td>2 g</td>
<td>20:2 g</td>
<td>2 g</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>2 g</td>
<td>—</td>
<td>2 g</td>
<td>—</td>
<td>2 g</td>
</tr>
<tr>
<td>KBr</td>
<td>0:2 g</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0:2 g</td>
</tr>
<tr>
<td>KI</td>
<td>0:2 g</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0:1 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>100 g</td>
<td>100 g</td>
<td>100 g</td>
<td>100 g</td>
<td>100 ml</td>
</tr>
<tr>
<td>Quantity of sol. A added to 1 l sea water</td>
<td>40 drops (2 ml)</td>
<td>2 ml</td>
<td>2 ml</td>
<td>0.55 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

**Solution B**

| Component | | | | | |
| MgSO₄ | — | — | — | — | — |
| Na₂HPO₄·12H₂O | 4 g† | 4 g | 4 g | 4 g | 4 g |
| CaCl₂·6H₂O | 4 g‡ | 4 g | 4 g | 4 g | 4 g |
| HCl conc. | 2 ml | 2 ml | 2 g | 2 ml | 1 ml |
| FeCl₃ (melted) | 2 ml | 2 ml | 2 g | 2 ml | — |
| H₂O | 80 ml | 80 ml | 80 ml | 100 ml | 100 ml |
| Quantity of sol. B added to 1 l sea water | 20 drops (1 ml) | 1 ml | 1 ml | 0.5 ml | 1 ml |

* Besides Solutions A and B, Matudaира adds: 1 ml Solution C (ferric chloride 1 g 100 ml⁻¹), 1 ml Solution D (3/20 n NaHCO₃), 0.5 ml Solution E (SiO₂, as Na silicate, 1 g l⁻¹), and 1 ml Solution F (MnCl₂, 0.4 mg; H₃BO₃, 0.4 mg; CuSO₄, 0.02 mg; H₂O, 100 ml) to 80 ml sterile sea water to which Solutions A and B have been added. Sterilize at 90° C for 30 mins, decant off the precipitate before adding Solutions C, D, E, and F.
† Anhydrous.
‡ 'Dry' CaCl₂.

Reconstituted sea-water salts

Culture media prepared with dried sea salts instead of natural sea water as a base offer some of the advantages of both a chemically defined sea-water medium and a natural sea-water medium. The expense and difficulty of compounding synthetic
formulations are avoided; this is especially important if large volumes are needed. The sea salt has a relatively stable composition which is given by the manufacturer (Rila Marine Mix: Rila Products, P.O. Box 114, Teaneck, N.J.; Instant Ocean: Aquarium Systems, Inc., 33208 Lakeland Blvd., Eastlake, Ohio, USA). The dried sea salts can be made up to any desired salinity with distilled, de-ionized or tap water, and enriched with whatever nutrient supplement is necessary. The prepared

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>100 mg</td>
<td></td>
<td>varied</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td></td>
<td>20 mg/mg</td>
<td></td>
<td>20 mg/mg</td>
<td>20 mg/mg</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td></td>
<td></td>
<td>varied</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>20 mg/mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td></td>
<td>varied</td>
<td>3 mg</td>
<td>3 mg</td>
<td>3 mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeCl₃</td>
<td></td>
<td></td>
<td>0.097 mg</td>
<td>1.4 mg</td>
<td></td>
</tr>
<tr>
<td>Ferric citrate</td>
<td></td>
<td>0.24 mg</td>
<td>0.01 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric EDTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnCl₂</td>
<td></td>
<td>0.013 mg</td>
<td>0.0045 mg</td>
<td>0.0075 mg</td>
<td></td>
</tr>
<tr>
<td>MnSO₄·7H₂O</td>
<td></td>
<td></td>
<td></td>
<td>3.38 mg</td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂HAsO₄</td>
<td></td>
<td>0.018 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoCl₂</td>
<td></td>
<td>0.013 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoSO₄·7H₂O</td>
<td></td>
<td></td>
<td></td>
<td>0.014 mg</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td></td>
<td></td>
<td></td>
<td>0.004 mg</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td></td>
<td></td>
<td></td>
<td>0.718 mg</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂SiO₃·9H₂O</td>
<td></td>
<td></td>
<td></td>
<td>28.4 mg</td>
<td></td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td></td>
<td></td>
<td></td>
<td>33.9 mg</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td></td>
<td></td>
<td>1.0 mg</td>
<td>127.3 mg</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td></td>
<td></td>
<td>1.0 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₁₂</td>
<td></td>
<td></td>
<td>1.0 µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine. HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>50 ml</td>
<td>25 ml</td>
<td>25 ml</td>
<td>1000 ml</td>
<td></td>
</tr>
<tr>
<td>Sea water to</td>
<td>1000 ml</td>
<td></td>
<td>1000 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aged sea water</td>
<td></td>
<td>100 ml</td>
<td>75 ml</td>
<td>75 ml</td>
<td></td>
</tr>
</tbody>
</table>
sea salt permits large-scale culture work to be carried out in areas remote from a supply of natural sea water. Another benefit is that it becomes possible to culture species inoculated into open tank vessels without introducing other unwanted phytoplankters (Ukeles, 1965b). The reviewer has cultivated marine chlorophytes, chrysophytes and diatoms in laboratory axenic cultures, as well as indoor and outdoor open tanks in artificial sea water made with Rila Marine Mix. Keck (1964) cultivated several species of Acetabularia on Rila salts, and North and Stephens

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>150 mg</td>
<td>10.0 mg</td>
<td>4.3 mg</td>
<td>250 mg</td>
<td>12.0 mg</td>
<td>310 mg</td>
<td></td>
</tr>
<tr>
<td>8-89 mg</td>
<td>0.6 mg</td>
<td>6.0 mg</td>
<td>20 mg</td>
<td>0.1 mg</td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>0.22 mg</td>
<td>0.51 mg</td>
<td>1.13 mg</td>
<td>0.02 mg</td>
<td>0.3 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.11 mg</td>
<td>0.52 mg</td>
<td>0.014 mg</td>
<td>0.02 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0196 mg</td>
<td>0.1 mg</td>
<td>0.025 mg</td>
<td>0.01 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.044 mg</td>
<td>0.1 mg</td>
<td>0.575 mg</td>
<td>0.04 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-60 mg</td>
<td>100 mg</td>
<td>84 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.012 mg</td>
<td>0.243 mg</td>
<td>0.01 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2 mg</td>
<td>0.37 mg</td>
<td>8.1 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 µg</td>
<td>2.0 µg</td>
<td>1.0 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 µg</td>
<td>1.0 µg</td>
<td>1.0 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2 mg</td>
<td>0.5 mg</td>
<td>25.0 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.0 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 ml</td>
<td>100 ml</td>
<td>1000 ml</td>
<td>1000 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>500 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4-12

Organic enrichments of natural sea water (Compiled from the sources indicated)

<table>
<thead>
<tr>
<th>Source</th>
<th>Enrichment mg l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lewin and Lewin (1960)</strong></td>
<td></td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.002 g</td>
</tr>
<tr>
<td>Na₂SiO₃·9H₂O</td>
<td>0.005 g</td>
</tr>
<tr>
<td>Fe as Fe(NH₄)₂(SO₄)₂·6H₂O</td>
<td>0.05 mg</td>
</tr>
<tr>
<td>Zn as ZnSO₄·7H₂O</td>
<td>0.03 mg</td>
</tr>
<tr>
<td>B as H₃BO₃</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Ca₂(NO₃)₂·7H₂O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Cu₂(SO₄)₃·6H₂O</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Mn₃(III)O₄</td>
<td>0.002 g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Na₂CO₃·9H₂O</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Fe,(NH₄)₂(SO₄)₂·6H₂O</td>
<td>0.05 mg</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.1 µg</td>
</tr>
<tr>
<td><strong>Gold and Baren (1966)</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>23.48 g</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>10.63 g</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>3.92 g</td>
</tr>
<tr>
<td>CaCl₂ (anhyd.)</td>
<td>1.11 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.66 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.19 g</td>
</tr>
<tr>
<td>KBr</td>
<td>0.1 g</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>0.03 g</td>
</tr>
<tr>
<td>SrCl₂·6H₂O</td>
<td>0.04 g</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.07 g</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na₂glycerophosphate</td>
<td>1.53 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>10 g</td>
</tr>
<tr>
<td>K₂PO₄</td>
<td>1500 µg</td>
</tr>
<tr>
<td>Glucose</td>
<td>3000 µg</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1500 µg</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>50 µg</td>
</tr>
<tr>
<td>Biotin</td>
<td>10⁻³ µg</td>
</tr>
<tr>
<td>Tris (hydroxymethyl)</td>
<td>3000 µg</td>
</tr>
<tr>
<td>aminomethane</td>
<td>1 µg</td>
</tr>
<tr>
<td>pH before autoclaving</td>
<td>6.4–6.6</td>
</tr>
<tr>
<td><strong>Provasoli (1968)</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>7 mg</td>
</tr>
<tr>
<td>Na₂glycerophosphate</td>
<td>1 mg</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>10 mg</td>
</tr>
<tr>
<td>Fe₉</td>
<td>5.0 µg</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Fe₉</td>
<td>50 µg</td>
</tr>
<tr>
<td>Complexed</td>
<td>0.34 mg</td>
</tr>
<tr>
<td>EDTA</td>
<td>100 µg</td>
</tr>
<tr>
<td>pH after autoclaving</td>
<td>7.8–7.8</td>
</tr>
<tr>
<td><strong>Starr (in: Aaronson, 1970)</strong></td>
<td></td>
</tr>
<tr>
<td>Sodium acetate·3H₂O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Trypticase</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Aged sea water to</td>
<td>100 ml</td>
</tr>
<tr>
<td>pH after autoclaving</td>
<td>7.8–7.8</td>
</tr>
</tbody>
</table>

(1967, 1971) used Instant Ocean sea salts for the culture of *Platymonas* sp. in studies of amino-acid uptake. Some examples of enrichments used with commercially available sea salts are given in Table 4-12.
Artificial sea water

The requirements for artificial sea-water medium used in cultivation of unicellular algae are that it be chemically defined, that it can be autoclaved, and that it support normal cell growth in subculture indefinitely. Artificial media, satisfying the nutritional requirements of most marine unicellular algae, have been formulated for a number of reasons: (i) to permit research on marine species where natural sea water is not available; (ii) to eliminate the intrinsic complexity and variability encountered in natural sea water; (iii) to develop non-precipitating media capable

### Table 4-12

<table>
<thead>
<tr>
<th>Sea-water media reconstituted from sea salts (Compiled from the sources indicated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KECK (1964)</strong></td>
</tr>
<tr>
<td>Rila Marine Mix</td>
</tr>
<tr>
<td>NaHPO₄</td>
</tr>
<tr>
<td>NaNO₃</td>
</tr>
<tr>
<td>H₂O</td>
</tr>
<tr>
<td><strong>LeWIN and co-authors (1966)</strong></td>
</tr>
<tr>
<td>Rila Marine Mix</td>
</tr>
<tr>
<td>NaNO₃</td>
</tr>
<tr>
<td>Na₂SiO₃·9H₂O</td>
</tr>
<tr>
<td>2 Na₂EDTA</td>
</tr>
<tr>
<td>A trace element mixture to give:</td>
</tr>
<tr>
<td>Fe</td>
</tr>
<tr>
<td>Zn</td>
</tr>
<tr>
<td>B, Cu, Co, Mn, Mo</td>
</tr>
<tr>
<td>Thiamine</td>
</tr>
<tr>
<td>Tryptone (Difco)</td>
</tr>
<tr>
<td>H₂O</td>
</tr>
<tr>
<td><strong>North and Stephens (1971)</strong></td>
</tr>
<tr>
<td>Instant Ocean</td>
</tr>
<tr>
<td>g-at N (as nitrate, glycine, glutamate, or arginine)</td>
</tr>
<tr>
<td>K₂HPO₄</td>
</tr>
<tr>
<td>FeCl₃</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>H₂O</td>
</tr>
<tr>
<td><strong>UKELES (1971)</strong></td>
</tr>
<tr>
<td>Rila Marine Mix</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>NaNO₃</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
</tr>
<tr>
<td>Thiamine·HCl</td>
</tr>
<tr>
<td>Tris</td>
</tr>
<tr>
<td>FeCl₃,6H₂O</td>
</tr>
<tr>
<td>Na₂EDTA</td>
</tr>
<tr>
<td>H₂O</td>
</tr>
</tbody>
</table>
of producing consistent results; (iv) to allow for the capacity to control and evaluate the significance of various chemical components and their interaction.

Allen (1914) recognized the complex nature of sea water. He stated that the goal in the search for a marine culture medium was to formulate an artificial sea-water medium such that the absence or diminution in quantity of any one of the constituents would have a profound effect on growth of the diatoms cultivated. The most interesting result of Allen's work was the observation that dissolving pure chemicals in distilled water, based on the composition of sea water as published by Dittmar (1884), yielded little or no growth of Thalassiosira gravida, unless 1% natural sea water or algal extract was added. In this early work the suggestion was made that cotton plugs not be used and media not be filtered through paper as in both cases injurious substances could be introduced to the medium. Also Dittmar's conclusion proved to be correct, that the difficulty in growing diatoms in artificial sea water was not due to a delicate balance between the different salts, but to the lack of a certain substance that occurs in natural sea water and is essential in very small amounts.

The early investigators used Dittmar's artificial sea water enriched with Miquel's nutrients (Allen, 1914; Peach and Drummond, 1924) or Barker's (1935) artificial sea water with or without soil extract. The artificial sea-water basal medium of McClendon and co-authors (1917) was used by Ryther (1954) for the culture of Nannochloris atomus, Stichococcus cylindricus and Phaeodactylum tricornutum. Chu (1942), Sverdrup and co-authors (1942) and Levrini (1946), following the sea-water analysis of Lyman and Fleming (1940), made up several artificial sea-water media. These early trials on the replacement of sea water with a chemically defined medium were often high in phosphate and citrate but low in calcium and magnesium and did not support growth of many species. With the discovery of the need for vitamins and the wide extent of auxotrophy, soil extract was eliminated by substitution of a known mixture of vitamins, amino acids and a mineral base compatible with high EDTA (Provasoli and Howell, 1952; Provasoli and Pintner, 1953). In the next phase of development the major elements of the Levrini (1946) formula were enriched with a modified trace metal mix, the EDTA level was reduced from 20 to 1 mg% and known mixtures of vitamins were added. The resulting medium, ASP, supported growth of Gyrodinium californicum, Platymonas sp., Syracosphaera carterae and a bacterized Gymnodinium splendens.

Further progress in media development occurred with the introduction of the buffer TRIS (tris(hydroxymethyl)-aminomethane) which minimized precipitation, a chelator (EDTA at 0.5–2 mg%) and lowered Ca and Mg concentrations. For most littoral unicellular algae calcium can be lowered to $\frac{1}{4}$ and Mg to $\frac{3}{4}$ the concentration in sea water. Many littoral species prefer 15% to 20% S, while tropical and pelagic species prefer salinities greater than 30% and an ionic composition similar to sea water. Nitrates, phosphates, vitamins and trace metals were included to satisfy the nutritional requirements of photosynthetic unicellular algae and a source of carbon for colourless species. These developments led to such artificial sea-water media as DC, RC and ASP2 (Provasoli and co-authors, 1957). Several of these media are satisfactory for the growth of all or a large number of algae and—although they do not provide maximum growth in every case—are useful for at least maintaining stock cultures in a uniform growth medium. RC is recommended for
Rhodomonas lens and other forms, like Prorocentrum sp. DC medium may have too much organic content for many species, but there is no precipitation at high phosphorus concentrations due to the solubilizing action of glycerophosphate/lactate; it was developed for Amphora perpusilla. ASP₂ provides the most favourable conditions for a large number of algae; it is of low salinity, low in N and P, and has high levels of chelator/trace-metal mix and vitamins.

The use of a Tris pH buffer in a diluted overchelated trace-metal mix resulted in non-precipitating media, such as ASP, ASP₂, ASP₆, designed and used as all-purpose media for diatoms, cryptomonads, chrysomonads, dinoflagellates, chlorophytes and red algae. These media were derived from DC, MGC and AAC—each developed from studies on a single organism: Amphora perpusilla, Gymnodinium splendens and Amphidinium carteri, respectively. In the reviewer's laboratory, ASP₂ is used as a standard medium for all stock cultures of chlorophytes, chrysophytes, cryptophytes, rhodophytes and diatoms. Another synthetic medium, ASP₆, was developed as a high-salinity medium for osmotically sensitive species. The increase in salinity is obtained by proportionate increases of Na, K, Ca and Mg over that contained in ASP₂. A conventional metal mix is adjusted to the chelator.

ASP₂ has been found to be a good medium for Syracosphaera elongata, Skeletonema costatum, Rhodomonas lens and Amphidinium klebsii.

A common plan recommended for the development of a synthetic sea water is as follows: (i) an artificial sea-water mix of low salinity and low calcium; (ii) a chelated trace-metal solution in the ratio of chelator:trace metal not less than 1 : 1, not more than 3 : 1; (iii) a pH buffering system; (iv) inorganic macronutrients, organic macro and micronutrients as required (PROVASOLI and co-authors, 1957). Medium S 22 developed by Droop (PROVASOLI and co-authors, 1957) is equivalent to about ½ strength sea water, but low in Mg and Ca. The buffering is considered insufficient and some precipitates occur. S 22 was developed for chrysomonads but it is now replaced by S 37, which is similar to S 32 but forms no precipitates and has an efficient buffer system, and organics are added. S 36 is similar to S 22 but with added silicon and thiamine and is considered equivalent to ASP₂ in composition and application.

If a given organism shows a reasonable amount of consistent growth in a given medium, the medium is generally assumed to be adequate. Some investigators consider natural sea water the ideal growth medium for marine plants (Kalle, 1958). McLachlan's (1960) work with Dunaliella tertiolecta, for example, showed that growth was not affected by a considerable variation in the composition of an artificial sea-water medium, but in no case did the growth exceed that of natural sea water. In another study (McLachlan, 1959), it was found that growth of several organisms in an artificial sea-water medium was equal to that obtained in an enriched natural sea-water medium, but several other organisms did not grow as well. McLachlan (1964) compared the population growth of 10 species of unicellular marine algae representing 7 different classes in an enriched sea-water medium with the growth obtained in several artificial marine media. Most species grew better in natural sea water, but addition of 2 mM sodium bicarbonate improved growth rates in artificial media so that results comparable to those obtained in natural enriched sea-water media were recorded. Examples of artificial sea-water media are shown in Table 4-13.
4. CULTIVATION OF PLANTS (R. UKELES)

Table 4-13

Artificial sea water media for cultivating marine unicellular algae

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Formula Details</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYTHER (1954)* for <em>Nannochloris</em> sp. and <em>Stichococcus</em> sp.</td>
<td>NaCl 28.726 g, NH₄Cl 0.053 g, MgSO₄·7H₂O 3.246 g, MgCl₂·6H₂O 2.260 g, CaCl₂ 1.153 g, KBr 0.068 g, NaHCO₃ 0.198 g, Distilled H₂O 1.0 l</td>
<td></td>
</tr>
</tbody>
</table>

*Modified from McCLENDON and co-authors (1917)

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Formula Details</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provasoli and co-authors (1957): ASP for bacteria-free cultures (several species)</td>
<td>NaCl 2.4 g, Vitamin Mix 8* 0.05 ml, MgSO₄·7H₂O 0.6 g, MgCl₂·6H₂O 0.46 g, CaCl₂ 0.06 g, KCl 0.05 g, Na₂EDTA 1.0 mg, Fe (as Cl⁻) 0.01 mg, Zn (as Cl⁻) 0.05 μg, Mn (as Cl⁻) 0.04 mg, Co (as Cl⁻) 0.1 μg, Cu (as Cl⁻) 0.04 μg, B (as H₃BO₃) 0.2 mg, H₂O 100 ml, pH 7.6</td>
<td></td>
</tr>
</tbody>
</table>

* Vitamin Mix 8, 1 ml, contains: thiamine, HCl, 0.1 mg; nicotinic acid, 0.1 mg; putrescine, 2HCl, 0.04 mg; Ca pantothenate, 0.1 mg; riboflavin, 0.05 μg; pyridoxine, 2HCl, 0.04 mg; pyridoxamine, 2HCl, 0.02 mg; p-aminobenzoic acid, 0.01 mg; biotin, 0.5 μg; choline, H₂ citrate, 0.5 mg; inositol, 1.0 mg; thymine, 0.8 mg; orotic acid, 0.26 mg; B₁₂, 0.05 μg; folinic acid, 0.2 μg; folic acid, 2.5 μg.
CHEMICAL ASPECTS

(Compiled from the sources indicated)

**Provasoli and co-authors (1957): ASP for bacteria-free cultures (many different species)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.8 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Ca (as Cl$^-$)</td>
<td>10 mg</td>
</tr>
<tr>
<td>Na$_2$NO$_3$</td>
<td>5 mg</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Na$_2$SiO$_3$.9H$_2$O</td>
<td>15 mg</td>
</tr>
<tr>
<td>Tris</td>
<td>0.1 g</td>
</tr>
<tr>
<td>B$_{12}$</td>
<td>0.2 µg</td>
</tr>
<tr>
<td>Vitamin Mix S3*</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

* Vitamin Mix S3, 1 ml, contains: thiamine.HCl, 0.05 mg; nicotinic acid, 0.01 mg; Ca pantothenate, 0.01 mg; p-aminobenzoic acid, 1.0 µg; biotin, 0.1 µg; inositol, 0.5 mg; folic acid, 0.2 µg; thymine, 0.3 mg.

**Provasoli and co-authors (1957): ASP$_5$ for high-salinity requiring species**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.4 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.8 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.07 g</td>
</tr>
<tr>
<td>Ca (as Cl$^-$)</td>
<td>15 mg</td>
</tr>
<tr>
<td>Na$_2$NO$_3$</td>
<td>30 mg</td>
</tr>
<tr>
<td>K$_2$glycerophosphate</td>
<td>10 mg</td>
</tr>
<tr>
<td>Na$_2$SiO$_3$.9H$_2$O</td>
<td>7 mg</td>
</tr>
<tr>
<td>Tris</td>
<td>0.1 g</td>
</tr>
<tr>
<td>B$_{12}$</td>
<td>0.05 µg</td>
</tr>
<tr>
<td>Vitamin Mix 8*</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

* Vitamin Mix 8 see ASP.

**Provasoli and co-authors (1957): DC for Amphora perpusilla**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.8 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Ca (as Cl$^-$)</td>
<td>10 mg</td>
</tr>
<tr>
<td>Na$_2$NO$_3$</td>
<td>50 mg</td>
</tr>
<tr>
<td>K$_2$glycerophosphate</td>
<td>40 mg</td>
</tr>
<tr>
<td>Na$_2$SiO$_3$.9H$_2$O</td>
<td>20 mg</td>
</tr>
<tr>
<td>Tris</td>
<td>0.5 g</td>
</tr>
<tr>
<td>B$_{12}$</td>
<td>0.3 µg</td>
</tr>
<tr>
<td>Vitamin Mix 8*</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Na lactate</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Na acetate.3H$_2$O</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

* Vitamin Mix 8 see ASP.
### Table 4-13—Continued

**PROVASOLI and co-authors (1957): RC for *Rhodomonas* sp.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.1 g</td>
<td>Na succinate</td>
<td>0.06 g</td>
</tr>
<tr>
<td>MgCl₂ 6H₂O</td>
<td>0.5 g</td>
<td>Na acetate 3H₂O</td>
<td>0.05 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.06 g</td>
<td>NaH glutamate</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Ca (as Cl⁻)</td>
<td>7 mg</td>
<td>Sucrose</td>
<td>0.07 g</td>
</tr>
<tr>
<td>Na₂SO₄ 10H₂O</td>
<td>0.3 g</td>
<td>Na₂ EDTA</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>10 mg</td>
<td>Fe (as Cl⁻)</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1 mg</td>
<td>Zn (as Cl⁻)</td>
<td>0.05 mg</td>
</tr>
<tr>
<td>Tris</td>
<td>0.5 g</td>
<td>Mn (as Cl⁻)</td>
<td>0.04 mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.1 mg</td>
<td>Co (as Cl⁻)</td>
<td>0.1 µg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.06 µg</td>
<td>Cu (as Cl⁻)</td>
<td>0.04 µg</td>
</tr>
<tr>
<td>B₁2</td>
<td>0.1 µg</td>
<td>B₃ (as H₂BO₃)</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Vitamin Mix 8†</td>
<td>0.02 ml</td>
<td>IS Metals*</td>
<td>1.0 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₂O</td>
<td>100 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>7.2–7.4</td>
</tr>
</tbody>
</table>

* IS metals, 1 ml, contains: Sr, 1.3 mg; Al, 0.05 mg; Rb, 0.02 mg; Li, 0.01 mg; I, 0.005 mg; Br, 6.6 mg.
† Vitamin Mix 8 see ASP.

**PROVASOLI and co-authors (1957): S for chrysomonads; S 36 for *Skeletonema* sp.; S 46 for *Hemiselmis* sp.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.5 g</td>
<td>I (as K⁺)</td>
<td>0.002 mg</td>
</tr>
<tr>
<td>MgCl₂ 6H₂O</td>
<td>0.25 g</td>
<td>Na₂ EDTA</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>0.04 g</td>
<td>Fe (as EDTA)</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Ca (as SO₄²⁻)</td>
<td>0.012 g</td>
<td>Zn (as SO₄²⁻)</td>
<td>0.23 mg</td>
</tr>
<tr>
<td>Br (as K⁺)</td>
<td>2.2 mg</td>
<td>Mn (as SO₄²⁻)</td>
<td>0.065 mg</td>
</tr>
<tr>
<td>Sr (as Cl⁻)</td>
<td>0.38 mg</td>
<td>Mo (as NaMoO₄)</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Al (as Cl⁻)</td>
<td>0.0028 mg</td>
<td>Co (as SO₄²⁻)</td>
<td>0.00063 mg</td>
</tr>
<tr>
<td>Rb (as Cl⁻)</td>
<td>0.0061 mg</td>
<td>Cu (as SO₄²⁻)</td>
<td>0.00013 mg</td>
</tr>
<tr>
<td>Li (as Cl⁻)</td>
<td>0.0006 mg</td>
<td>Distilled H₂O to</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

**Enrichments of S:**

<table>
<thead>
<tr>
<th></th>
<th>S 22</th>
<th>S 32</th>
<th>S 36</th>
<th>S 37</th>
<th>S 46</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>50 mg</td>
<td>50 mg</td>
<td>50 mg</td>
<td>50 mg</td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0 mg</td>
<td>1.0 mg</td>
<td>1.0 mg</td>
<td>1.0 mg</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.1 mg</td>
<td>0.1 mg</td>
<td>0.1 mg</td>
<td>0.1 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>B₁₂</td>
<td>10 µg</td>
<td>10 µg</td>
<td>10 µg</td>
<td>10 µg</td>
<td>10 µg</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.0 mg</td>
<td></td>
<td></td>
<td>4.0 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 mg</td>
</tr>
<tr>
<td>Asparagine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 mg</td>
</tr>
<tr>
<td>Guanine</td>
<td>4.0 mg</td>
<td></td>
<td></td>
<td></td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.4 mg</td>
<td></td>
<td></td>
<td></td>
<td>0.4 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 mg</td>
</tr>
<tr>
<td>Na acetate (anhyd.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 mg</td>
</tr>
<tr>
<td>Na₂SiO₃ 9H₂O</td>
<td></td>
<td></td>
<td>10 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### CHEMICAL ASPECTS

Table 4-13—Continued

<table>
<thead>
<tr>
<th>Kain and Fogo (1968a)</th>
<th>AR₄ for Asterionella japonica</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>2:0 mM MgCl₂.6H₂O</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0:2 mM Na₂SO₄</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>5:5 μM CaCl₂</td>
</tr>
<tr>
<td>Na₂SiO₃</td>
<td>100 μM KCl</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>2:5 μM NaHCO₃</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>20 μM KBr</td>
</tr>
<tr>
<td>CoSO₄</td>
<td>0:05 μM SrCl₂.6H₂O</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0:02 μM NaF</td>
</tr>
<tr>
<td>H₂BO₄</td>
<td>970 μM EDTA</td>
</tr>
<tr>
<td>NaCl</td>
<td>401:5 mM Tris</td>
</tr>
</tbody>
</table>

**MCLAUGHLIN and co-authors (1960):** MMK for *Katodinium dorsalisulcum*

| NaCl | 2:5 g Pyridoxine HCl | 1:0 μg |
| MgSO₄.7H₂O | 0:9 g K₂HPO₄ | 1:0 mg |
| KCl | 0:07 g L-Alanine | 50:0 mg |
| Ca (as Cl⁻) | 30:0 mg Na₂ fumarate | 5:0 mg |
| Na₂SiO₃.9H₂O | 1:0 mg D-Ribose | 5:0 mg |
| P-II metal mix* | 3:0 ml Sorbitol | 5:0 mg |
| Nitritotriacetic acid | 2:0 mg Tris | 0:1 g |
| Biotin | 0:1 μg H₂O | 100 ml |
| Thiamine .HCl | 0:01 mg pH adjusted to | 7:4 |

*P-II Metal Mix, 1 ml, contains: EDTA, 1:0 mg; Fe, 0:01 mg; B, 0:2 mg; Mn, 0:04 mg; Zn, 0:006 mg; Ca, 0:001 mg; adjusted to pH 7:5 with NaOH.*

**MCLAUGHLIN and co-authors (1960):** MKD for *Gyrodinium sp., Gymnodinium sp., Melosira sp.*

| NaCl | 2:4 g Pyridoxine HCl | 2:0 μg |
| MgSO₄.7H₂O | 0:9 g Folic acid | 2:0 μg |
| KCl | 0:07 g K₂HPO₄ | 1:0 mg |
| Ca (as Cl⁻) | 30:0 mg Urea | 0:1 mg |
| NaNO₃ | 5:0 mg D-L-Alanine | 5:0 mg |
| Na₂SiO₃.9H₂O | 1:0 mg Na₂ fumarate | 5:0 mg |
| P-II metal mix* | 0:3 ml (NH₄)₂SO₄ | 0:1 mg |
| Nitritotriacetic acid | 20:0 mg D-Ribose | 5:0 mg |
| B₁₂ | 1:0 μg Tris | 0:1 g |
| Biotin | 1:0 μg H₂O | 100 ml |
| Thiamine .HCl | 0:01 mg pH | 7:6-7:8 |

*P-II Metal Mix sea MMK.*
4. CULTIVATION OF PLANTS (R. UKELES)

Table 4-13—Continued

**ALDRICH and WILSON (1960): for Gymnodinium breve**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>29.0 g</td>
<td>KNO₃</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>6.0 g</td>
<td>KNO₃</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>4.5 g</td>
<td>Thiamine</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.7 g</td>
<td>Vitamin B₁₂</td>
<td>1.0 µg</td>
</tr>
<tr>
<td>KCl</td>
<td>0.6 g</td>
<td>Biotin</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Tris</td>
<td>20.0 mg</td>
<td>Sulphide solution*</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Na₂S·9H₂O</td>
<td>3.0 mg</td>
<td>Metal solution†</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0 mg</td>
<td>Triple-distilled H₂O</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

* Sulphide solution, 5 ml, contains: NH₄Cl, 1.0 mg; NaHCO₃, 1.0 mg; Na₂S·9H₂O, 0.8 mg; KH₂PO₄, 0.5 mg; MgCl₂·6H₂O, 0.2 mg.
† Metal solution, 20 ml, contains: EDTA, 3.0 mg; Mn (as MnCl₂·4H₂O), 0.2 mg; Rb (as RbCl), 0.2 mg; Al (as AlCl₃·6H₂O), 0.1 mg; Co (as CoCl₂·6H₂O), 0.1 mg; Ca (as CaCl₂·2H₂O), 0.2 mg; Se (as H₂SeO₃), 0.1 mg; Cr (as K₂Cr₂O₇), 0.1 mg; Mo (as Na₂MoO₄·2H₂O), 0.1 mg; Sr (as SrCl₂·6H₂O), 0.1 mg; Ti (as TiO₂), 0.1 mg; Zn (as ZnCl₂), 0.1 mg; Zr (as ZrOCl₂·6H₂O), 0.1 mg; Ba (as BaCl₂), 0.02 mg; Cd (as CdCl₂·2H₂O), 0.02 mg; Cu (as CuCl₂), 0.02 mg; Fe (as FeCl₃·3H₂O), 0.02 mg; Co (as (NH₄)₂CO(NO₃)₂), 0.02 mg; V (as NH₄VO₃), 0.02 mg; Ni (as NiCl₂·6H₂O), 0.02 mg; Ru (as RuCl₃), 0.02 mg; Sn (as SnCl₂·2H₂O), 0.02 mg.

**DROOP (1962a): S76 for Skeletonema costatum**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>15 g</td>
<td>Mn</td>
<td>0.05 mg</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>2.5 g</td>
<td>Zn</td>
<td>5.0 µg</td>
</tr>
<tr>
<td>CaSO₄·2H₂O</td>
<td>0.5 g</td>
<td>Cu</td>
<td>5.0 µg</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5 g</td>
<td>Co</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Glycylglycine</td>
<td>0.5 g</td>
<td>Br</td>
<td>25 mg</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.25 g</td>
<td>Sr</td>
<td>4.0 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.1 g</td>
<td>Al</td>
<td>3.0 mg</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.01 g</td>
<td>Rb</td>
<td>0.05 mg</td>
</tr>
<tr>
<td>Na₂SiO₃·9H₂O</td>
<td>0.1 g</td>
<td>I</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>B₁₂</td>
<td>0.1 µg</td>
<td>Li</td>
<td>6.0 µg</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>0.05 g</td>
<td>Mo</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Fe</td>
<td>0.5 mg</td>
<td>Distilled H₂O</td>
<td>1 l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH adjusted to</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**PROVASOLI and MCLAUGHLIN (1963): MCC for Gymnionium splendens**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>100.0 mg</td>
<td>Zn (as Cl)</td>
<td>0.015 mg</td>
</tr>
<tr>
<td>Monoethyl PO₄</td>
<td>5.0 mg</td>
<td>Mn (as Cl)</td>
<td>0.12 mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.6 g</td>
<td>Co (as Cl)</td>
<td>0.003 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.4 g</td>
<td>B</td>
<td>0.6 mg</td>
</tr>
<tr>
<td>Ca (as Cl)</td>
<td>10.0 mg</td>
<td>Nitrilotriacetic acid</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>60.0 mg</td>
<td>Tris</td>
<td>100.0 mg</td>
</tr>
<tr>
<td>EDTA</td>
<td>3.0 mg</td>
<td>B₁₂</td>
<td>0.01 µg</td>
</tr>
<tr>
<td>Fe (as Cl)</td>
<td>0.03 mg</td>
<td>pH</td>
<td>7.8-8.0</td>
</tr>
<tr>
<td>Fe (as Si)*</td>
<td>0.2 mg</td>
<td>Distilled H₂O</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

* Sulphosalicylic acid used to dissolve FeSO₄(NH₄)₂SO₄.
**Chemical Aspects**

**Table 4-13—Continued**

<table>
<thead>
<tr>
<th>Provasoli and McLaughlin (1963): AC for <em>Amphidinium cartieri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
</tr>
<tr>
<td>K₂HPO₄</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Ca (as Cl)</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>Fe (as Cl)</td>
</tr>
<tr>
<td>Zn (as Cl)</td>
</tr>
<tr>
<td>Mn (as Cl)</td>
</tr>
</tbody>
</table>

**Provasoli and McLaughlin (1963): for *Gyrodinium californicum***

| KNO₃                     | 10.0 mg | Mo (as Na) | 0.05 mg |
| KH₂PO₄                   | 2.0 mg  | Co (as Cl) | 0.003 mg |
| MgSO₄·7H₂O                | 30.0 mg | Cu (as Cl) | 0.0063 mg |
| MgCl₂·6H₂O                | 0.3 g   | Si Metal mix* | 0.5 ml |
| NaCl                      | 2.4 g   | B           | 0.2 mg   |
| Ca (as Cl)                | 5.0 mg  | NaH glutamate | 2.0 mg |
| KCl                       | 30.0 mg | L-Lysine    | 1.0 mg   |
| EDTA                      | 20.0 mg | L-Leucine   | 0.2 mg   |
| Fe (as Cl)                | 0.3 mg  | B₁₂         | 0.01 µg  |
| Zn (as Cl)                | 0.4 mg  | pH          | 7.6      |
| Mn (as Cl)                | 1.0 mg  | Distilled H₂O | 100 ml |

*Si Metal Mix, 1 ml contains: Sr (as Cl), 1.3 mg; Al (as Cl), 0.05 mg; Rb (as Cl), 0.02 mg; Li (as Cl), 0.02 mg; K (as KI), 0.006 mg; Br (as NaBr), 0.5 mg.*

**Takano (1963, 1964): SC for diatoms**

| NaCl                      | 24.0 g  | Citric acid | 3 mg   |
| MgSO₄·7H₂O                | 8.0 g   | H₃BO₃      | 1.5 mg |
| CaCl₂·2H₂O                | 0.37 g  | MnCl₂·4H₂O | 1 mg   |
| KCl                       | 0.7 g   | ZnSO₄·7H₂O | 22 µg  |
| NaNO₃                    | 300 mg  | CuSO₄·5H₂O | 79 µg  |
| K₂HPO₄                   | 10 mg   | (NH₄)₄MoO₇·4H₂O | 15 µg |
| NaHCO₃                   | 200 mg  | NH₄VO₃     | 23 µg  |
| Na₂SiO₃                  | 100 mg  | CoCl₂·6H₂O | 15 µg  |
| EDTA                      | 10 mg   | EDTA       | 250 µg |
| B₁₂                      | 0.015 µg | Distilled H₂O | 1 l   |
| FeSO₄·7H₂O                | 3 mg    |            |        |

**Swift and Taylor (1966): for *Cricosphaera elongata***

| NaCl                      | 25.0 g  | Cobalamin | 1 µg   |
| MgSO₄·7H₂O                | 5.8 g   | Na₂EDTA   | 30 mg  |
| MgCl₂·6H₂O                | 2.8 g   | FeCl₃·6H₂O | 1.45 mg |
| CaCl₂·2H₂O                | 150 mg  | MnCl₂·4H₂O | 4.32 mg |
| KNO₃                     | 500 mg  | ZnCl₂     | 312 µg |
| Na glycerophosphate      | 30 mg   | CoCl₂·6H₂O | 121 µg |
| H₃BO₃                    | 35 mg   | Distilled H₂O | 1 l   |
| Thiamine·HCl              | 1 mg    |            |        |

*
**Table 4-13—Continued**

LEWIN (1966a): SW for diatoms

<table>
<thead>
<tr>
<th></th>
<th>SW-1</th>
<th>SW-2</th>
<th>SW-1</th>
<th>SW-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>20.0 g</td>
<td>14.5 g</td>
<td>0.1 g</td>
<td>0.1 g</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>1.0 g</td>
<td>6.15 g</td>
<td>1.0 g</td>
<td>1.2 g</td>
</tr>
<tr>
<td>MgCl₂ · 6H₂O</td>
<td>3.0 g</td>
<td>Na₂SiO₃ · 9H₂O</td>
<td>0.9 g</td>
<td>1.8 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1.0 g</td>
<td>Tris</td>
<td>0.25 mg</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>CaCl₂ · 2H₂O</td>
<td>1.0 g</td>
<td>Na₂Tartarate</td>
<td>0.25 mg</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>B₁₂</td>
<td>0.5 mg</td>
<td>MgCl₂ · 6H₂O</td>
<td>0.005 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.5 mg</td>
<td>MnCl₂ · 4H₂O</td>
<td>0.005 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>0.37 g</td>
<td>Mo</td>
<td>0.005 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0.25 g</td>
<td>Zn</td>
<td>0.005 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>K₂HPO₄ · 3H₂O</td>
<td>0.027 g</td>
<td>Distilled H₂O</td>
<td>1 l</td>
<td>1 l</td>
</tr>
<tr>
<td>Na glycerophosphate</td>
<td>0.1 g</td>
<td>pH adjusted to pH</td>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

GOLD and BAREN (1966): for Gyrodinium colinii

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>23.48 g</td>
<td>Na₂glycerophosphate 6H₂O</td>
<td>150 mg</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ · 6H₂O</td>
<td>10.63 g</td>
<td>K₂HPO₄</td>
<td>10 mg</td>
<td></td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>3.92 g</td>
<td>Glucose</td>
<td>3000 mg</td>
<td></td>
</tr>
<tr>
<td>CaCl₂ (anhyd.)</td>
<td>1.11 g</td>
<td>Glutamic acid</td>
<td>1500 mg</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.66 g</td>
<td>(NH₄)₂SO₄</td>
<td>50 mg</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.19 g</td>
<td>Biotin</td>
<td>10⁻³ mg</td>
<td></td>
</tr>
<tr>
<td>KBr</td>
<td>0.1 g</td>
<td>Thiamine</td>
<td>1 mg</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.03 g</td>
<td>Tris</td>
<td>3000 mg</td>
<td></td>
</tr>
<tr>
<td>SrCl₂ · 6H₂O</td>
<td>0.04 g</td>
<td>Distilled H₂O</td>
<td>1 l</td>
<td></td>
</tr>
<tr>
<td>Metal Mix*</td>
<td>3 mg</td>
<td>pH adjusted</td>
<td>6.4⁻6.6</td>
<td></td>
</tr>
<tr>
<td>FeCl₃ · 6H₂O</td>
<td>10 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Metal Mix, 1 ml, contains: Na₂EDTA, 10 mg; FeCl₃ · 6H₂O; 0.5 mg; H₃BO₃, 1.0 mg; MnCl₂ · 4H₂O, 1.5 mg; ZnCl₂, 0.1 mg; CoCl₂ · 6H₂O, 0.05 mg.

BARRAT and DOR (1967): for Prymnesium parvum

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.0 g</td>
<td>NaMoO₄ · 2H₂O</td>
<td>1.0 mg</td>
<td></td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>0.3 g</td>
<td>ZnSO₄ · 7H₂O</td>
<td>15 μg</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>10 mg</td>
<td>CoCl₂ · 6H₂O</td>
<td>0.3 μg</td>
<td></td>
</tr>
<tr>
<td>CaCl₂ · 2H₂O</td>
<td>10 mg</td>
<td>Tris</td>
<td>100 mg</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1 mg</td>
<td>Thiamine · HCl</td>
<td>1 mg</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>5 mg</td>
<td>Vitamin B₁₂</td>
<td>10 μg</td>
<td></td>
</tr>
<tr>
<td>MnCl₂ · 4H₂O</td>
<td>0.5 mg</td>
<td>Distilled H₂O</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>NaNO₃</td>
<td>20 mg</td>
<td>pH</td>
<td>8.2⁻8.4</td>
<td></td>
</tr>
<tr>
<td>FeCl₃ · 6H₂O</td>
<td>0.1 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

YANASE and IMAI (1988): for Monochrysis lutherii, Platymonas sp., Nitzschia closterium, Chaetoceros calcitrans

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>18 mg</td>
<td>Metal Mix*</td>
<td>30 ml</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>600 mg</td>
<td>Fe (as Cl⁻)</td>
<td>100 μg</td>
<td></td>
</tr>
<tr>
<td>NaNO₃</td>
<td>500 mg</td>
<td>Tris</td>
<td>1 g</td>
<td></td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>5 g</td>
<td>Vit. B₁₂</td>
<td>3 μg</td>
<td></td>
</tr>
<tr>
<td>Ca (as Cl⁻)</td>
<td>100 mg</td>
<td>Na₂SiO₃</td>
<td>80 mg</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>30 mg</td>
<td>Vitamin Mix †</td>
<td>1 ml</td>
<td></td>
</tr>
</tbody>
</table>

* Metal Mix, 100 ml, contains: Na₂EDTA, 100 mg; Fe, 1 mg; Zn, 0.5 mg; Mn, 4 mg; Co, 0.01 mg; Cu, 0.004 mg; B, 20 mg.
† Vitamin Mix, 50 ml, contains: B₁₂, 10 μg; biotin, 50 μg; B₁, 5 mg.
Table 4-13—Continued

**Shephard (1969):** for *Acetabularia* sp.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>24 g</td>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>1 mg</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>12 g</td>
<td>FeCl$_3$.6H$_2$O</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>1 g</td>
<td>MnCl$_2$.4H$_2$O</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Tris</td>
<td>1 g</td>
<td>CoCl$_2$.6H$_2$O</td>
<td>2 μg</td>
</tr>
<tr>
<td>KCl</td>
<td>0.75 g</td>
<td>CuSO$_4$.5H$_2$O</td>
<td>2 μg</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>40 mg</td>
<td>Distilled H$_2$O</td>
<td>1 l</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1 mg</td>
<td>Thiamine.HCl*</td>
<td>300 μg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>100 mg</td>
<td>p-aminobenzoate*</td>
<td>20 μg</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>12 mg</td>
<td>Ca-pantothenate*</td>
<td>10 μg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>2 mg</td>
<td>Vitamin B$_{12}$*</td>
<td>4 μg</td>
</tr>
</tbody>
</table>

* These solutions are made up separately and added to the salt solution through a sterilizing membrane filter after autoclaving.

**Riley and Roth (1971):** for wide range of species

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>18 g</td>
<td>NaNO$_3$</td>
<td>0.05 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>5 g</td>
<td>Trace metals*</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>CaCl$_2$.6H$_2$O</td>
<td>0.6 g</td>
<td>Growth factors*</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>KCl</td>
<td>6.0 g</td>
<td>Distilled H$_2$O</td>
<td>1 l</td>
</tr>
<tr>
<td>Na glycerophosphate</td>
<td>0.01 g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Added aseptically following u.v. sterilization.

Trace metals: EDTA (disodium salt), 10 g; nitrilotriacetic acid (NTA), 4 g; ferric-iron (as chloride); 1 g; zinc (as chloride), 60 mg; manganese (as chloride), 800 mg; cobalt (as chloride), 600 μg; copper (as chloride), 240 μg; boron (as boric acid), 1.2 g; redistilled water to 200 ml.

Growth factors: tris-(hydroxymethyl)-methylamine A.R., 100 g; di-potassium hydrogen phosphate A.R., 400 mg; thiamine, 160 mg; nicotinic acid, 20 mg; calcium pantothenate, 20 mg; biotin, 0.2 mg; inositol, 1 g; folic acid, 0.4 mg; thymine, 300 mg; vitamin B$_6$, 8 mg; vitamin B$_{12}$, 0.8 mg; redistilled water to 400 ml.

(c) Physical Aspects

**Illumination**

Photosynthetic species have often been incubated in a north window using sunlight as energy source; but artificial illumination systems are now available that offer special advantages. These devices range from simple bench lighting with artificial light of 300 to 600 W tungsten lamps, sometimes cooled by running water (Warburg, 1919; Hartmann, 1921; Pringsheim, 1926), culture rooms, cabinets or refrigerators electrically wired to accommodate tungsten or fluorescent bulbs, to sophisticated designs for programmed illumination that are commercially available or individually designed. One model (Knaggs, 1968) allows for considerable control over the spectral light characteristics, as well as the irradiance levels of light incident upon the cultures (see also Volume I, Chapter 2). Jitts and co-authors (1964) described an apparatus for exposing phytoplankton cultures to 64 different combinations of light and temperature of known spectral energy resembling sunlight. Other designs for lighting systems and reference to published material are to be found in Downs and Bailey (1967).

The problem with tungsten illumination, to maintain an even distribution of
illuminance at low temperature, was overcome with the introduction of the fluorescent lamp which emits little heat. Artificial light sources have emission spectra not necessarily similar to natural sunlight; each type of lamp has its own particular emission spectrum (Fig. 4-21). Cool-white fluorescent lamps are the most popular, but warm-white and daylight lamps are also used for cultivating unicellular algae. The relative merits of special lamps (Sylvania Gro-Lux, Westinghouse Plant Gro and Duro-Test Corp. Vita-Lite) designed especially for the requirements of photosynthetic plants have not yet been assessed. Quartz-lined iodine vapour filament type lamps are becoming popular for photosynthetic studies; they produce a continuous spectrum with no ultra-violet and an intensity of

Fig. 4-21: Approximate spectral energy distribution of General Electric lamps. (a) White fluorescent; (b) warm-white fluorescent; (c) soft-white fluorescent; (d) cool-white fluorescent; (e) daylight fluorescent; (f) plant light fluorescent. (Data from General Electric.)
photosynthetically active light, about three times as high as that obtained from fluorescent lights (JYTTs and co-authors, 1964).

In most cases illumination is provided so that light falls on the outside of the culture container; however, bulbs are available to fit into the inside of a culture carboy or fermentation vessel. This submerged high-intensity illumination (manufactured by the New Brunswick Scientific Co., New Brunswick, New Jersey, USA) is water jacketed to cool the lamp surface. The light output can be varied by an intensity controller to provide 100 to 6000 W.

Tanks in which the culture depth is limited to 1 or 2 m can be adequately illuminated by sunlight or several overhead fluorescent lamps; however, illuminating a 10-m deep tank presents special problems. STRICKLAND and co-authors (1969) used a 152-cm diameter searchlight with a spectral distribution close to sunlight; they directed this searchlight at a plane mirror above the tank; the mirror was assembled on a steel frame tower that could be tipped at a 47.7° angle with the horizontal. In this way, searchlight and mirror produced a collimated light beam that covered the tank top, and near parallel light passed down the length of the tank (Fig. 4-22).

Light quality may affect growth, reproduction and morphology of the unicellular algae cultivated. BAATZ (1941) reported considerable differences in cell-division rates and morphology when marine diatoms were cultivated under lights of equal energy but different colours (Volume I, p. 141); population growth was significantly higher in green than in blue or red light. In contrast, WALLEN and GEEN (1971a, b) found significant higher growth rates of Cyclotella nana and Dunaliella tertiolecta under blue light and lower growth rates under green light than in white light of the same irradiance. SAXS and LEE (1971) reported that growth of algae from salt marsh communities varied widely with different spectra. One group of three chlorophytes required broad-band white light to maintain growth, but growth of two diatoms (Cylindrotheca closterium and Nitzschia sp.) was repressed.
by white light and increased in individual spectra of blue, green, yellow and red light. Other diatoms, *N. acicularis* and *Amphora* sp., preferred yellow and red light to blue or green and *N. brevisirostra* grew better in blue than in yellow or red light. *Skeletonema costatum* cultivated under 4 different light sources (incandescent, incandescent with reflector lamps—contains 10% more emission in the blue light than in the incandescent lamps—and 2 types of Phillips fluorescent lamps) exhibited no differences in photosynthetic rate (Jørgensen, 1970). Pravasoli (in: Halldal, 1966) observed no differences in the population density of various flagellates when incubated under cool white, deluxe white, daylight or natural white lamps. On the other hand, there are reports that daylight fluorescent lamps are detrimental for algae over a long period (Algoëus, 1951). *Prorocentrum micans* flourished under incandescent illumination but ceased to develop under white fluorescent light after 2 divisions (Kain and Fogg, 1960). Algoëus (1951) attributed inhibition to an incorrect ratio of red-to-blue irradiance; inhibition could be reversed by increasing the red component. Proper irradiance is necessary for maximum toxin production in *Prymnesium parvum* (Daffn and co-authors, 1972) and for nitrate and nitrite assimilation in several other species of unicellular marine algae (Grant and Turner, 1969).

In a comparison of the population growth rates of 6 species of marine unicellular algae at various irradiances (incandescent filamentous or daylight fluorescent), maximum species-specific growth was the same under each artificial light source at saturating irradiances. Under suboptimal irradiances, however, *Dunaliella primolesta* and *Brachiomonas submarina* required higher irradiances from a fluorescent source than from an incandescent source to maintain the growth rate. This was not the case with *Phaeodactylum tricornutum*, *Monochrysis lutheri* and *Chlorella ovalis* (Quraishi and Spencer, 1971). Growth characteristics in relation to illuminances (20 W daylight fluorescent) were similar in any of the four algae studied by Yanase and Imai (1968); growth rate increased rapidly up to 2500 lux and stayed at this value until about 8000 lux in *M. lutheri*, *Prymnesium* sp., *Nitzschia closterium* and *Chrysochromulina calcitrans*.

There is little agreement on the best method of making meaningful measurements of radiant energy (Volume I, Chapter 2). White light is often measured as illumination in foot-candles; but foot-candle meters are corrected to match the sensitivity of the human eye with a peak sensitivity at 555 nm which has no relationship to the absorption characteristics of plants. Units based on the standard candle should be avoided now that instruments for measuring radiant energy are available (Strickland, 1965). Information should be given by stating irradiance in terms of the energy unit langlies per minute (ly min⁻¹ = g cal. cm⁻² min⁻¹) or μW cm⁻²⁻¹, together with a statement of the wavelength of the radiation used and band width if a polychromatic light source is used. Factors have been given for the conversion of candle units to energy units by Strickland (1958) which can vary according to the type of lamp used.

Sometimes, closely related species show variations in response to illuminance. *Dunaliella primolesta* declines from a peak of 1000 ft-c to 2000 ft-c (about 3500 μW cm⁻²⁻¹), while *D. tertiolecta* is much less sensitive to high illuminance (Eppley, 1963). Maximum growth rates of *Phaeodactylum tricornutum* populations occur at 12,000 lux and are reduced only by 12 to 14% at half this illuminance (Hayward,
1968c). Duro (1961c) incubated Monochrysis lutheri under warm-white fluorescent lamps at 2000 lux which is close to saturation; population growth increases 10% at double this illuminance. Population growth of the planktonic diatoms Ditylum brightwellii and Nitzschia turboïdula is limited at low illuminance in a 16-hr light, 8-hr dark regime and D. brightwellii is strongly inhibited in continuous light (Paasche, 1968b). The importance of the night-day cycle has been reviewed by Rabinowitz (1951), Sweeney and Hastings (1962) and others. Optimum illumination for population growth of Porphyridium aerugineum is 400 ft-c; P. cruendum, 800 ft-c; Amphidinium sp., 500 ft-c; Cryptomonas ovata, 1000 ft-c; and N. closterium, 400 to 1100 ft-c as reported in one study (Brown and Richardson, 1968).

Jitts and co-authors (1964) observed a 'stress' response in phytoplankton. Population growth rate was more susceptible to extremes of illumination when cells were near the extremes of temperature, and cells were most susceptible to extremes of both temperature and light if the chemical nature of the medium was not satisfactory. Jones (in: Halldal, 1966) showed an interrelationship of response to illumination and nutrient levels in Nitzschia closterium and Carteria sp.; increase in the nutrient level results in an increased growth response at higher illumination. N. closterium and Tetraselmis sp. exhibit a lower optimum illuminance for maximum growth with low concentrations of nitrate and phosphate than in higher concentrations, where an increased illuminance is required (Maddux and Jones, 1964).

Adaptation to illumination occurs through two mechanisms: In one type, known as the 'Chlorella type', the pigment content changes so that individuals adapted to a higher illuminance have a lower chlorophyll content per cell than those adapted to lower illuminance; in another, the 'Cydolatella type', the pigment component is the same at high and low illuminance, but the actual photosynthetic rate is higher in cells grown at high illuminance (Steemann Nielsen and Jørgensen, 1968; Jørgensen, 1969). The volume of both cells and chloroplast changes with variation in illuminance during growth of several species (Brown and Richardson, 1968).

Temperature

Incubation temperatures for cultivation of marine unicellular algae often range between 15° and 22° C; this range is acceptable for a variety of species (Volume I: Gessner, 1970). Incubation at room temperature in lieu of a temperature-controlled incubator is economical and still in use; however, this practice is not reliable and in direct sunlight may be harmful because of high temperature and abundance of short ultra-violet wavelengths (Butcher, 1951). Incubators in a wide variety of sizes with various degrees of sophistication in programming for light, temperature and humidity are now commercially available. Such culture rooms, incubators or modified refrigerators generally provide a temperature-controlled environment, with illumination of 100 to 500 ft c, that is generally suitable for algae. Further control of temperature and humidity can be provided by placing each small culture dish in a larger Petri dish containing distilled water (Sweeney, 1951). An emergency cutoff should always be included in the light current so that failure of the temperature thermostat does not result in a lethal heat output of the illuminating lamps.
Detailed descriptions are available for the construction of growth chambers and cabinets provided with artificial light, temperature and humidity control (Davies and Hoagland, 1928; Hudson, 1957; Evans, 1959; Hiesy and Milner, 1962; Droop, 1969). Objectives in such constructions should be (i) economy, (ii) flexibility in range of control factors and (iii) independence of individual units. Where several temperature settings are required simultaneously, a multiple series of thermoregulated water baths can be employed (Ukeles, 1961) or, as an alternative, an apparatus consisting of an aluminum block, heated at one end and cooled at the other (Thomas and co-authors, 1963; Jitts and co-authors, 1964). Foco and co-authors (1959) described an apparatus in which a large number of algae could be maintained simultaneously at 4 different temperatures with uniform illumination.

Response to temperature may vary even within one and the same species due to genetic differences, acclimation, concomitant illumination and nutritional circumstances (Volume I, Chapter 4). Planktonic unicellular algae adapt to temperatures primarily by varying the concentration of their pigments, as well as their photosynthetic and other enzymes (Jørgensen, 1968). Uni-algal cultures indicate that there are southern and northern strains of the same species with varying temperature tolerances. While strains of diatoms from South American waters were unable to grow at low temperatures, clones of the same strain from waters around Cape Cod grew well at 3° to 4° C (Hulbert and Guillard, 1968). Species isolated from tropical waters are unable to grow at low temperatures, e.g., a small Gymnodinium sp. failed at 15° C and Chaetoceros sp. and Nannochloris sp. failed at 10° C but not at 15° C to 37° C (Thomas, 1968). A clone of Cyclotella nana from the Sargasso Sea grows at 15° to 25° C but not at 0° to 4° C although clones of the same species taken from more northern waters grow at 4° to 10° C (Guillard and Rytner, 1962).

Since it was observed that the upper temperature limit of the freshwater flagellate Ochromonas malhamensis can be extended from 36° to 38° C by inclusion of extra trace metals, amino acids and vitamins in the medium (Hutner and co-authors, 1957a), nutritional composition of the culture medium must be considered in relation to all reports of temperature tolerances in laboratory cultures. Nutrient-dependent temperature responses have also been observed in marine strains. The optimum temperature for 72-hr assays with Gyrodinium colhii was 30° C and growth was inhibited at 38° C. However, if the basal medium was supplemented with growth factors supplied by natural products (yeast extract, lettuce fusion), which were eventually replaced by amino acid and vitamin B12, this heterotrophic dinoflagellate could be cultured at 35° C with yeast extract or tryptophane and proline (Gold and Baren, 1966). While Kain and Fogo (1958b) reported that exposure of Isochrysis galbana to 30° C for 1 week was not lethal (cells divided normally when returned to a normal temperature of 20° C), Ukeles (1961) found this species to be not viable after exposure to 30° C for 8 days. This difference may be attributable to the inclusion of complex nutritional factors (soil-extract enriched medium), the use of a slightly bacterized culture and a strain adapted to 25° C in the former study; in the latter paper a mineral-enriched sea-water axenic culture and a strain adapted to 20° C was used. In Skeletonema costatum, cultivated under nutrient-deficient conditions, the optimum temperature range for rapid cell division becomes narrower (Curl and McLeod, 1961).

In a continuous culture apparatus, Maddux and Jones (1964) found that the
levels of both temperature (10°-31° C) and light intensity (800-3900 lux) yielding maximum growth rates of *Nitzschia closterium* are higher with higher nutrient concentrations. Schöne (1972) investigated the generation time of *Thalassiosira rotula* using 11 different combinations of temperature and light; high temperature (20° C) combined with light saturation (2400 lux) causes very short chains; low light illuminance (1200 lux) results in long chains. The doubling time varies between 9 hrs (2400 lux, 22° C or 5500 lux, 12° C) and 40 hrs (2400 lux, 40° C or 500 lux, 12° C).

![Graph showing growth rates of various algae](graph.png)

**Fig. 4-23**: Population growth of unicellular algae in laboratory cultures as a function of temperature. Solid black area—no growth; dotted area—population growth less than that at 20-5°C; hatched area—growth equal to or greater than 20-5°C. (Original.)

Effects of temperature on the population growth and physiological responses of laboratory strains of unicellular algae have been studied by Spencer (1954), Ukeles (1961), Guillard and Ryther (1962), and Yanase and Imai (1968). Barker (1935) cultivated dinoflagellates at 18° to 20° C. Temperatures suitable for laboratory cultures of other species in a mineral-enriched natural sea-water medium, and the upper temperature tolerances for population growth are shown in Fig. 4-23 and Table 4-14.
In *Coccolithus huxleyi*, coccolith formation depends on temperature (Watabe and Wilbur, 1966). The percentage of coccolith-forming cells is 2 to 3 times greater between 18° and 24°C (optimum temperature for population growth) than at 7°, 12°, or 27°C; there is a possibility, however, that near the upper or lower temperature limits naked cells might simply outgrow the coccolithus-forming cell types. In a clone in which all cells formed coccoliths (Paasche, 1968a), coccolith calcium production per unit cell proved to be less dependent on temperature (12.5°-28.5°C) than the former report would indicate.

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth*</th>
<th>No growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nitzschia</em> sp. O-17</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td><em>Nitzschia</em> sp. O-1</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td><em>Chaetoceros</em> sp.</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td><em>Cyclotella</em> sp.</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td><em>Dentonula</em> sp.</td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td><em>Pheidactylum tricornutum</em></td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td><em>Syracosphaera carterae</em></td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td><em>Isochrysis galbana</em></td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td><em>Monochrysis lutheri</em></td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td><em>Cryptomonas</em> sp.</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td><em>Platymonas</em> sp.</td>
<td>34</td>
<td>37</td>
</tr>
<tr>
<td><em>Chlamydomonas</em> sp.</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td><em>Carteria</em> sp.</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp.</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td><em>Dunaliella tertiolecta</em></td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td><em>Dunaliella primolecta</em></td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td><em>Pyramimonas</em> sp.</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td><em>Chlorella**tugtlata</em></td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td><em>Chlorella</em> autotrophica</td>
<td>37</td>
<td>41</td>
</tr>
<tr>
<td><em>Stichococcus</em> sp.</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td><em>Nanochloris</em> sp.</td>
<td>37</td>
<td>40</td>
</tr>
</tbody>
</table>

* Population growth in enriched natural sea water.

**Salinity**

The salinity of a marine environment is obviously the most unique requirement to be considered in culture of marine unicellular algae. In general, oceanic salinities range from 33‰ to 37‰; but salinity varies widely in estuaries, tidal pools, areas of high rainfall and extensive runoffs (Volume I: Kalff, 1971). Tolerance to salinity change is generally wide; most neritic marine flagellates can grow in 12‰ to 40‰ S, with an optimum at 20‰ to 24‰ S; but Caribbean and Gulf of Mexico species prefer 33‰ S (Provasoli, 1963a; Volume I: Gessner and Schramm, 1971). The response to culture salinity is not merely a result of osmolality but also a response to dissolved salts in solutions and their particular action on the cell. Droop (1958a) suggested that the response of *Cyclotella nana* to salinity was due to its Na require-
ment. Many marine planktonic algae do not grow well or die when the sea water is diluted more than 50% (Braarud, 1961; Lewin and Guillard, 1963; Pintner and Provasoli, 1963). However, certain unicellular algae will grow in culture solutions whose total ionic content and chemical composition (Droop, 1958b) are different from natural sea water and may grow more rapidly in 1⁄4-strength sea-water media than at 35% S.

The centric diatom Cyclotella nana was found to grow in sea water at salinities ranging from about 15% to 50%, but could also survive at dilutions of 1⁄4 full-strength sea water when the osmotic pressure was adjusted with sucrose. These findings suggest the maintenance of an internal ionic composition, despite variation in composition of the external environment. Hayward (1970) showed that the cellular concentration of cations did not vary if Phaeodactylum tricornutum was cultivated in full-strength or 1⁄4-strength artificial sea water (ASP), although the growth rate during the exponential phase improved in a more dilute medium. According to McLachlan (1960), the flagellate Dunaliella tertiolecta has clearly distinguishable osmotic and ionic requirements. In a medium having the proportions of sea water, growth of D. tertiolecta became first limited by sodium as the total salinity was decreased by dilution. This flagellate could tolerate an osmotic pressure as low as that equivalent to 1⁄10 of sea water, but a sodium requirement not lower than 1⁄50 of sea water (Volume I, Chapter 4).

Among the chrysomonads, Coccolithus huxleyi and Oicosphaera neapolitana are oceanic stenohaline, but Syracosphaera sp., Hymenomonas sp. and Pavlova gyraea are euryhaline. P. gyraea, the most euryhaline representative, lives in brackish water while C. huxleyi is found in the sea and in polluted waters at salinities above 18% (Pintner and Provasoli, 1963). Since the experimental salinities were obtained by varying Na, K, Ca and Mg concentrations (keeping the proportions constant), the metal ions may play a part in these results. Isochrysis galbana also showed a wide tolerance in culture, with no variation in population growth between 15% and 40% S but a reduction in growth at 10% S; this is in keeping with the occurrence of this species in oceanic water and in littoral and estuarine areas. Work on the chrysomonad Monochrysis lutheri has also shown that a distinct salinity optimum does not exist, but there may be preferences for lower salinities (Droop, 1958b, McLachlan, 1961); salinity extremes are more likely to result in decreased growth rates upon increasing light intensities (Craigie, 1969). It has been demonstrated experimentally that this tide-pool organism can rapidly adjust to a new osmotic milieu by adjusting the concentration of free cyclitol (Craigie, 1969).

Cricosphaera species that grow in the laboratory in the motile phase tolerate salinities as low as 4% to 8% (Braarud, 1951; McLachlan, 1961; Pintner and Provasoli, 1963) and as high as 45% to 50% (Braarud, 1951; Pintner and Provasoli, 1963). The non-motile stage can even withstand evaporation to crystallization of sea water, and continuous growth of coccolithophorids has been observed at salinities ranging from 6% to 90% (Boney and Burrows, 1966). Coccolithus huxleyi, however, cannot be cultivated at salinities below 16% or above 45%; the minimum value for optimum culture is 20% to 35% S (Mjaaland, 1956; Guillard, 1963; Pintner and Provasoli, 1963).

Braarud (1951) studied the effect of salinity on the reproductive rate of a cultivated chrysomonad, a cryptomonad and 3 dinoflagellates. He observed four types of
responses that were essentially the same in different sea waters and under different light and temperature conditions: (i) the Syracosphaera response is characterized by a high rate of reproduction in high salinities (25% to 45% S) with an optimum at 30% S; (ii) the cryptomonad response reveals a well-defined optimum at low salinities (15% to 20% S) with a steep decrease in salinities exceeding 35% S; (iii) the Amphidinium response shows growth within a broader range of salinities than the cryptomonad, but also an optimum at low salinities (15% to 20% S); Peridinium trochoideum responds similarly; (iv) the dinoflagellate Exuviaella baltica exhibits a low optimum at 10% S, but a broad range of salinities (10% to 40% S) in which population growth is good. PROVASOLI and McLAUGHLIN (1963) also reported that this organism is euryhaline; growth rates increase in 1/2-strength artificial sea water (ASP₂ and ASP₃). *Gonyaulax catenella* growth was supported in salinities of 21.3% to 35% when adjusted by NaCl additions (CUMMINS and co-authors, 1966). Dinoflagellate salinity tolerances indicate a euryhaline tendency. However, some species have a well-defined optimum: Gymnodinium breve at 27% to 37% S (ALDICH and WILSON, 1960); Amphidinium sp. and *P. trochoideum* at 15% to 20% S (BRAARUD, 1961); *P. balticum* and *P. chattoni* divide only in dilute media (8% to 20% S), but retain motility in sea water. Low-salinity preference has been reported for Eutreptia sp. with optimum salinities between 8% and 17% and for Exuviaella (11% S).

Two genera of chlorophytes, *Stichococcus* and *Chlorella*, are known for the wide range of their habitats. GEORGE (1957) observed that the marine forms of *Stichococcus* grew equally well in fresh or sea water; the three marine strains of *Chlorella* also had no strong preference for sea water compared to fresh water. Girson’s (1956) isolates from a solar evaporation pond used in salt manufacture showed that the *Stichococcus* strain was a brackish-water organism. A strain of *Platymonas* was more resistant to salt but did not grow in highly concentrated solutions. *Dunaliella viridis* and *D. salina* tolerated salinities 6 to 7 times that of sea water; *Stephanopera gracilis* tolerated a salinity 10 times higher than that of sea water but not below twice sea water. Other studies with *Dunaliella* also demonstrated tolerances to wide ranges of salinities. According to McLACHLAN (1960), *D. tertiolecta* can grow in salinities ranging from 3.5% to 120% S; but a narrower salinity range is necessary for maximum pigment synthesis. Fresh water cannot be tolerated, but similar species have been cultivated in a range of fresh water to full-strength sea water (ALLEN, 1956; GEORGE, 1957). In artificial sea water *Platymonas* sp. and *Porphyridium cruentum* exhibit wide salinity-tolerance ranges; relative growth constants do not vary over a range from 4.5% to 35% S, but the chlorophyll content per cell is affected by changes in salinity (McLACHLAN, 1961).

Cultivated *Delonula confervacea* exhibits optimal population growth at salinities from 15% to 30%, irrespective of the light and temperature levels tested. In nature, *D. confervacea* has been found at salinities ranging from about 10% to 34%, indicating general agreement in the salinity response between culture and natural populations (GUILLARD and RYTHER, 1962; SMAYDA, 1969). The physiological differences between clones of *Cyclotella nana* compared roughly to the condition in nature where each clone was obtained (GUILLARD and RYTHER, 1962). Estuarine clones remained virtually unchanged over a range from 0.5% to 35% S. Clones isolated from the Continental Shelf water grew more rapidly at salinities over 8% S.
while Sargasso Sea clones did not survive below 17.5\% S and grew well only at 24\% S. This clone resembles marine species such as *Asterionella japonica* (Kain and Fogg, 1958a), *Thalassiosira decipiens* (Mclachlan, 1961) and *Nitzschia* sp. (Ryther, 1954). Hayward (1968c) studied salinity responses in several isolates of *Phaeodactylum tricornutum* from tidal pools. Cultivation experiments with three isolates showed that a decrease in salinity (artificial sea water) from 52.5\% to 17.5\% S had little effect; but an increase from 52.5\% to 78-80\% S resulted in a steady decline of population growth, except for one isolate that was significantly different in its response at 8.75\% and 35\% S. Droop (1958a) suggested that basic physiological differences between neritic and supralittoral, or estuarine species may rest in the tolerance to Na.

**pH and Eh**

The methods employed to control the pH in an algal culture include aeration with CO₂-enriched air at different partial pressures, use of chemicals that tend to counteract the normal pH variations, and pH buffers added to the culture medium. In an unbuffered medium, utilization of a particular nitrogen source will affect the pH and the availability of certain metals. Nitrate uptake tends to increase the pH which influences the availability of certain ions (Ca, Mg, Fe); utilization of ammonia has the opposite result (Threlfall and Threlfall, 1935); urea effects no alteration in pH. Wherever possible, a judicious use of nitrogen sources will assist in keeping the pH steady. Solters and Lye (1967) described the construction and operation of an apparatus that provides precise pH control in carboys of algal cultures in which output of a laboratory pH meter, with a combination pH electrode inserted in the culture vessel, was used to control the addition of CO₂/air mixtures. The CO₂ additions act as effective buffers when aerating cultures with known CO₂ concentrations, thus controlling the carbonate–bicarbonate buffer system. A similar system in which the pH and Eh were controlled by adjustments in the CO₂ tension and oxygen delivered to the system was described by Dobson and Bullen (1964). The 'dummy'-tube method was devised by Kain and Fogg (1958a) to control pH of the medium during growth of the diatom *Asterionella japonica*. Two series of culture tubes (an experimental and a 'dummy') were prepared at 6 different pH's, each treated in the same way. No attempt was made to keep the 'dummy' tubes sterile, so that the 'dummy' was opened at intervals and the pH of the medium adjusted to its initially set values. The quantity of acid or alkali needed for this adjustment was then added aseptically to the experimental series.

An ideal buffer system should be (i) effective at a moderately low concentration, (ii) non-utilizable and (iii) non-toxic. Phosphate buffers are unsatisfactory for culture media because they are utilized for growth, are needed in concentrations high enough to be toxic (Provasoli and co-authors, 1957) and precipitate with bivalent cations in alkaline medium. The carbonate–bicarbonate buffer system of sea water is useless for autoclaved media because of the loss of large volumes of CO₂ in sterilization. These buffers also develop precipitates on autoclaving although they can probably be used with filter-sterilized media or the pH adjusted by adding the required amount of bicarbonate as a filter-sterilized solution after autoclaving (Swift and Taylor, 1966). Precipitates may or may not be harmful in the medium; in any event it is just about impossible to reproduce the same kind of
precipitate (Provasoli and co-authors, 1957). The addition of natural materials, as soil extract, to media is helpful as it will hinder formation of precipitates in autoclaved media by formation of chelate complexes.

Since the pH rises during autoclaving, a buffer capacity is needed that will be effective at a slightly higher pH than the initial pH at which the medium was adjusted. Thus, for media buffered with glycoglycine (pK₂ 8.1) at pH 8.0, addition of other compounds as valine (pK₂ 9.6), histidine (pK 9.7) or glycine (pK₂ 9.7) would be beneficial (Droop, 1961b). However, there are objections to the inclusion of organic buffers since they can act as chelators and the regulation of pH becomes complicated if the compound is metabolized. Introduction of the amine buffer, tris(hydroxymethyl)-aminomethane (Tris or THAM), has been partially satisfactory and its use in marine media is widespread. It is an effective agent in arresting the alkaline shift to some extent even when adjusted to pH 7.0 at the lower limit of the buffer range. Tris is an amino alcohol, stable at room temperature; it absorbs minimal amounts of CO₂ from air and may be dried by heating at 100°C; in one report it is regarded as unstable at 110°C (Nahas, 1963). Tris is considered non-utilizable for chlorophytes (Wiedeman, 1964), although membrane penetration has been demonstrated (Omachi and co-authors, 1961), and Mahler (1961) found that it could not be considered unreactive in a wide variety of enzymatic reactions. Duggan and co-authors (1964) recommended that the assumption not be made that Tris is an ideal inert buffer but recognition be made of the possible interactions of Tris with enzymes and substrates, particularly where there is a carbonyl group. Some organisms are able to tolerate relatively high Tris concentrations (100 mg/; Provasoli and Mclaughlin, 1963) and none of the 39 isolates (chlorophytes) studied by Smith and Wiedeman (1964) showed harmful effects of Tris buffer; however, Kain and Fogg (1968b), Packer and co-authors (1961), Mclachlan and Gorham (1962) and Mclachlan (1963) have reported toxic effects of Tris, as well as of other amino alcohols on bacteria, algae and protozoans. The inhibition associated with Tris appeared to be rate limiting and may be due at least in part to the reduction in available carbon, as Tris can form complexes with CO₂. Tris may antagonize the uptake of potassium (MacLeod and Onofrey, 1954), thus exaggerating its requirements (Provasoli and co-authors, 1957), and also complexes with silver, aluminium, copper and zinc (Nahas, 1963).

Glycylglycine has been used as a buffer in marine culture media and considered much less toxic than Tris although the expense precludes its use in mass cultures (Droop, 1961b; Mclachlan, 1964). The response of 10 species of unicellular marine algae to three buffers—Tris, glycylglycine, and Tricine (tris(hydroxymethyl)methylglycine) were compared (Mclachlan, 1964). All organisms grew better in glycylglycine than in Tris, with the exception of Dunaliella tertiolecta; Tricine gave no special advantage while being more toxic to some species. Tris has a deficiency as a buffer when a wide range of pH values is to be studied because of its limited buffering range. Therefore, different buffer systems must be used to cover an extended pH range, each buffer having its own individual effect. The advantage of the CO₂-bicarbonate buffer is that a wide range of pH values can be studied with the same buffer system, eliminating the confusion between buffer and pH response.

The observed pH response may be a reflection of the availability of essential components in the media that are altered at certain pH values, or of the chemical
response of cellular constituents to certain pH ranges, or to the buffer constituent itself. Alterations in the medium that occur in certain pH ranges depend upon mass action equations controlling behaviour of heavy metals with chelating agents and the degree of ionization of weak acids and bases, hence utilization and toxicity. The stability of any given chelate depends largely upon pH, and the pH at which the metallic ion is no longer chelated varies from metal to metal. At lower pH values, more chelator can be added or metals reduced; at higher pH's chelators can be reduced since there is a relative reduction in free metal ions with increasing precipitation. A synthetic medium needs to be designed for utilization at a particular pH setting, an approach used by McLaughlin (1958) for growing *Prymnesium parvum* in the acid range. Although marine flagellates are maintained in the laboratory in alkaline media, *Dunaliella tertiolecta* could be adapted to grow in a histidine-buffered medium at pH 6.5 with a rate exceeding the standard artificial sea water medium (EPILEY, 1963). Evidence for effects of the buffer system that are independent of pH is found in observations made by Grant (1968) on nitrogen assimilation by *D. tertiolecta*. While the buffer system had a significant effect on the rate of nitrogen assimilation, the pH did not. Phosphate buffer, in particular, depressed assimilation rates in light. Similar, although much smaller, effects were observed in Tris and bicarbonate buffers.

Most marine species have a wide tolerance to pH in the alkaline range. It has sometimes been observed that the pH in tidal pools may increase as high as pH 10.0. Kylin (1917) studied a wide variety of marine algae in sea water at pH's adjusted to 3.6 to 10.0 and observed that the majority survive for at least a few days in pH 6.8 to 9.6. Bachrach and Lucciardi (1932) reported a narrow pH range for the diatoms *Navicula* sp. and *Nitzschia* sp. cultivated in natural sea water with urea as a nitrogen source. There was no population growth at pH 6.4; growth was poor at pH 7.0 and optimum at pH 8.9; no growth occurred at pH 9.0. Hayward (1938c) experimented with *Phaeodactylum tricornutum* controlling the pH by Tris (2 g l\(^{-1}\)) between pH 6.0 and 10.0, back titrating to the required pH for the lower values. Growth increased from a minimum at pH 6.0 to a maximum at pH 8.0, and in a few instances over pH 9.0. In studies by Gibor (1956) with 0.4 M Tris, only *Stichococcus* sp. and *Platymonas* sp. showed a preference for high pH, while *Dunaliella viridis*, *D. salina* and *Stephanopelis gracilis* were indifferent from pH 7.5 through 9.2.

With the technique of daily adjustments made to the enriched medium by the 'dummy-tube' method, best growth of *Isochrysis galbana* was obtained at pH 8.0 and inhibition occurred over 8.25 (Kain and Fogg, 1958b). The marked inhibition of *I. galbana* in Tris at 8.25 mM led the authors to conclude that a different strain of *I. galbana* was used by Provasoli and co-authors (1967) who found this species to be tolerant to 0.1% (8.25 mM) of the buffer. The red alga *Porphyridium cruentum* grew well in artificial sea water in a wide range of pH values (pH 5.2–8.3), growth rates and maximum population sizes being similar; Tris was used as a buffer above pH 7.0; at lower values, potassium phthalate-NaOH buffer was employed. At all pH values no significant pH changes occurred before and after the experiments (Jones and co-authors, 1963).

Swift and Taylor (1966) employed the CO\(_2\)-bicarbonate buffer system to study growth responses of the unicellular motile coccolithophorid *Cricosphaera elongata* over a wide pH range. Cell-division rate was not related to changes in partial
pressure of CO₂, bicarbonate or carbonate concentration. Optimum pH for cell division occurred at pH 7.8, a value commonly found in the brackish-water supra-littoral pools from which C. elongata was isolated. Most marine and estuarine forms studied in the laboratory have a similar pH tolerance, an optimum at about pH 8.0 and a decrease in growth rate at more acid and alkaline pH values (Bachrach and Lucciardi, 1932; Barker, 1935; Droop, 1955; Kain and Fogg, 1958b; Paasche, 1964). Droop (1955) reported no growth of C. elongata and two other chrysophytes if the initial pH was less than 7.0, but in Swift and Taylor's (1966) experiments there was growth of C. elongata at pH 6.4. According to McLachlin (1958), Prymnesium parvum can grow equally well at pH 6.0 and pH 8.0 only if very high concentrations of trace metals are added to the medium. Lowering the pH of the medium to less than 5.7 removes external coccoliths from the coccolithophorids (Isenberg and co-authors, 1964; Paasche, 1964; Bone and Burrows, 1966; Swift and Taylor, 1966).

With the exception of Droop (1961a, b, 1962a), little attention has been paid to the redox phenomenon in algal culture—perhaps due to the fact that with photosynthesizing algae, media are oxygen saturated. The redox potential in natural sea water is controlled solely by the oxygen system; at pH 8.0 the redox potential is +400 mv (Cooper, 1937b) and is likely to be similar in culture media. The Eh of the medium is depressed 200 to 300 mv by adding 1.0 mg 1⁻¹ of sodium sulphide immediately before inoculation. There is evidence that diatoms benefit from the addition of small amounts of compounds with divalent sulphur or other reducing agents (Harvey, 1939; Matudaïra, 1942; Lewin, 1954). The time interval between autoclaving and inoculation of culture media influences the growth response of Skeletonema costatum (Droop, 1962a) and Cyclotella nana (Guillard and Cassie, 1963). This phenomenon is correlated with the re-entry of oxygen to the medium following autoclaving; the growth response shows a sharp optimum if inoculation occurs about 2 to 3 days after autoclaving; thereafter, growth rate declines. This decline can be reversed by aseptic addition of a small amount of sulphide. Such aging of culture media and the involvement of sulphide clearly indicate a role of oxygen in these responses. There is no reason to consider reducing conditions per se important to the growth response as the diatoms inhabit oxygenated environments. Droop (1962a) concluded that the action of sulphide must lie in the reduction of some component that can only be slowly re-oxidized, such as the ferrie/ferrous equilibrium and, consequently, in the uptake of iron. Another possibility is that there are sulphhydryl groups on the cell surface, significant in active transport, that are sensitive to the redox potential.

(3) Outdoor Cultures

(a) Pool and Tank Cultures

The practice of outdoor mass cultivation must be regarded as a type of agricultural undertaking; like crops, these cultures are subjected to changes in natural conditions, although some control can be managed. For some experimental investigations in situ cultures are considered the only means of evaluating the behaviour
of unicellular algae in nature. For other purposes the goal is to profit from sunlight as an inexpensive energy source in culturing large quantities of algae, or to enrich some natural body of water (produce abundant phytoplankton) and thus increase fish productivity. In recent times it is the general conviction that production of unicellular algae in outdoor cultures is no more difficult than that of other vegetable crops (Oswald and Golueke, 1968a); this is in contrast to the complex production systems envisioned several years ago (Fisher, 1955).

The major considerations in the design of outdoor culture systems are: cost of construction, method of temperature control, provision of nutrients, control of predators and design of units that can withstand meteorological changes. Tamiya (1956) described and compared the performance of 2 types of outdoor cultures: (i) a closed circulation system in which the culture is circulated either in a liquid plastic tube or in a shallow concrete tank covered with plastic; (ii) an open bubbling system in which the culture, contained in an open shallow trench, is aerated with CO₂-enriched air from plastic pipes. The open system was quite durable and required almost no attention, but the closed system was difficult to operate; vinyl sheeting became brittle and developed holes, the plastic tubing was not stable in steady winds breaking at fixed points, the cells dried from the spray on the upper surface of the tube, and precipitation of cells could not be eliminated even when the flow rate was increased. In the open system precipitation could be avoided by increasing the number of aeration tubes and rate of bubbling. In a 2000-I round pool, with a small island at its centre, the culture fluid was circulated by a pump through an underground pipe connected to the two arms extending from the centre to the periphery of the pool. Due to water flow from holes in the arms that rotated slowly (180° min⁻¹), the solution circulated evenly in the pond, stirring and sweeping the algal cells. Complications arose from foreign organisms (bacteria, fungi, protozoans, and rotifers) that appeared in such outdoor cultures. Agents, such as 2,4-dinitro-6-cyclohexyl-phenyl acetate and pentachlorphenyl acetate, were used to inhibit the predators.

Cummins and co-authors (1966) investigated the feasibility of culturing Gonvaelax catenella in outdoor pools. Their outdoor culture system consists of 2 plastic swimming pools (pool liners, clips and outlets all of plastic or rubber), 8 ft (2.4 m) diameter, 2 ft (0.6 m) deep, installed on a level sand base (Fig. 4-24). Convex polyethylene covers provide protection in case of excessive rainfall. Temperature in the two pools is controlled by two mercury thermoregulators placed in 1 master pool. Although conditions in the pools did not support growth of G. catenella, the pools supported growth of several other species, primarily diatoms, that occurred naturally in the sea water of the basal medium. There may have been some inherent toxicity in the plastic construction that was inhibitory to the growth of the more sensitive species (Chapter 7).

In an earlier study, a 3000-gal (11,355 l) cedarwood shallow oval tank was used to cultivate unicellular algae outdoors in glass enclosures (Fig. 4-25; Loosanoff, 1951). To maintain the temperature above freezing during the winter, an automatic oil stove is put into the enclosure, and to supplement light during dark winter days fluorescent lights are installed over the tanks. Sea water is pumped into the tanks and enriched with a type of complete fertilizer. The best fertilizer is a mixture of 5-3-5 and 6-3-6 (ratios of nitrogen, phosphate, potash) in about 1 to 2 g 1000 l⁻¹ sea
water or less (Loosanoff and Engle, 1942). A natural phytoplankton bloom appeared of such species as Chlorella sp., Prorocentrum triangulatum and Nitzschia closterium. Copepod predators were brought under control with organic phosphates and chlorinated hydrocarbons (Loosanoff and co-authors, 1957). The advantages of this type of culture are its simplicity, low cost and ease of maintenance. The dis-

Fig. 4.24: (a) Outdoor culture pools for culturing large populations of unicellular algae; (b) diagram of temperature control system. (After Cummins and co-authors, 1966; modified; reproduced by permission of J. M. Cummins.)

advantages are the sporadic occurrence of predators that rapidly graze the entire algal population, the lack of control of population density or species composition, and the inability of some algae to survive under these conditions.

Raymont and Adams (1958) reported on cultures of Phaeodactylum tricornutum in outdoor painted cement tanks (2400 l volume) partially sterilized by soaking 48 hrs in NaOCl and covered with a sloping roof of 'windowlite' or plastic suspended
POOL AND TANK CULTURES

on wooden frames. No attempt was made to regulate temperature, which was about 25° C over the year, and only natural sunlight or sunlight supplemented with nighttime illumination was used. The culture medium was prepared from coarsely filtered sea water, inorganic phosphorus and nitrogen salts. Ansell and co-authors (1963b) later cultivated \textit{P. tricornutum} in 1000-l concrete tanks, in which sea water was filtered through a plankton net with no attempt at sterilization, and nitrogen and phosphate added as enrichments. The decline of cultures was associated with an increase in the heterotrophic flagellate \textit{Monas} sp. Concrete tanks with 15,000 l sea water were used by Edmondson and Edmondson (1947) to study the dynamics of a natural plankton bloom. Sea water, filtered to remove large animals, was pumped from the adjacent harbour into the tank and enriched with \( \text{KH}_2\text{PO}_4 \) and \( \text{NaNO}_3 \).

![Fig. 4-25: Outdoor culture of \textit{Chlorella} sp. and other species in a wooden vat. (Original.)](image)

At the beginning of the experiment the dominant alga was \textit{Leptocylindricus} sp., later, \textit{Proorocentrum micans} and then \textit{Amphiprora} sp.

While these outdoor mass cultures, employing natural sea water enriched with fertilizer or inorganic nutrients, produced good crops of phytoplankton, there was little control of the species composition (important for some purposes, e.g., feeding of young invertebrate larvae; Chapter 5) and they were all plagued by a heavy predator invasion. To deal with these problems an outdoor culture system was devised that is suitable for northern climates during summer months and for warmer climates all year round (Ukeles, 1965b). In these experiments the growth medium was a commercially prepared sea salt diluted with tap water and enriched with nutritive supplements. This artificial sea-water medium permits some control of the nutritional composition of the medium and minimizes the introduction of grazing
predators and unwanted algae (Figs 4-26 and 4-27). Each tank was inoculated with a dense suspension of the species to be cultivated taken from laboratory cultures brought outdoors or from the previous tank culture. Temperature control was effected by circulating cold water pumped from the nearby bay into an outer tank which served as a water bath for the culture tank. Under these simple and inexpensive conditions the following species were cultivated, harvested as a batch culture, and used for invertebrate feeding: Monochrysis lutheri, Isochrysis galbana, Dunaliella euglena, Phaeodactylum tricornutum, Chlorella autotrophica and C. stigmatophora.

For studies to evaluate growth and reproduction under natural conditions, tanks have several deficiencies in regard to high light penetration, a large surface-to-volume ratio and undesirable temperature changes. Many of these difficulties were overcome by the construction of a deep tank at the Hydraulics Laboratory of Scripps Institution of Oceanography (La Jolla, USA). The tank (3 m in diameter x 10 m high) is constructed of welded steel coated on the inside with a matte-finish black plastic and on the outside with a polyurethane insulating material (Fig. 4-22). Details of the scaffolding stairway, Plexiglas windows, sampling tubes and photo cells have been described by Strickland and co-authors (1969). The sea water (33.4‰ S) used in the tank is filtered through coarse sand and then through a large swimming-pool filter covered with diatomaceous earth; it removes zooplankton and phytoplankton and reduces bacterial counts. The water in the tank can be cooled or heated by submerged heating elements and stirred by a stream of air bubbles introduced at the tank bottom. The sea water is enriched with vitamins, chelated iron, acidified meta-silicate, \( \text{KH}_2\text{PO}_4 \) and \( \text{KNO}_3 \), and inoculated with 130 l of

![Fig. 4-26: Outdoor cultures of unicellular algae in fibreglass tanks. Left sides: valves regulating the supply of cooling water. (Original.)](image)
culture (Ditylum brightwellii, Cachonina niei, or a mixed culture of Phaeocystis sp. and Gonyaulax polyedra).

For practical purposes, large-scale mass cultures require a considerable supply of basic nutrients; hence the combination of mass cultures with waste reclamation has engendered considerable interest. ANSELL and co-authors (1963a) enriched the harbour water used with sewage effluent. Their results, in general, confirm the earlier work of RAYMOND and ADAMS (1958) performed in outdoor battery-jar cultures.

With or without salinity adjustments (sea salts), purified sewage effluent is as good a source of nutrients as inorganic fertilizer. In both studies species other than Phaeodactylum tricornutum were common although in small number, but colourless species, like Monas sp. and Amoeba sp., appeared in large numbers and eventually caused a decline of the culture. According to GOLUBEK and OSWALD (1962), a medium consisting of a mixture of sea water and sewage, enriched with urea and several salts, promotes good rates of growth and reproduction in a mass culture of Porphyridium cruentum.
Another form of inexpensive enrichment is the use of cold nutrient-rich water from the deep ocean. ROELS and co-authors (1971) demonstrated the feasibility of pumping offshore nutrient-rich deep waters through a glass-lined centrifugal pump into concrete-lined tanks onshore, which are then inoculated with *Cyclotella nana*. The phytoplankton bloom is then pumped at controlled rates through production tanks of *Crassostrea virginica* and *Mercenaria mercenaria*. Excellent population growth of *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Dunaliella tertiolecta* and *C. nana* was obtained in water from 500 m or deeper.

(b) In Situ Cultures

Some investigators take the position that *in situ* rather than laboratory cultivation of phytoplankton provides conditions closer to those encountered in nature. IMAI (in: WALNE, 1969) placed a raft of 20-1 polyethylene cans inoculated with phytoplankton in the sea and recorded satisfactory temperature and sunlight penetration to the cultures. STRICKLAND and TERHUNE (1961) studied growth, reproduction and decay processes in mixed plankton populations under *in situ* conditions: they enclosed coastal sea water in a free-floating thin plastic sphere 20 ft (6 m) in diameter, suspended 5-5 m below the sea surface and connected to the atmosphere by a narrow neck (Fig. 4-28). The sea water was taken from near the sea floor, pumped to land, filtered and filled into the plastic bag. It was then inoculated with surrounding sea water (taken from a depth of about 4 m) which was pumped through an all-plastic assembly and strained through 320 μm mesh nylon netting to remove the larger zooplankters. McALLISTER and co-authors (1961) and ANITA and co-authors (1963) described studies on primary production in coastal waters in such plastic spheres.

In another effort to study the culture of unicellular algae under conditions as close as possible to those found in the natural environment, LLOYD and MORRIS (1971) developed an apparatus consisting of a glass tube closed at the top, with a sintered glass filter at the lower end. This is moored beneath the sea surface after inoculation with the micro-algae under examination. Water enters and leaves the tube due to hydrostatic pressure differences created by tides and winds, providing water exchange while not allowing undesirable organisms to enter. After a period of submersion the amount of population growth is estimated.

(c) Pond and Embayment Cultures

Attempts have been made to increase the productivity of coastal areas by fertilization. The ensuing increase in phytoplankton production may be considered an enrichment culture, although the natural, rather than an inoculated, population is expected to increase. The value of fertilizing enclosed or semi-enclosed water bodies to increase the yield of food for fishes is not well understood, and inorganic fertilizers do not always produce a bloom of the expected phytoplankton species. Heavy fertilization poses a danger because of the rapid growth of filamentous algae and heavy blooms that may destroy the entire area (IVERSEN, 1968).

The early attempts to raise the productivity of enclosed or semi-enclosed bodies of sea water by fertilization were directed towards increasing the yield of oysters and
fishes (GaaRDER, 1932; GROSS and co-authors, 1946; GROSS, 1947; MARSHALL, 1947). Experiments on fertilization of Scottish sea lochs have shown that the added nitrates and phosphates disappear from the water paralleled by an increase in phytoplankton. EDMONDSON and EDMONDSON (1947) described the results of their experiments in salt-water fertilization as a tool in the study of food-chain dynamics (Volume IV). Some experiments were carried out in concrete tanks and others in salt-water embayments. Commercial nitrate and phosphate fertilizers added indicate a close correlation between the rate of phosphate utilization and oxygen production. In a salt-water pond selected for fertilization studies by PRATT (1949), commercial fertilizer was applied during periods of small tidal exchange. The artificially increased concentrations of phosphate and nitrate increased the standing crop of phytoplankton to several times the prefertilization maximum after an initial lag of a few days to two weeks. The predominant genera that occurred in the pond

![Diagram of plastic bag used for in situ cultivation of phytoplankters. Cross-section plan. (After STRICKLAND and TERHUNE, 1961; modified; reproduced by permission of American Society of Limnology and Oceanography.)](image)
were as follows in decreasing order of frequency: *Navicula*, *Peridinium*, *Nitzschia*, *Pleurosigma*, *Exuvialia*, *Amphiprora*, *Eutreptia*, *Prorocentrum*, *Skeletonema* and *Chetoceros*.

Experiments in a small salt-water loch, about 18 acres in area, showed that addition of nutrients sometimes causes immediate increase in phytoplankton density (Marshall, 1947; Orr, 1947). Utilization of added fertilizer is extremely rapid and, although nutrients can be temporarily lost to circulation, they are rapidly regenerated. In almost every case and at every depth, addition of fertilizer was followed by increases in number of nannoplankton within 1 to 3 days, but the effect of fertilization on diatoms and dinoflagellates was not as clear. In later experiments, Marshall and Orr (1948) replaced the sodium nitrate and super-phosphate by commercial ammonium sulphate, ammonium nitrate and monoammonium phosphate. The monoammonium phosphate is more readily soluble than the super-phosphate, which probably went to the bottom as a solid. Buljan (1957) first solubilized cheap sources of phosphate (bone meal, crushed phosphate) in sulphuric acid. Super-phosphate and soil sea-water extracts were dissolved in acid and allowed to drain slowly from a moving boat. Blooms of surface algae and increases in the phyto-benthos resulted from this fertilization of a shallow inlet of the Adriatic Sea.

(4) Harvesting

(a) Methods

The economics of a large-scale production of unicellular algae often depend on the efficiency of the harvesting process rather than on the culture itself. Several methods are available. Selection of the most suitable depends on the ultimate use of the algal cells and on the cost and efficiency of the process.

For complete utilization of the nutritive value of the algae cultivated, the most direct harvesting approach is to feed the algae to a herbivorous animal (e.g., crustaceans or fishes) raised for aquacultural purposes (Chapter 5). More often, harvesting of algal populations involves concentrating the cell suspension, perhaps de-watering the slurry and drying the concentrated cells. Methods used for removal of nuisance algae from water systems, such as chemical flocculation, centrifugation, filtration, flotation, ion exchange and sedimentation, can also be applied to harvesting procedures. For industrial algae production flocculation is considered a promising method because it is one of the least expensive and reasonably dependable processes. Although unicellular algae are larger than colloids, they possess many similar surface properties. Discrete algal cells from a stable suspension have a chemically reactive cell surface and a negative surface charge (Ives, 1959). Chemically induced algal flocculation occurs with the addition of cationic poly electrolytes but not with anionic or non-ionic polymers (Cohen and co-authors, 1958; Golseke and Oswald, 1965; Tenney and co-authors, 1969). The degree of flocculation depends upon the extent of coverage of active sites on the cell surface and parameters affecting flocculation (pH, cell concentration, cationic concentration and algal growth phase). A natural process of auto-flocculation, which manifests itself as the clumping and settling of algae, may prove useful. This occurs most often in shallow ponds when, due to algal consumption of CO₂, the pH increases above 9.5.
GOLUEKE and OSWALD (1962) used the technique of removing both cells and dissolved metabolites simultaneously by adding an equal volume of 80 to 90% ethyl alcohol to a culture of *Porphyridium cruentum*. The mixture was stirred vigorously until a stringy coagulum was formed that could be wrapped around a straight wire and removed from the liquid. The water is removed from the algal mass by squeezing it through the fingers or in an appropriate device. In this procedure the initial cost of the alcohol and the cost of recovering it constitute the major expenses of harvesting.

An efficient froth flotation procedure was developed for harvesting algae from dilute suspensions. The cell concentration of the harvest is a function of pH, aeration rate, aerator porosity and height of foam in the aerating column. The economic aspects of the process seem favourable for harvesting mass cultures (LEWIN and co-authors, 1962).

For analytical purposes algal cells may be separated by centrifuging and then dried in a vacuum to constant weight (KETCHUM and co-authors, 1949). Continuous centrifugation in a small Sharples centrifuge, with the stainless-steel collecting cylinder lined with plastic, was found very satisfactory for harvesting cells from small-scale (15–30 l) mass cultures (UKELES, unpublished). OSWALD and GOLUEKE (1968b) described the use of an industrial centrifuge with a 30-in. (75 cm) bowl, which removes 80% of the algae from pond cultures at a rate of 100 gal (378.5 l) min⁻¹. The major problem with industrial centrifugation is the cost of power.

Molecular filters are often used for the collection and staining of fixed algae samples. CLARK and SIGLER (1963) suggest the application of molecular filters for collecting plankton from natural samples or for harvesting from small cultures. Some of the more delicate marine flagellates, however, may be damaged by filtration, as well as by centrifugation techniques. In contrast, the concentrator developed by DODSON and THOMAS (1964) is gentle in its action. It consists of a stiff tube of clear lucite to which the filter (nylon phytoplankton netting) is attached with ethylene dichloride. The tube is dipped slowly into the beaker containing the sample and the water that flows into the tube is removed with a large pipette or by gentle suction. This method is particularly useful for separating large naked dinoflagellates (e.g., species of *Gyrodinium* and *Noctiluca*) or diatoms with spines (e.g., species of *Chaetoceros*).

Preservation and storage of algal suspensions may be required for short or long periods of time. Concentrated algal suspensions (chrysophytes, chlorophytes, diatoms) can be stored for several days at ordinary refrigerator temperatures (+5°C) without destroying the usefulness of the algae as food for molluscan species (UKELES, unpublished). Cells of *Cylindrotheca fusiformis*, inoculated into sea water enriched with succinate or lactate and incubated under a mixture of paraffin and ‘Vaseline’ to exclude air, did not multiply during 2 months; however, upon re-illumination, aerated cells began to multiply immediately and population growth was apparent in 4 days (LEWIN and HELLEBUST, 1970). According to BELCHER (1968), palmella stages of *Prymnesiopsis reticulata* containing many cysts are unaffected by drying at room temperature for 4 days. Some species can withstand long-term storage and be kept in the form of standby stock cultures for many years: *Acetabularia mediterranea* and *A. crenulata*, about 1 mm in length, kept in sterile sea water in the dark with intermittent illumination (3 days/3 months) remained alive for 5 years (KECK,
In a study on pure cultures of unicellular marine algae, Antia and Cheng (1970) showed that 6 species resumed growth in the light after 24 weeks (maximum period tested) storage in the dark. Survival of other species exposed to darkness varied from 3 to 18 weeks.

The silica-gel technique for long-term preservation of Neurospora (Perkins, 1962) may be applicable to algae. Of 20 algal cultures placed on silica gel, 13 were alive after 2 days and 11 after 3½ years. Efforts towards long-term preservation of unicellular algae by lyophilization have been made by Daily and McGuire (1954), Watanabe (1959) and Holm-Hansen (1964). Lyophilized marine species have shown some promise as foods for invertebrates (Hidu and Ukeles, 1962; Brown, 1972) but have otherwise remained unexplored. Hwang (1968) preserved strains under liquid nitrogen.

For commercial application, the algal slurry obtained from centrifugation or coagulation must be dewatered before further drying. The three technically and economically feasible methods are: (i) filtration on an industrial gravity filter; (ii) percolation and evaporation from a sand bed (drying and dewatering combined in one process); (iii) centrifugation in a solid bowl centrifuge. Drum drying is a satisfactory but expensive method. Surface drying in sunlight is very economical. Algae dry quickly when spread about ½-in. (0.62 cm) thick on a lightly oiled surface. The algal chips are raked from the bed and the sand removed by screening. The sand-bed method combines two operations; it is simple and economical. The economics of growing, harvesting and processing of algae have been discussed in detail by Oswald and Golueke (1968b).

(b) Yield Determination

Culture yield can be measured in different ways and often more than one method is used simultaneously. Among the most important methods are determination of cell numbers, dry weight, optical density, ATP, chlorophyll or other pigments, ¹⁴C uptake, and oxygen production. For details on analytical techniques consult Strickland and Parsons (1968).

The method most frequently used to evaluate yield is counting of cell numbers; it can be accomplished in various ways. Several types of counting chambers are available for microscopic enumeration of cell numbers. A sample to be counted is withdrawn from the culture container and fixed with 0·1% formalin, Lugol's solution or solid iodine crystals (the latter offers special benefits as it does not introduce a dilution factor, increases the specific gravity and stains the organism, thus indicating the extent of storage products and cell morphology). The following counting chambers have been used: Petroff-Haussser bacteriological chamber, Sedgwick-Rafter cell, Palmer plankton counting slide, Neuman-Kollowitz cell, Levy haemocytometer blood counting chamber, chambers (16 and 25 mm) for counting in the inverted microscope.

Errors in pipette sampling arise from the fact that unicellular algae vary in specific gravity, may attach themselves to the pipette wall, and motile forms respond to light gradients. A 2-stage sampling method was developed by McAlice (1971), who also analyzed the assumption that individual microscope counts from a single aliquot conform to Poisson distribution. Whipple (1927) used the Sedgwick-Rafter
counting chamber, together with an ocular grid; 1 ml pipetted into the counting chamber is enumerated by strip counting with an accuracy of 5 to 10%. Difficulty in the use of the haemocytometer and Sedgwick-Rafter counting chambers arises if very small cells are counted; the chambers are too deep for high magnification, making it difficult to distinguish small forms from debris or bacteria. Special thin haemocytometer cells are now available for use with phase illumination to overcome this problem but haemocytometers remain deficient because of the small counting chamber. The Palmer counting slide consists of a disc-shaped chamber that holds 0.1 ml of sample, and permits use of a higher magnification than the Sedgwick–Rafter cell. However, the latter is still to be recommended where large organisms are to be counted and a greater volume of sample is desirable (Palmer and Maloney, 1954). Some investigators prefer to suspend culture samples in a Petroff–Hausser bacterial counting chamber because it has a thin slide and allows better illumination (Wood, 1962). Carpenter and co-authors (1972) employed the Utermöhl (1936) technique for cell enumeration, as recommended by Lund and co-authors (1958), in which the preserved phytoplankton is allowed to settle on the glass bottom of a cylindrical vessel and examined from below through an inverted microscope.

Electronic devices, originally designed for automation in counting red blood cells, are now frequently applied to the enumeration of algal cells. The suitability of the counter in determining the number and size of algal cells has been demonstrated in numerous publications (e.g., Hastings and co-authors, 1962; Maloney and co-authors, 1962; El-Sayed and Lee, 1963). Two difficulties are apparent with this apparatus: the medium must be prepared carefully so that it is free of particles other than algal cells and the cells counted cannot be seen. Millipore Filter Co. has recently marketed a counter that visualizes the counted particle, but the cost of the instrument makes it inaccessible to many laboratories. On the plus side is the speed and capability of counting many more cells than would be possible in microscopic counts; in certain models, the automatic cell-size distribution plotters offer additional information.

The dry-weight technique is probably the most reliable in cultures with heavy growth. However, insoluble precipitates and salts that cannot be washed out without damaging the cells present problems for accurate determination. Grant (1968) recommends the following method: centrifuge cells, resuspend the pellet in distilled water, transfer to a tared vessel, dry overnight at 45°C in a vacuum oven and, finally, weigh after storage (24 hrs) over P₂O₅ in a desiccator.

For dilute suspensions, optical density (turbidometric) methods offer both speed and accuracy. Beer's law is not obeyed when the algal suspension has an absorbance greater than 1.0; in such cases the cell suspension is diluted or other counting methods are used. Optical density (OD) is used as an approximate measure of 'population growth'; however, the OD is also influenced by cell size, pigment content and cell particulates. Repeated OD measurements can easily be carried out on the same culture over a period of time if test tubes are selected that fit the cuvette compartment (or special adapter). OD of the experimental tube is read against a blank tube consisting of the same growth medium as the inoculated tube.

As ATP is found in all living organisms, it has been suggested that quantitative determinations of ATP might yield useful biomass and harvesting estimates. It is necessary to know the cellular concentration of ATP relative to such a biomass
criterion as dry weight or organic carbon for a variety of organisms. The cellular content of ATP, relative to the cell size and cellular organic carbon, was investigated by Holm-Hansen (1970) in 24 species of marine unicellular algae. During exponential growth the cellular ATP content remained fairly constant, averaging 0.35% of the cellular organic carbon.

Acceptable estimates of biomass can be found from a determination of chlorophyll or other pigments for which very sensitive fluorometric methods (Yentsch and Menzel, 1963; Holm-Hansen and co-authors, 1965), as well as other analyses (Strickland and Parsons, 1968), are available. One problem is to find suitable factors for converting estimates of plant chlorophyll to plant carbon; ratios of plant carbon to chlorophyll have been assigned specific values (Strickland, 1965).

Culture yield can also be in terms of photosynthesis, 14C uptake or oxygen production, but caution should be exercised since cell-division rates do not necessarily respond to changes in growth conditions in the same way as photosynthesis.

The general advantage of visual counting methods over biochemical and automated procedures for measuring the harvest is that visual observation decreases the possibility of measuring artifacts.
4. CULTIVATION OF PLANTS

4.2 Multicellular Plants

S. Bonotto

(1) Introduction

On land, plants are the most conspicuous and most numerous organisms. They derive their nourishment from carbon dioxide present in the air and from water and dissolved minerals of the soil. In the sea, no plants reveal themselves to the casual observer, except seaweeds on the coasts of continents and islands. Only in rare cases have large plants been seen in the open ocean, e.g. floating Sargassum species in the Sargasso Sea. Marine grasses and mangroves are restricted to specific habitats. These multicellular plants obtain their nutrients mostly from substances dissolved in sea water. They are of ecological importance in littoral zones, but in the overall ecological dynamics of oceans and coastal waters (Volume IV), their role is rather limited. The enormous productivity of the sea (e.g., McConnaughey, 1970) is based on gigantic numbers of unicellular plants, known collectively as phytoplankton (e.g. Orr and Marshall, 1969; Chapter 4.1).

Multicellular marine plants are of significance both for research and for commercial cultivation. They serve as food, directly or indirectly, for a multitude of invertebrates, fishes, birds, and marine mammals. Coastal seaweed forests (e.g. in USA, Japan, Peru) play an important role for spawning and shelter of invertebrates and fishes. Coralline Rhodophyceae species, play also an important role in the formation of atolls and coral reefs (e.g. Tanaka, 1964; Bakus, 1966; Hackett, 1969; Levrini and co-authors, 1969).

(a) Importance of Multicellular Algae for Scientific Purposes

Multicellular marine algae are used for studying biological phenomena, especially in the fields of photobiology, active transport, cellular and molecular biology, biochemistry and biophysics. They are important ecological indicators of local water-movement patterns (Volume I: Schwenke, 1971) and of water quality (Volume V). Hence an increasing number of marine ecologists use multicellular algae as assay organisms for assessing water pollution. Life-cycle studies on multicellular algae have revealed important insights into ontogenetic development and evolutionary genetics.

(b) Importance of Multicellular Algae for Economic Purposes

Food for Man

Most edible seaweeds (species of Laminaria, Porphyra, Undaria, etc.) contain amino acids, carbohydrates, fats, proteins, vitamins, trace elements and an ap-
preciable amount of iodine. Their nutritive value to man, however, is not very high:
some of the carbohydrates cannot be assimilated, due to lack of suitable enzymatic
systems. Nevertheless, since ancient times, a number of marine algae have served
man as direct or indirect source of food. Species of Laminaria and Gracilaria were
used as food by the Chinese several thousand years ago (Dawson, 1966). Species of
the red seaweed Porphyra have been eaten for more than 2000 years in Japan
(Okazaki, 1971), as well as in China, America (by the Indians) and by the inhabi-
tants of Hawaii, Philippines, Malaysia and Indonesia (Dawson, 1966). In European
countries, seaweeds are of much less importance for food. Nevertheless, a few
species (e.g. Chondrus crispus, Rhodymenia palmata, Ulva lactuca) have been, and
still are, eaten—mainly in Iceland, Ireland and Scotland (Hallsson, 1964; Daw-
son, 1966).

Several green, brown and red seaweeds are particularly appreciated as human
food (see the recipes compiled by Pennelly, 1971), for example, species of the red
alga Porphyra, the brown algae Laminaria and Undaria, and the green algae
Enteromorpha and Monostroma (Kurogi, 1963a; Cheng, 1969; Leving and co-
authors, 1969; Haszgawa and Sambonsuga, 1971; Okazaki, 1971). In recent years,
farming of seaweeds has reached considerable proportions involving millions of
people (Dawson, 1966). While the nutritional value of seaweeds lies at present
primarily in their vitamin (Kanazawa, 1963) and mineral contents, future tech-
nical innovations might permit a better utilization of the world’s seaweed re-
sources for human nutrition (Michaene, 1971; Volume V). Enzymatic hydrolysis
of algal polysaccharides (alginate, carrageenan, fucoidan, laminarin, etc.) could
provide simple carbohydrates assimilable by man (Boney, 1965). Sodium alginate
and the propylene glycol ester of alginic acid are widely used in the alimentary
industry (Okazaki, 1971). Several marine algae have been shown to contain
mannitol (Caraes, 1969; Umamaheswara Rao, 1969) and amino acids (Fowden,
1962; Lewis, 1962; Huve and Pellegrini, 1969; Schlichting and Purdom,
1969; Tsuchiya, 1969). Aqueous extracts of seaweeds might thus prove useful for
growing cultures of micro-organisms with a high nutritional value to man. Extracts
of Ecklonia species have been used, for example, to grow yeast cultures (Tomiyasu
and Zentani, 1952). Further research along this line might open promising
prospects for human nutrition.

Food for Animals

Marine algae are used as animal fodder in many coastal areas of the world,
especially in European countries. During World War I, scarcity of grain in Europe
led to the use of seaweeds as food for cows and horses (Verhas, 1925; Dawson
1966). Following the success in Europe, several seaweeds were commercially
processed for animal fodder; at present, seaweed meals are used for cattle, sheep,
hogs and poultry in several countries (Boney, 1965; Dawson, 1966; Nebb and
Jensen, 1966). In some coastal regions of Ireland and Scotland, cattle and sheep
are fed almost exclusively on species of Alaria and Rhodymenia; the milk has not
acquired the taste of algae (Dawson, 1966). Flesh-tainting may follow seaweed
feeding, but disappears after a period of grass feeding (Boney, 1965). Marine algae,
although mainly used for stock feed, can be used for a variety of smaller animals.
Benthic algae may serve as food for invertebrates such as *Gammarus oceanicus* and *Idotea baltica* (e.g. Ravanko, 1969). Improvement of seaweed production by means of modern cultivation techniques will certainly increase their utilization as animal fodder.

**Medicine**

Marine algae and some of their derivatives are used for a number of medical purposes. From ancient times the Chinese utilized species of *Sargassum* and *Laminariales* for treatment of goitre and other glandular diseases (Dawson, 1966). *Gelidium* species were used for stomach disorders and for heat-induced illness, whereas *Laminaria* stipes were employed in surgery (opening of wounds) and in obstetrics (expansion of cervix). Stomach disorders were treated also with ‘agar-agar’, a name of Malay origin which means ‘jelly’. Agar was employed further as a laxative and slenderizing dietetic (Dawson, 1966). It was produced from species of *Gelidium, Gracilaria* and *Pterocladi*a, mainly in China; but later (about 1662), the Japanese took over the production and maintained the monopoly until the Second World War. Since 1881, when Robert Koch discovered that bacteria may be cultivated on agar, it has become very important throughout the world for medical, pharmacological and industrial research laboratories and for hospitals.

Other seaweeds have been employed against human diseases: *Chondrus crispus* (the Irish moss) for treatment of urinary disorders, diarrhoea, chronic pectoral infections, pulmonary distress and as a remedy for consumption; *Corallina officinalis*, and later *Alsidium helminthochorton*, for their vermifuge properties (Chapman, 1950; Dawson, 1966; Levy and co-authors, 1969); more recently, an algal phytocolloid (Algasol T 331) was found beneficial in oncologic therapy (Claudio and Stendardo, 1965, 1966).

A seaweed dietary seems particularly useful in preventing Basedow’s disease. According to Okazaki (1971), in Japan—where marine algae are often used as human food—very few people suffer from this illness. Algal iodine is most probably responsible for this beneficial effect. In *Macrocystis pyrifera* trace elements are probably implicated in countering anaemia (Stefert and Wood, 1958). Seaweeds are believed in Japan to exert beneficial effects against atherosclerosis. Tsuchiya (1968) excellently reviewed his own work and the research performed (mainly in Japan) by other authors on the hypocholesterolemic activities of seaweeds. Unfractionated extract of *Laminaria* species given orally to hypertensive patients effected a lowering in blood pressure. In rabbits, feeding with *Laminaria* species caused hypocholesterolemic and hypotensive effects and prevented the formation of ethromatous plaques of the aorta. Further work on *Laminaria angustata* led to the discovery of a new basic amino acid, laminine [trimethyl-(5-amino-5-carboxypentyl)-ammonium]; laminine depresses the blood pressure in rabbits. Experiments with extracts from *Fucus gardneri*, *Heterochordoria abietina* and *Sargassum muticum* suggest that their hypocholesterolemic power is probably due to highly unsaturated fatty acids. Tsuchiya reported also that some seaweed chlorophyll derivatives, such as phaeophytin and phaeophorbide, have the ability to lower the blood cholesterol level in rats. Moreover, several algae derivatives (alginic acid, carrageenan, iodine-containing protein) were found to be effective in preventing the development of hypercholesterolemia in animals.
The carrageenans, which are naturally occurring sulphated polysaccharides (Anderson, 1959), were the object of many studies. Immunochemical experiments performed on rabbits led to the conclusion that lambda and kappa carrageenans represent two distinct molecular fractions (Johnston and McCandless, 1969). Carrageenans showed anti-ulcer and antisecretory effects in animals (Anderson and Soman, 1963) and were administered in a degraded form to peptic ulcer patients (Anderson, 1969). Carrageenans from Chondrus crispus and Eucheuma spinosum possess also anticoagulant and antilipaemic activities (Murata, 1961; Anderson, 1969). Degraded Eucheuma carrageenan (known as ‘Ebirmar’; average molecular weight about 25,000) was absorbed from the intestinal tract of the guinea-pig, but no gastro-intestinal absorption was found in the case of high molecular-weight (about 800,000) lambda carrageenan (Anderson, 1969).

Some seaweed polysaccharides find many other pharmacological and medical applications. Alginic acid might be used as a tablet disintegrating agent (Gibaldi and Kaciz, 1966) and different alginates were found effective in reducing the intestinal absorption of $^{85}$Sr, $^{137}$Ba and $^{226}$Ra (Skoryna and co-authors, 1966; Skoryna and Tanaka, 1969; Van der Borqht and co-authors, 1971, 1972a; Humphreys and co-authors, 1972).

Alginate treatment is now considered a potentially useful therapeutic against strontium and radium poisoning (Sutton, 1967; Carr and co-authors, 1968; Van der Borqht and co-authors, 1972b). Fucoidan, an acidic polysaccharide prepared from several Fucaceae species, was found to precipitate in vitro more ferrous than ferric iron (Skoryna and Tanaka, 1969). If this property persists in vivo, fucoidan could be useful for clinical treatment of siderosis.

Manure

In coastal areas large marine algae have been used as manure for a long time (Verhas, 1925); this practice has increased in the last decade (Boney, 1965). The seaweeds most often used as manure belong to the brown algae (e.g. species of Ascophyllum, Laminaria, Macrocystis). They may be utilized directly (dug into the soil), composted, or reduced to dried meals. Although dried meals are still marketed by a number of firms, liquid seaweed extracts are more and more preferred (Milton, 1964; Challen and Hemingway, 1966).

Liquid products are marketed as ‘Maxicrop’, ‘Baby Bio’, ‘Biohumus’, ‘Marinure’, ‘Seahorn’, ‘Seahorse’, ‘SM-3’, ‘Trident’ or ‘Algifert’ (Stephelson, 1966; Booth, 1969; Povolny, 1969); they can be used as direct soil dressing or applied as foliar sprays (Skelton and Senn, 1969). Seaweed fertilizers supply trace elements (Booth, 1964) and are particularly convenient for poor or alkaline soils where deficiency diseases are observed (Myklestad, 1964; Booth, 1969). Direct soil dressing improves plant-root growth and increases both crumbling and water-retaining properties of the soil (McDowell, 1966; Blunden and Woods, 1969). The success in the application of seaweeds depends on their manurial value, which is not the same for all species used, and on the properties of the soil treated (Boney, 1967).

The manurial value of a seaweed is based on its content not only in mineral elements but also in organic matter (Francki, 1958; Blunden and Woods, 1969): liquid
seaweed extracts supply to the soil minerals as well as organic substances. They increase growth and metabolic activity in several plants, enhance frost resistance and induce resistance to fungal and insect pests (Booth, 1966, 1969; Challen and Hemingway, 1966; Stephelson, 1966; Blunden and Woods, 1969; Povolny, 1969; Senn and Skelton, 1969; Sælton and Senn, 1969). Recent work by Senn and Skelton (1969) and by Brain and co-authors (1973) suggests that many of the metabolic stimulations observed in plants treated with seaweed extracts may be due to growth regulators which occur in several species of marine algae (Mowat, 1963, 1964; Moss, 1965; Buigeln and Craig, 1971). Brain and co-authors (1973) demonstrated a cytokinin activity in a commercial aqueous seaweed extract (SM-3, Chase Organics) prepared from species of Laminariaceae and Fucaceae.

Inhibitory effects on plants by some carbohydrate derivatives commonly found in seaweed extracts (i.e., alginate, carrageenan, laminaran) were reported by Blunden and Woods (1969). But mannitol, which is present in appreciable amounts in brown algae (Caraës, 1969), had a beneficial effect on plants (Blunden and Woods, 1969). Further research on the effects of seaweed extracts on plants is required before safe conclusions on their mechanism of action may be reached.

Industrial Uses

Seaweed utilization for many different purposes led to industrialization of their production. Although large amounts of algae are still collected manually by fishermen, mechanical harvesting methods have undergone rapid development. In California, *Macrocystis pyrifera* is harvested by a motor-driven barge equipped with a machine which cuts the kelp about 1 m below the water surface and collects the cut material (Dawson, 1966). In Canada, large amounts of *Chondrus crispus* are storm-tossed on the beaches; a mechanical system has been developed for separating unwanted material (sand, stones, debris, dust, etc.) from the seaweed (Johnston, 1969).

Seaweeds collected on beaches or cut in the sea are now used in many different countries for industrial purposes (Levrince and co-authors, 1969). In Europe, seaweeds were used for the industrial production of 'kelp', ash rich in soda and potash obtained from burning different plants (e.g. species of *Ascophyllum*, *Chorda*, *Fucus*, *Himanthalia*, *Laminaria*, *Saccorhiza*). In non-European countries, the term 'kelp' has been applied to the large brown alga. Soda and potash, initially obtained mainly in Britain from seaweeds, were later substituted by a higher quality soda obtained from *Salicornia* species. Similarly, the production of seaweed iodine in Europe was discontinued when iodine from Chilean nitrate sources became available. Only in Japan and China is seaweed iodine still produced for commercial uses (Dawson, 1966; Cheng, 1969).

The seaweed industry has known ups and downs during the last 100 years (Levrince and co-authors, 1969). It became particularly important during World Wars I and II, when seaweeds were utilized for a number of purposes. At present, seaweed utilization continues in an economically satisfactory way and it is expected to increase in the future as numerous kelp beds in different parts of the world are still unexploited. Most seaweeds of industrial interest are listed below. Particularly important are numerous polysaccharides, extracted from brown and red sea-
4. CULTIVATION OF PLANTS (S. BONOTTO)

weeds: agar, agaroid, alginate, carrageenan, fucoidan, funorin, iridophycinate, etc. The practical applications of these algal derivatives in many fields of modern industry have received detailed attention from CHAPMAN (1950), ANDERSEN (1964), MACFARLANE (1964), BONEY (1965), DAWSON (1966), GORDON and co-authors (1966), KRISHNAMURTHY (1967), IVERSEN (1968), LEVRINO and co-authors (1969), VENKATARAN (1969) and OKAZAKI (1971).

(c) Importance of Spermatophyta (Sea Grasses, Mangroves and Salt-marsh Plants)

Some angiosperms have successfully occupied marine habitats. As they usually occur in areas seldom visited by 'terrestrial botanists', only a few specialists have thus far paid attention to them. According to Dawson (1966), a more thorough exploration of marine habitats will probably reveal additional species; in muddy bays of Pacific Central America he found two new species in 1953. Most of these plants are of ecological importance in coastal areas.

(2) Cultivation of Multicellular Algae in Nature and Under Laboratory Conditions

(a) Cultivation in Nature

Seaweed cultivation in nature began some 300 years ago (KURIGI, 1963a; OKAZAKI, 1971) and has been improved considerably in the last 50 years by new techniques. Technological progress was due mainly to the discovery of new material and to investigations in the field and in the laboratory. Field studies have shown that the growth of several seaweeds (Ascophyllum nodosum, Fucus vesiculosus, Himanthalia elongata, Laminaria cloustonii, L. digitata, L. saccharina and Pelvetia canaliculata) varies in an 11-year cycle, which seems related to the well-known 11-year cycle in sunspot activity (WALKER, 1956; ARDRÉ, 1969, 1970). Although of ecological interest, such potential correlation has, at present, no practical value for commercial seaweed cultivation.

A variety of methods have been employed to cultivate green, red and brown seaweeds (see below). Man has succeeded in farming suitable areas of the sea. Efforts have been made also to improve cropping in poor grounds by fertilization (CHENG, 1969; YAMADA, 1972). The utilization of manure is at present widely in practice in China for the cultivation of Laminaria japonica (CHENG, 1969) and in Japan for the cultivation of species of Gelidium, Laminaria, Porphyra and Undaria (YAMADA, 1972). Floating or fixed rafts are used to extend culture grounds into the open sea (OKAZAKI, 1971; JAMISON and BESWICK, 1972). Prevention or control of diseases (Chapter 8) will further increase annual harvests. Additional studies are required to improve our basic knowledge on nutritional requirements, growth promotion and diseases of multicellular algae.

(b) Cultivation under Laboratory Conditions

Cultivation of marine algae under laboratory conditions is a basic prerequisite in all fields of modern phycology (KORNMAN, 1970). Important new insights into
the ecology, taxonomy and physiology of multicellular algae have been gained by means of laboratory cultures. Examples are the discovery of a heteromorphic life cycle in *Saccorhiza bulbosa* (SAUVAGEAU, 1915); of a diplahaplohaplohaploid cycle of isomorphic generations in species of *Enteromorpha*, *Chaetomorpha*, *Cladophora* and *Ulva* (Føyn, 1929, 1934a, b; HARTMANN, 1929); of genotypic sex determination in *Laminaria saccharina* (SCHREIBER, 1930), of heteromorphic alternation in *Derbesia marina* and *Halicypsis ovalis* (KORNmann, 1938), *D. tenuissima* and *H. parvula* (Feldmann, 1950), *D. neglecta* and *Bryopsis halymeniae* (Hustedt, 1964); of the complicated life cycle of *Urospora wormskioldii* (Kornmann, 1961a); and of the *Conchocelis* phase in the life cycle of *Porphyra umbilicalis* (Drew, 1949, 1954) and of *P. tenera* (Kurogi, 1953, 1961). This latter finding has greatly contributed to the development of new methods for commercial cultivation of *Porphyra* (see below).

In recent years, the life histories of numerous other marine algae have been analyzed in laboratory cultures (Dawson, 1960; Dube, 1967; Sanbonsuga and Hasegawa, 1967, 1969; Hilton and McLean, 1971; Hoshaw and West, 1971; Chen and McLachlan, 1972).

While the ecological validity of information on life histories obtained under laboratory conditions must be evaluated with care (Smith and Jones, 1970; Hilton and McLean, 1971), culture experiments under controlled laboratory conditions constitute a basic tool for analyzing life cycles and phenomena related to morphology, taxonomy, ultrastructure, genetics, photosynthesis, biochemistry, biophysics and radiobiology (Bonotto and co-authors, 1972). The success obtained during the last few decades in cultivating marine algae under laboratory conditions has been rendered possible by: (i) advances in the preparation of natural and artificial sea-water media (see also Chapter 2); (ii) availability of antibiotics, bacteriocides and other chemicals permitting the control of infections in the culture; (iii) improved methods for isolation of algae; (iv) better control of culture conditions (light, temperature, pH); (v) development of more sophisticated apparatus.

(c) Culture Media

The most frequently used medium for cultivating multicellular marine algae remains Erdschreiber: sea water enriched with N, P and soil extract. Erdschreiber was initially utilized by Hämmerring (1931) for cultures of the giant unicellular alga *Acetabularia mediterranea*, and by Føyn (1934a, b) for cultures of *Cladophora suhriana* and *Ulva lactuca*. Erdschreiber is a chemically undefined medium; its constituents (organic and inorganic compounds) vary in different sea waters (Provasoli, 1963, 1971; Hoyt, 1970) and with the type of earth extract used. The variability can be reduced when the same type of sea water (collected and stored in large amounts) and the same type of earth extract are used for long periods of time. Earth extracts contain practically all of the most common amino acids (Fig. 4-29). The amino-acid composition of extracts obtained from two different types of earth (from Brussels and from Naples) are listed in Table 4-15. Important differences in amino-acid composition exist even between two extract preparations from the same earth. Moreover, the extract obtained from earth collected in Naples contains a much higher level of phenylalanine, than that of Brussels; in cultivation experiments it gives better results, assuring rapid growth of *Acetabularia mediterranea*.
Fig. 4.29: Amino acids found in extract of earth samples from Brussels, eluted at pH 3.25 (above), at pH 4.25 or 5.28 (below). Ammonia is also present. (After Latzeur and Bonotto, 1973; modified; reproduced by permission of Société Royale de Botanique de Belgique.)
It is not yet known, however, if phenylalanine alone stimulates growth or in combination with other substances present.

Table 4-15

Amino-acid composition (% µM) of extracts obtained from two different types of earth; important differences exist even between two extract preparations (Exp. 1, Exp. 2) from the same earth (After LATEUR and BONOTTO, 1973; reproduced by permission of Société Royale de Botanique de Belgique)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Earth from Brussels Exp. 1</th>
<th>Earth from Naples Exp. 1</th>
<th>Earth from Brussels Exp. 2</th>
<th>Earth from Naples Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>12.12</td>
<td>14.76</td>
<td>11.51</td>
<td>12.99</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.38</td>
<td>3.91</td>
<td>2.02</td>
<td>3.15</td>
</tr>
<tr>
<td>Serine</td>
<td>7.11</td>
<td>4.25</td>
<td>4.02</td>
<td>4.48</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.18</td>
<td>17.46</td>
<td>10.44</td>
<td>13.93</td>
</tr>
<tr>
<td>Proline</td>
<td>6.46</td>
<td>8.92</td>
<td>2.30</td>
<td>1.76</td>
</tr>
<tr>
<td>Glycine</td>
<td>16.55</td>
<td>11.01</td>
<td>8.35</td>
<td>8.05</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.69</td>
<td>6.54</td>
<td>3.51</td>
<td>4.15</td>
</tr>
<tr>
<td>Valine</td>
<td>5.91</td>
<td>8.94</td>
<td>4.44</td>
<td>6.12</td>
</tr>
<tr>
<td>Methionine and derivatives</td>
<td>1.44</td>
<td>0.60</td>
<td>0.79</td>
<td>0.10</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.23</td>
<td>6.69</td>
<td>6.61</td>
<td>6.24</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.83</td>
<td>10.01</td>
<td>11.33</td>
<td>10.26</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.08</td>
<td>2.62</td>
<td>2.98</td>
<td>2.81</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.01</td>
<td>5.30</td>
<td>33.67</td>
<td>25.94</td>
</tr>
</tbody>
</table>

Presumably, the amino acids present in the earth extract are utilized by the algae. A variety of amino acids occur in sea water (HOBBIE and co-authors, 1968; BÖHLING, 1970, 1972) and affect the growth of marine algae (PUKES-M-DAO, 1962; IMADA and SAITO, 1971; IMADA and co-authors, 1971). In the case of Acetabularia mediterranea, radio-active amino acids added to the culture medium are rapidly taken up and incorporated (BONOTTO and co-authors, 1968, 1969; CHAUDHURI and SPENCER, 1968). Amino acids are also taken up by the marine flagellate Platymonas subcordiformis (NORTH and STEPHENS, 1967) and by several macroscopic forms (NORTH and STEPHENS, 1969; see also Volume II: GILLES, 1975). NORTH and STEPHENS suggest that dissolved free amino acids in nearshore waters may have considerable significance in the nitrogen nutrition of marine algae.

Some of the above-mentioned drawbacks are avoided when artificial sea water (Volume I, p. 687; this volume p. 29) is used for preparing culture media. For unicellular marine algae, artificial sea-water media have been developed by PROVASOLI and co-authors (1957) and others (Chapter 4.1). Several unicellular algae, including Acetabularia mediterranea (SHEPHERD, 1970), have been cultivated axenically in a synthetic medium. Axenic cultures of seaweeds are more difficult to obtain (PEDERSEN, 1968). The artificial sea-water media developed by PROVASOLI (1963) are very convenient for a number of Rhodophyceae. Some algae, however, grow better—or grow only—in media with natural sea water. Thus, Ectocarpus confervoides or Porphyra omoi prefer enriched natural sea water (PROVASOLI, 1963). Exposed to artificial media, the sea lettuce Ulva lactuca exhibits only slow, callus-
like growth; near-normal morphology of this green alga was obtained by Provasoli (1958) only when the enriched natural sea water was complemented by trace metals, vitamins, adenine and kinetin. Ectocarpus fasciculatus shows a slow callus-like growth in bacteria-free cultures, but develops a normal habitus in the presence of kinetin. Likewise, Pylaiella littoralis, which is incapable of growing in artificial media without the addition of natural sea water, shows normal growth and appearance if kinetin is used (Pedersen, 1968). However, the zoospores of P. littoralis, when cultivated in an artificial medium (Fries, 1963) with kinetin added, had an absolute requirement for vitamin B$_{12}$ (Pedersen, 1969a). The Conchocelis of Porphyra tenera, if cultivated in axenic culture, needs vitamin B$_{12}$ for growth (Iwasaki, 1965). Several other multicellular algae were found to require vitamins (Provasoli, 1963; Pedersen, 1969a).

It is now well established that a number of seaweeds not only accumulate a variety of metals but need some particular elements (Black and Mitchell, 1952; Klempner, 1957; Provasoli, 1963; von Stosch, 1963; Fries, 1966; Pedersen, 1969b). Even though some elements are very dilute in sea water they may be physiologically important for the algae, which show a concentration factor for heavy metals ranging from 100 to 30,000 (Black and Mitchell, 1952). Ion uptake

---

**Fig. 4-30:** Thalassiotron, an apparatus for simulating tidal movements. A: fixed container, where the algae are placed; B: movable and graduated container, containing refrigerated (10° C) sea water—the sea water flows from B to A and vice versa through flexible tubes; C: refrigerant container—A and B are each 37 cm high, 28 cm wide and 58 cm long at their inner sides. 1: refrigeration unit; 2: screw for adjusting tide cycle; 3: motor (1725 rpm); 4: first adjustable reduction; 5: second double reduction (900-1); 6: third reduction (4-1); 7: fourth reduction (70-1); 8: lever rotating twice in 24 hrs and 55 mins; 9: cable moving container B; 10: pump for sea water circulation; 11: tubes for refrigerant circulation in B. (After Tremblay and Mehman, 1964; modified; reproduced by permission of Revue canadienne de Biologie.)
by marine algae may be enhanced by water movement (waves, currents, tides; PROVASCOTT, 1963). Hence, an apparatus such as the thalassiotron (Fig. 4-30) which imitates tidal movements in the laboratory, constitutes a useful tool for studies on uptake of inorganic elements as well as of organic molecules by seaweeds (see also Chapter 2, p. 246). All these findings underline the difficulty of imitating natural sea water adequately. They suggest that the inadequacy of the present artificial media for growing certain seaweeds probably reflects the lack of some organic (hormones, vitamins, amino acids or other unknown organic molecules) or inorganic (elements) factors, which occur in natural sea water.

A number of organic molecules have been detected in sea water: auxins (BENTLEY, 1958), vitamins (HARVEY, 1957; DAISLEY and FISHER, 1958), amino acids (BOHLING, 1970, 1972) and more complex substances (HOYT, 1970). The major ions are sodium, magnesium, calcium, potassium, chloride, sulphate, bromide and carbonate, which together account for more than 99.9% of the salts (ORR and MARSHALL, 1969). The concentrations of the major constituents of sea water and those of 45 minor elements have been listed in Volume I (pp. 66–71, 684).

The major constituents of sea water, and an increasing number of the minor ones, play a role in growth processes of unicellular algae (ORR and MARSHALL, 1969). Presumably, these elements are also of importance in the growth of multicellular algae. The development of more adequate artificial media for multicellular algae requires better knowledge of their mineral metabolism and general physiology.

(d) Apparatus for Cultivation and Experimentation

While it is possible to cultivate some multicellular algae successfully in the laboratory in small vessels containing enriched natural sea water or artificial media, satisfactory growth of the larger algae is seldom obtained under these conditions. Many authors have attempted to overcome these difficulties by employing open systems with a continuous supply of fresh natural sea water, or re-circulation systems containing natural sea water. Several large brown algae (species of Alaria, Laminaria, Macrocystis, Undaria) have been cultivated in this way (NEUSHUL, 1963; NEUSHUL and HAXO, 1963; ANDERSON, 1965; NORTH and co-authors, 1969; SOUTH, 1970).

The apparatus devised by SOUTH (1970) for cultivating Alaria esculenta (Fig. 4-31) can be built easily and may be used also for other seaweeds. In this apparatus, fresh sea water is filtered before it reaches the culture tanks, and light from fluorescent tubes may be experimentally manipulated. The closed re-circulating seawater system used by JONES and DENT (1970) for growing red and brown algae, is illustrated in Fig. 4-32. Sea water is pumped from the storage tanks (where sedimentation occurs) to a header tank, from which it flows by gravity to the experimental benches. Filters may be inserted between the taps and the culture dishes in order to remove suspended matter.

Although both open systems and closed systems with re-circulating natural sea water have been successfully employed for algae cultivation, there are disadvantages: (i) even filtered sea water may contain bacteria, diatoms, small flagellates and algal spores, which contaminate the cultures; (ii) re-circulating water may accumulate metabolic waste products released by the cultured seaweeds, thus
Fig. 4.31: Apparatus for cultivation of *Aelaria esculenta*. a: tap for sea water entry; b and c: filters retaining particles down to 15µm and 6µm, respectively; d and e: tap and plastic tube for entry of sea water to upper culture tank; f: fluorescent tubes; g: nylon-filter plug; h: plates for attachment of fluorescent tubes; i: nuts for fixation of plates on a wooden groove. (After *South*, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)

Fig. 4.32: Closed re-circulating sea water system for cultivation of seaweeds. B₁ and B₂: experimental benches; I₁ and I₂: inlet pipes to storage tanks 1 and 2; V₁, V₂, V₃, V₄, and V₅: valves. (After *Jones and Dent*, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)
changing the composition of the culture medium (Jones and Dent, 1970). To minimize these potential drawbacks, more effective filtration and frequent changes of the sea water are necessary.

The thalassiotron (Fig. 4-30), initially devised by Tremblay and Mehran (1964), was later improved by the same authors (Mehran and Tremblay, 1965a, b, 1966a, b). With the latest model it is possible to conduct 4 experiments simultaneously (Mehran and Tremblay, 1966b). The thalassiotron is a useful tool for investigating the uptake of radionuclides ($^{59}$Fe, $^{65}$Zn) by seaweeds (Fig. 4-33) and can be used for a variety of studies on physiology and mineral metabolism of algae exposed to defined tidal regimes.

Light and temperature conditions can be controlled in a cultivation apparatus described by Edwards and van Baalen (1970). The apparatus (Fig. 4-34), origin-

---

**Fig. 4-33**: Fucus spiralis. Absorption of $^{59}$Fe in two compartments (I and II) of the new thalassiotron, with different radionuclide concentrations (I $= 1708 \times 10^6$ dpm l$^{-1}$; II $= 3915 \times 10^6$ dpm l$^{-1}$). (After Mehran and Tremblay, 1966b; modified; reproduced by permission of le Naturaliste canadien.)
ally designed for cultures of unicellular green and coccoid, and filamentous blue-green algae (Hàlldal, 1958; hàlldal and FrenCh, 1958), has been modified for use with uni-algal cultures of benthic marine algae. It permits to obtain a temperature gradient at a right angle to an irradiance gradient and was used for studying effects of temperature and irradiance on growth and reproduction of several brown and red algae.

Fig. 4-34: Cultivation apparatus for benthic marine algae. Irradiance and temperature gradients can be programmed. The algae are cultivated in Petri dishes on the aluminium plate (1). 2: warm water bath; 3: cold water bath; 4: movable fluorescent lamps. (After Edwards and van Baalen, 1970; reproduced by permission of Walter de Gruyter and Co.)

(3) Green Algae

(a) Commercial Importance

The green algae or Chlorophyceae are of clear green appearance; the chlorophyll of their plastids is not masked by other pigments. The most familiar marine green algae belong to the so-called confervoid algae. The classification of the green algae has been summarized by LevrinQ and co-authors (1969).

Green algae of commercial importance are listed alphabetically in Table 4-16. Most of the Chlorophyceae listed in Table 4-16 are widespread and grow abundantly in protected areas in the intertidal and upper subtidal zones (McConnaughey, 1970); they attach to various substrates. Along the Belgian coast we have frequently found species of Enteromorpha and Ulva attached to shells of living mussels. Attachment to mollusc shells has been observed also in larger brown algae such as Fucus sp. (Fig. 4-35).
Green algae of commercial importance. Predominant use (after Boney, 1965) is indicated by: hf: human food, af: animal food, mp: medical purposes, ma: manure, in: industrial use. The list is based on papers by Verhas (1925), Kurogi (1963a), Boney (1965), Levrino and co-authors (1969) and Okazaki (1971).

<table>
<thead>
<tr>
<th>Codium sp.</th>
<th>hf, mp</th>
<th>Monostroma crassissimum</th>
<th>hf, af</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. dichotomum</td>
<td>hf</td>
<td>M. grevillei</td>
<td>hf, af</td>
</tr>
<tr>
<td>C. divaricatum</td>
<td>hf</td>
<td>M. latissimum</td>
<td>hf, af</td>
</tr>
<tr>
<td>C. fragile</td>
<td>hf</td>
<td>M. nitidum</td>
<td>hf, af</td>
</tr>
<tr>
<td>C. intricatum</td>
<td>hf</td>
<td>M. tubuliforme</td>
<td>hf, af</td>
</tr>
<tr>
<td>C. lindenbergii</td>
<td>hf</td>
<td>Prasiola japonica</td>
<td>hf</td>
</tr>
<tr>
<td>C. muelleri</td>
<td>hf</td>
<td>Ulva fasciata</td>
<td>hf</td>
</tr>
<tr>
<td>C. tenue</td>
<td>hf</td>
<td>U. lactuca</td>
<td>hf, af, mp, ma</td>
</tr>
<tr>
<td>C. tomentosum</td>
<td>hf, in</td>
<td>U. latissima</td>
<td>hf, af</td>
</tr>
<tr>
<td>Enteromorpha compressa</td>
<td>hf, af</td>
<td>U. nematoida</td>
<td>hf</td>
</tr>
<tr>
<td>E. flexuosa</td>
<td>hf</td>
<td>U. penniformis</td>
<td>mp</td>
</tr>
<tr>
<td>E. intestinalis</td>
<td>hf, af</td>
<td>U. portula</td>
<td>hf, af, mp</td>
</tr>
<tr>
<td>E. linza</td>
<td>hf, af</td>
<td>U. reticulata</td>
<td>mp</td>
</tr>
<tr>
<td>E. proliferosa</td>
<td>hf</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Cultivation

Although several green algae are of economic significance (mainly as food for man and animals), only few species are at present cultivated commercially.
4. CULTIVATION OF PLANTS (S. BONOTTO)

Genus Monostroma

Species of Monostroma are collected for food mainly in Japan and China (Boney, 1965; Leveridge and co-authors, 1969). They are cultivated on artificial substrata, the so-called 'Hibi' (Yamada, 1969). This consists mainly of bamboo brush and of nets made from natural or synthetic materials. Frequently, species of Monostroma grow as contaminants on the substrata offered for Porphyra (Iwamoto, 1960; Kurogi, 1963a). The zoospores and the zygotes (Kornmann, 1962) produced are distributed according to the direction of the main surface currents during the flood (e.g. Boney, 1965). Hence certain areas of the culture grounds (the so-called 'Taneba') become crowded. The location of these favoured areas appears to shift with the years, a fact which could be avoided by artificial spreading of spores obtained in laboratory cultures.

Genus Ulva

Ulva lactuca and U. pertusa are common inhabitants of intertidal areas. They exhibit isomorphic alternation of diploid and haploid plants. Mobile zoospores, released by diploid plants, produce gametophytes, which generate gametes. U. thuretii inhabits the shores of the Mediterranean Sea and the Atlantic Ocean and is known from Spain, Portugal and France (Foyn, 1955). Axenic cultures of U. lactuca and U. taeniata have been obtained by Provasoli (1968, 1969; Chapter 5.11), who observed that, in the absence of bacteria, plant hormones are required for normal development of a foliaceous thallus. These important findings suggest that the growth of seaweeds cultivated in coastal waters may be influenced by hormones which are found there in appreciable amounts.

Genus Enteromorpha

Release of zoospores and gametes from Enteromorpha plants occurs periodically (Christie and Evans, 1962); it is light dependent (Lersten and Voth, 1960). Although Enteromorpha plants are at present obtained as by-product in Porphyra cultures (Kurogi, 1963a), sufficient data on their life histories and environmental requirements exist to permit intensive cultivation.

(4) Brown Algae

(a) Commercial Importance

The brown algae or Phaeophyceae constitute the most familiar large seaweeds. Their brown or olive-brown colour is due to chlorophyll masking by several other pigments. The Phaeophyceae comprise a large group: about 1500 species have been described (Louis, 1967). A recent classification has been provided by Levrini and co-authors (1969).

The commercially most important brown algae belong to the orders Laminariales and Fucales. The main species used by man are listed alphabetically in Table 4-17. In some cases species synonyms have been added in parentheses.
### Table 4-17

Brown algae of commercial importance. Predominant use (after Boney, 1965) is indicated by: hf: human food, af: animal food, mp: medical purposes, ma: manure, iu: industrial use. The list is based on papers by Verras (1925), Boney (1965), Levrino and co-authors (1969), Hasegawa (1971a, b) and Okazaki (1971).

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Use(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarum cribrosum</td>
<td>iu</td>
</tr>
<tr>
<td>A. fimbriatum</td>
<td>iu</td>
</tr>
<tr>
<td>A. euculelia</td>
<td>hf, af, ma</td>
</tr>
<tr>
<td>A. fistulosa</td>
<td>hf</td>
</tr>
<tr>
<td>A. marginata</td>
<td>ma</td>
</tr>
<tr>
<td>A. vohotensis</td>
<td>hf</td>
</tr>
<tr>
<td>A. yezoensis</td>
<td>hf</td>
</tr>
<tr>
<td>Arthrocnmus bifidus</td>
<td>hf</td>
</tr>
<tr>
<td>A. kuritensis</td>
<td>hf</td>
</tr>
<tr>
<td>Ascophyllum nodosum</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>Chondria pacifica</td>
<td>hf</td>
</tr>
<tr>
<td>Chordaria flagelliformis</td>
<td>hf, mp</td>
</tr>
<tr>
<td>Costaria costata</td>
<td>hf</td>
</tr>
<tr>
<td>Cystophora sp.</td>
<td>af</td>
</tr>
<tr>
<td>Cystophyllum sp.</td>
<td>hf</td>
</tr>
<tr>
<td>Dictyophoros polyoides (Syn: D. membranacea)</td>
<td>hf, mp</td>
</tr>
<tr>
<td>D. platigioumma</td>
<td>hf</td>
</tr>
<tr>
<td>Dictyota acutiloba</td>
<td>hf</td>
</tr>
<tr>
<td>D. apiculata</td>
<td>hf</td>
</tr>
<tr>
<td>Durvillea antarctica</td>
<td>hf, af, mp, ma, iu</td>
</tr>
<tr>
<td>Ecklonia bicyclus</td>
<td>iu</td>
</tr>
<tr>
<td>E. cava</td>
<td>iu</td>
</tr>
<tr>
<td>E. kurome</td>
<td>hf</td>
</tr>
<tr>
<td>E. latifolia</td>
<td>hf</td>
</tr>
<tr>
<td>E. maxima</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>Eckloniopsis sp.</td>
<td>hf, iu</td>
</tr>
<tr>
<td>Egregia menziesii</td>
<td>ma, iu</td>
</tr>
<tr>
<td>Eisenia bicyclus</td>
<td>hf, ma, iu</td>
</tr>
<tr>
<td>Endarachne sp.</td>
<td>hf</td>
</tr>
<tr>
<td>Fucus serratus</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>F. vesiculosus</td>
<td>af, mp, ma, iu</td>
</tr>
<tr>
<td>Heterochordaria abietina</td>
<td>hf</td>
</tr>
<tr>
<td>Himanthalia elongata (Syn: H. lorna)</td>
<td>ma, iu</td>
</tr>
<tr>
<td>Hispida fusiforme</td>
<td>hf</td>
</tr>
<tr>
<td>Hormonira banksei</td>
<td>af</td>
</tr>
<tr>
<td>Hydroclathrus clathratus (Syn: H. cancellatus)</td>
<td>hf, ma</td>
</tr>
<tr>
<td>Kjelmanniella gyrata (Syn: Laminaria gyrata)</td>
<td>hf</td>
</tr>
<tr>
<td>Laminaria angustata</td>
<td>hf, iu</td>
</tr>
<tr>
<td>L. ochroleioides</td>
<td>hf, iu</td>
</tr>
<tr>
<td>L. diabolica</td>
<td>hf, iu</td>
</tr>
<tr>
<td>L. digitata</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>L. fragilis</td>
<td>iu</td>
</tr>
<tr>
<td>L. hyperborea</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>(Syn: L. cloustonii)</td>
<td>hf, mp, iu</td>
</tr>
<tr>
<td>L. japonica</td>
<td>hf, mp, iu</td>
</tr>
<tr>
<td>L. longipedalis</td>
<td>hf</td>
</tr>
<tr>
<td>L. longissima</td>
<td>hf, iu</td>
</tr>
<tr>
<td>L. religiosa</td>
<td>hf</td>
</tr>
<tr>
<td>L. saccharina</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>Lessonia sp.</td>
<td>iu</td>
</tr>
<tr>
<td>L. variegata</td>
<td>ma</td>
</tr>
<tr>
<td>Macrocytis integrifolia</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>M. pyrifera</td>
<td>af, ma, iu</td>
</tr>
<tr>
<td>Mesogloia crassa</td>
<td>hf</td>
</tr>
<tr>
<td>M. decipiens</td>
<td>hf</td>
</tr>
<tr>
<td>Nemacystis decipiens</td>
<td>hf</td>
</tr>
<tr>
<td>Nereocystis hasegawae</td>
<td>hf, af, ma, iu</td>
</tr>
<tr>
<td>Padina australis</td>
<td>af, ma</td>
</tr>
<tr>
<td>Pelagophycus porra</td>
<td>ma, iu</td>
</tr>
<tr>
<td>Pelvetia canaliculata</td>
<td>af, ma</td>
</tr>
<tr>
<td>Petalonia fascia</td>
<td>hf</td>
</tr>
<tr>
<td>Sargassum aequifolium</td>
<td>hf</td>
</tr>
<tr>
<td>S. echinocarpum</td>
<td>hf</td>
</tr>
<tr>
<td>S. ensers</td>
<td>hf, iu</td>
</tr>
<tr>
<td>S. fusiforme</td>
<td>hf, mp, ma, iu</td>
</tr>
<tr>
<td>S. granuliferum</td>
<td>hf</td>
</tr>
<tr>
<td>S. hemiphylhum</td>
<td>ma</td>
</tr>
<tr>
<td>S. horneri</td>
<td>iu</td>
</tr>
<tr>
<td>S. linifolium</td>
<td>iu</td>
</tr>
<tr>
<td>S. microcanthum</td>
<td>iu</td>
</tr>
<tr>
<td>S. natans</td>
<td>mp</td>
</tr>
<tr>
<td>S. serratifolium</td>
<td>hf, iu</td>
</tr>
<tr>
<td>S. siliculosum</td>
<td>hf</td>
</tr>
<tr>
<td>S. sp.</td>
<td>hf, af, ma</td>
</tr>
<tr>
<td>S. vernerrimum</td>
<td>ma</td>
</tr>
<tr>
<td>S. thumbergii</td>
<td>af, mp, ma</td>
</tr>
<tr>
<td>S. vulgare</td>
<td>hf</td>
</tr>
<tr>
<td>S. wrightii</td>
<td>ma</td>
</tr>
<tr>
<td>Scytostithon sp.</td>
<td>hf</td>
</tr>
<tr>
<td>Splachnidium rugosum</td>
<td>hf</td>
</tr>
<tr>
<td>Tinsclodia sp.</td>
<td>ma</td>
</tr>
<tr>
<td>Turbinaria sp.</td>
<td>ma</td>
</tr>
<tr>
<td>U. ornata</td>
<td>iu</td>
</tr>
<tr>
<td>Undaria peterseniana</td>
<td>hf</td>
</tr>
<tr>
<td>U. pinnatifida</td>
<td>hf</td>
</tr>
<tr>
<td>U. undarioides</td>
<td>hf</td>
</tr>
</tbody>
</table>
Brown algae occur on the coasts of all oceans but are most abundant in the temperate-to-cold waters (Volume I: GESSNER, 1970). Most of these large algae are found near shores in water not more than 20 m deep (McCONNAUGHEY, 1970). Several species, however, are able to grow in deeper waters when light is sufficient. *Laminaria rodriquezii*, for example, was found on the Apollo Bank (Tyrrhenian Sea) at 75 m, and *L. ochroleuca* in the Messina Straits at 95 m (GIACCONE, 1971).

(b) Cultivation

Most of the more than 70 species used by man are harvested from natural habitats, but a few important brown algae are cultivated, especially in Japan and China, where the technology of cultivation is advanced (CHENG, 1969; HASEGAWA, 1971a, b; OKAZAKI, 1971). The two genera *Laminaria* and *Undaria* comprise, at present, the commercially most important representatives; several species are farmed on a large-scale basis. Other genera, however, would lend themselves to commercial cultivation, providing an adequate technology is developed. Recent research by NORTH and co-authors (1969) has established that dispersion of *Macro-cystis* embryos in suitable sea areas may help to increase natural kelp stands. The three genera *Laminaria*, *Undaria* and *Macro-cystis* will be considered here in detail.

**Genus Laminaria**

*Laminaria* seaweeds are commercially important not only for their food value but also for specific constituents such as alginic acid (HELLEBUST and HAUG, 1969, 1972a, b). In Japan and China, they are primarily used as foodstuffs. The yearly production of *Laminaria* in Japan amounts to about 30,000 tons in dried weight (HASEGAWA and SANBONSUGA, 1971).

*Laminaria* is harvested between May and November, with a maximum at the end of July and during August (CHAPMAN, 1960; OKAZAKI, 1971). Since the plants grow abundantly in rocky areas with cold water, even in summer, harvesting by diving, which is used for *Gelidium* species, is impracticable. As *Laminaria* seaweeds attain considerable sizes, they may be easily collected by fishermen by means of long-handled rakes. These rakes are no longer needed for harvesting raft-cultured plants, which can be removed by hand.

The main methods used to facilitate cultivation of *Laminaria* are: (i) planting of stones; (ii) blasting of reefs; (iii) removing of competing plants (*Sargassum*, *Phyllospadix* and *Zostera*); (iv) raft cultures (CHENG, 1969; HASEGAWA, 1971a).

The large plants harvested by fishermen are the macroscopic sporophytes, which alternate during the life cycle with small filamentous gametophytes. On the blade of the macroscopic sporophyte develop numerous sporangia which release mobile zoospores (KAIN, 1963). The zoospores settle on solid natural or artificial substrata and produce a germ-tube which gives rise to the gametophyte. After fertilization of the oosphere within the oogonium, new sporophytes develop from the microscopic gametophyte (SMITH, 1955).

Several studies are concerned with the effects of abiotic environmental factors (especially light and temperature; Volume I: GESSNER, 1970; HELLEBUST, 1970) and nutritional factors on growth rates of gametophytes and sporophytes (HARRIES,
1932; KAIN, 1963; CHENG, 1969; LÜNING, 1970; HASEGAWA, 1971b; HASEGAWA and SANBONSSUA, 1971). Experimentation on the microscopic gametophytes and on the early sporophytes (up to a length of a few cm) is relatively easy. Technical difficulties arise with the large sporophytes.

Diving techniques (NORTH, 1961; NEUSHUL and HAXO, 1963) have assisted in overcoming these difficulties by permitting field experiments with large sporophytes. A useful method for transplantation experiments with Laminaria hyperborea, growing in the sublittoral zone of Helgoland (southern North Sea) has been developed by LÜNING (1969). The plants are carefully removed from the rocks by a diver; they are then mounted on PVC plates with plastic network and rubber bands (Fig. 4-36). According to LÜNING, PVC plates are preferable to wooden plates, since the latter are destroyed by woodboring organisms. The PVC plates are then mounted on iron frames (Fig. 4-37) and submerged to the desired water depth. Such 'underwater growth stations', which may be easily hoisted on board, allowed LÜNING (1970) to make regular recordings on the growth of transplanted L. hyperborea at different depths (Fig. 4-38) and to perform interesting observations on several factors which control development.

Transplantation experiments with species of Laminaria and other Laminariales (Alaria, Macrocystis, Undaria) were also performed by other authors (NORTH, 1964; SUNDENE, 1964; SANBONSSUA and HASEGAWA, 1967; CHENG, 1969; HASEGAWA, 1971a, b). HASEGAWA (1971b) reared Laminaria sporophytes in laboratory cultures by the so-called 'forcing cultivation technique' and then transferred them to fishing grounds.

Fig. 4-36: Laminaria hyperborea. Lower part of stipe and holdfast mounted on a PVC plate with plastic network and rubber bands. (After LÜNING, 1969; reproduced by permission of Springer-Verlag, Berlin.)
Fig. 4-37: Underwater growth station (iron frame, 2 x 1 m) with mounted PVC plates bearing two-year-old specimens of Laminaria hyperborea. The station has been hoisted on board a boat for photographic recording. (After LÜNING, 1970; reproduced by permission of Biologische Anstalt Helgoland.)

Fig. 4-38: Laminaria hyperborea. Records of the growth of two specimens during third year of life. (a) Plant growing at 2 m water depth; (b) plant growing at 6 m depth. (After LÜNING, 1970; modified; reproduced by permission of Biologische Anstalt Helgoland.)
Collectors for settling zoospores are often employed to obtain young Laminaria. Frequently, spatial competition is observed on the collectors between young Laminaria sporophytes and fast-growing smaller algae (e.g. Ectocarpus sp.). Such unwanted competition may be avoided if very young sporophytes are kept at 10°C under controlled laboratory conditions and transferred to the sea when the water temperature falls below 20°C. Growth of young sporophytes may also be improved by using chemical fertilizers (Boney, 1965; Cheng, 1969; Yamada, 1972). Development of L. hyperborea gametophytes may be hastened by enriching natural sea water with nitrate, phosphate and bicarbonate (Walker and Smith, 1948).

Fertilization of sporophytes growing on floating rafts, with sodium nitrate or ammonium nitrate, has become current practice in China (Cheng, 1969). Since the demand for fertilizers increases, the Chinese are considering the possibility of large-scale introduction of nitrogen-fixing algae and bacteria in waters, such as those of the Yellow Sea, which are poor in nitrate. A new strain of Laminaria japonica ("Hai-Ching 1"), which is better adapted to high temperatures, has permitted the extension of kelp culture to South China; this may be considered one of the major achievements in modern mariculture. These findings suggest that fertilization and breeding techniques, which have been employed successfully on land, could increase the crop of commercially farmed algae.

**Genus Undaria**

*Undaria* is a well-known genus, endemic to Japanese waters. The species harvested for commercial uses are: *Undaria peterseniana*, *U. pinnatifida* and *U. undarioides* (Okazaki, 1971). They are produced on all Japanese coasts except in the South-east and North-east of Hokkaido (Fig. 4-39). Annual Undaria harvests for the period 1955-1980, are listed in Table 4-18. The main harvesting season differs according to the region. May and June are the best months for harvesting Undaria blades North of the Miyagi Prefecture, including Hokkaido (Table 4-19); South of the Chiba Prefecture, harvesting activities reach a maximum 1 to 2 months later (Okazaki, 1971). The methods of harvesting Undaria plants from natural or artificial cultures are similar to those employed for Laminaria species (see above).

Three major methods are employed by Japanese fishermen for increasing the annual production of Undaria species: (i) providing additional areas with natural solid-substrata for attachment; (ii) removal of competing seaweeds; (iii) raft cultures.

Additional natural attachment areas are provided by throwing stones into the sea or by dynamiting sea-bottom rocks. These measures make more solid substrata available for zoospore settlement. Fishermen hope that removal of commercially non-valued competing marine plants (Phyllospadix, Sargassum, Zostera) will be mechanized (Hasegawa, 1971a).

Raft cultures have, in recent years, gained in importance. Freshly harvested Undaria plants are soaked in shallow water where rope collectors are placed to attract settling zoospores (Fig. 4-40). After shedding and settling of zoospores, the ropes are tied to the culturing rafts. Zoospore emission by Undaria sporophylls
Fig. 4-39: *Undaria peterseniana*, *U. pinnatifida*, *U. undarioides*. Geographical distribution of commercially used *Undaria* species in Japan. (After OKAZAKI, 1971; reproduced by permission of the author.)

Table 4-18

*Undaria peterseniana*, *U. pinnatifida*, *U. undarioides*. Total commercial production in Japan (After OKAZAKI, 1971; modified)

<table>
<thead>
<tr>
<th>Year</th>
<th>Amount harvested (tons)</th>
<th>Dry matter (tons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1955</td>
<td>45,053</td>
<td>8,743</td>
</tr>
<tr>
<td>1956</td>
<td>42,818</td>
<td>8,554</td>
</tr>
<tr>
<td>1957</td>
<td>51,960</td>
<td>9,361</td>
</tr>
<tr>
<td>1958</td>
<td>46,542</td>
<td>8,982</td>
</tr>
<tr>
<td>1959</td>
<td>50,711</td>
<td>9,067</td>
</tr>
<tr>
<td>1960</td>
<td>62,864</td>
<td>11,111</td>
</tr>
</tbody>
</table>

Table 4-19

*Undaria peterseniana*, *U. pinnatifida*, *U. undarioides*. Harvested amounts (tons of wet plants) as a function of season in Hokkaido (After OKAZAKI, 1971; modified)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>75</td>
<td>622</td>
<td>3282</td>
<td>3743</td>
<td>996</td>
<td>45</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
has been studied by Suto (1962). When the sea temperature reaches 14° C, zoospores are released at 2-hr intervals between 9 a.m. and 6 p.m.; they settle on natural or artificial solid substrata. Detailed knowledge of these processes has provided the basis for improving the methods of cultivation. Further increase of sea temperature to about 20° C during spring creates optimal conditions for the development of the culture (Okazaki, 1971).

**Genus Macrocystis**

Species of the giant kelp *Macrocystis* constitute a direct or indirect food source for a large number of nearshore animals. They are of considerable economic value, mainly for the production of alginic acid and alginates (North, 1961; Boney, 1965; Dawson, 1966). Biological studies have concentrated on *Macrocystis pyrifera* and, to a lesser extent, on *M. angustifolia* and on *M. integrifolia* (Cribb, 1964; North, 1961, 1964; Clendenning, 1964; Anderson and North, 1969; Chirife and Gardner, 1969; Guzman del Proo, 1969; North and co-authors, 1969; Teplitzky 1969). *M. pyrifera*, which reaches an age of 8 to 10 years, attains average lengths of 50 to 70 m. Young plants of this species show remarkable growth rates of about 30 cm per day (Levrino and co-authors, 1969).

In Californian as well as in Tasmanian waters, the plants are harvested by cutting their branches (see above: Industrial Uses) about 1 m below the water surface, at 4-month intervals. This method of harvesting assures greater penetration of sunlight to the plants below, enhancing photosynthesis and growth. New branches soon reach the surface, and the 4-month harvesting cycle is repeated. Under favourable conditions, the fast growth of the plants permits a good harvest 3 to 4 times a year.

The large *Macrocystis* kelps represent the diploid (2n = 32) sporophytic generation. The sporangia, located on the sporophylls in the basal region of the plant, release zoospores; these germinate and develop into the filamentous gametophyte (Walker, 1952; Neushul, 1963). Growth of the gametophyte, fertilization, and development of young sporophytes under controlled laboratory conditions were obtained by several authors (Neushul, 1963; Anderson, 1965; Anderson and North, 1966; North and co-authors, 1969).
Transplantation experiments have shown that growth of *Macrocystis* on submerged rafts is rather fast during autumn and winter (Neushul and Haxo, 1963). Transplantation techniques were largely used to obtain kelp restoration in Californian waters (North, 1968a, b, c; North and Mitchell, 1968). An underwater nursery, constructed of used tuna netting, was utilized to protect kelp transplants from grazing by herbivorous fishes (North, 1968c). Grazing by sea-urchins (mainly *Strongylocentrotus franciscanus*) was prevented by buoying the plants about 1 m from the bottom (North, 1965b). Large-scale lime treatments were also used to free the bottom from sea-urchins (North, 1965a). A massive development of *Macrocystis pyrifera* was observed within areas inhabited by sea otters, which are effective sea-urchin predators (e.g. North, 1965c). More recent investigations by North and his group, have shown that release of mass-cultured *Macrocystis pyrifera* into suitable sea areas constitutes a practical means of increasing natural kelp stands (North and Mitchell, 1968; North and co-authors, 1969).

North and his group (e.g. North, 1972) obtained in the laboratory mass cultures of *Macrocystis pyrifera* sporophytes from zoospores. The zoospores were allowed to settle on solid substrata (microscope slides, Plexiglas strips, glass cloth, or nylon ropes) and then placed under continuous illumination in running sea water. The gametophytes attained sexual maturity within 10 to 20 days; another 5 to 20 days were necessary for sporophyte development. At this stage (young sporophytic embryos), the algae were carefully detached from their substrate and dispersed into the sea. North observed that swirling the embryos in a suspension of lead dust before dispersing provokes adherence of the heavy particles and increased sporophyte settling. But also without lead-particle treatment, the young sporophytes may attach, as their basal parts are sticky. A preliminary estimate by North indicates that about $10^5$ embryos must be dispersed to obtain one attached plant—a very low yield. However, in view of the large numbers of embryos obtained (up to $10^5$ to $10^6$ embryos per cm$^2$ of culture substrate) the low yield does not constitute a serious obstacle for increasing kelp stands in the sea.

(5) Red Algae

(a) Commercial Importance

The red algae or Rhodophyceae derive their typical colour from the fact that they contain, in addition to chlorophyll, the red pigment phycoerythrin and sometimes the blue pigment phycocyanin. A total of 3744 red-algae species has been described (Louis, 1967), and a classification presented by Levrin and co-authors (1969).

The red algae most important for commercial purposes belong to the genus *Porphyra* ('purple laver') and to a member-rich group known as Agarophites. Table 4-20 lists, in alphabetical order, the main red-algae species of economic value. In some cases, synonyms have been added in parentheses.

The Rhodophyceae are almost exclusively marine; less than 100 species are found in fresh water. They occur in all, including polar, seas; but they find optimum habitat conditions in the tropics. Most red algae grow in shallow waters or inter-

<table>
<thead>
<tr>
<th>Species</th>
<th>hf, iu</th>
<th>hf, iu</th>
<th>hf, iu</th>
<th>hf, iu</th>
<th>hf, iu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthophthalmopsis japonica</td>
<td>Acanthophora spicifera</td>
<td>A. nitidissima</td>
<td>A. goudiella tenera</td>
<td>A. hystrix conspicua</td>
<td>A. plicata</td>
</tr>
<tr>
<td>Alsidium helminthochorton</td>
<td>Asparagopsis sandfordiana</td>
<td>Beckerella sp.</td>
<td>Bostrychia radicans</td>
<td>Caloglossa adnata</td>
<td>C. leprieurii</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
<tr>
<td>A. plicata</td>
<td>A. plicata</td>
<td>A. pingui</td>
<td>C. pusilla</td>
<td>C. boydenii</td>
<td>C. pusilla</td>
</tr>
</tbody>
</table>
| A. plicata | A. plicata | A. pingui | C. pusilla | C.
| G. polyctenum   | IU   | Gracilariaopsis chorda | IU   |
| G. prionides   | IU   | G. rhodetricha | IU   |
| G. pulchellum  | IU   | G. vermiculophylla | IU   |
| G. pulvinum    | IU   | Grateloupia divaricata | IU   |
| G. purpurascens| IU   | G. filicina | IU   |
| G. pusillum     | IU   | G. imbricata | IU   |
| G. pyramidalis | IU   | G. okamurai | IU   |
| G. rigens      | HF   | Gymnogongrus flabelliformis | HF, IU |
| G. sp.         | HF, MP, IU | G. griffithsiae | IU   |
| G. subcostatum | IU   | G. javanicus | HF   |
| G. subfastigiatum | IU   | G. pinnulata | HF, IU |
| G. tenue       | IU   | Haloseaion glandiforme | HF   |
| G. vagum       | IU   | Halymenia durvilleae | HF   |
| Gigartina chamaissoi | IU   | Hypnea boergensis | IU   |
| G. clavifera   | IU   | H. cenomyces | HF, IU |
| G. decipiens   | IU   | H. ceratophylla | HF, IU |
| G. internodia  | IU   | H. chordea | IU   |
| G. ochotensis  | IU   | H. cornuta | IU   |
| G. pacifica    | IU   | H. divaricata | HF   |
| G. pistillata  | HF, IU | H. esperi | IU   |
| G. stellata    | IU   | H. flabelliformis | IU   |
| (Syn: G. mamillosa) | IU   | H. hamulosa | IU   |
| G. teedii      | IU   | H. japonica | IU   |
| G. tenella     | IU   | H. musciformis | IU   |
| Gloiopteris complanata | IU   | H. nidifica | HF, AF, MP |
| G. furcata     | IU   | H. nidulans | IU   |
| G. furcata     | IU   | H. panacea | IU   |
| G. furcata     | IU   | H. saudana | IU   |
| G. furcata     | IU   | H. epicifera | IU   |
| G. furcata     | IU   | H. valentineae | IU   |
| G. furcata     | IU   | H. variabilis | IU   |
| G. furcata     | IU   | Iridea cornucopiae | IU   |
| G. furcata     | IU   | I. edulis | HF   |
| G. furcata     | IU   | I. flaccida | IU   |
| (Syn: I. laminaroides) | IU   | Laurencia botryoides | HF   |
| G. corinipodia | IU   | L. obtusa | HF   |
| G. crassa      | IU   | L. papulosa | HF   |
| G. denticulata | IU   | L. perforata | HF   |
| G. dura        | IU   | L. pinnatifida | HF   |
| G. edulis      | HF, IU | Liagora farinosa | HF   |
| G. eucymeoides | HF   | L. decussata | HF   |
| G. gigas       | IU   | Lithothamnion calcareum | MS   |
| G. incurvata   | IU   | L. crassum | MS   |
| G. multipartita | IU   | Mastocarpus klenzianus | HF   |
| (Syn: G. lacinulata, G. foliifera) | IU   | Meristotheca papulosa | HF, IU |
| G. punctata    | IU   | Nemalion multifidum | HF   |
| G. purpurascens| IU   | Neodictya yendoana | IU   |
| G. salicornia  | IU   | Odontalia corymbifera | IU   |
| G. subtilissima| IU   | Pachymenia himantophora | MS   |
| G. taenioides  | HF, IU | Pachymenia sp. | IU   |
| G. textorii    | IU   | Pachymeniopsis elliptica | HF, IU |
| G. verrucosa   | IU   | Phyllophora brodiae | IU   |
tidal zones and rarely go down to depths in excess of 30 to 40 m. However, in the tropics, red algae have been dredged as deep as 170 m (McConnaughey, 1970). The Rhodophyceae possess an uncalcified thallus, except the red coralline algae, which accumulate large amounts of lime.

(b) Cultivation

Most of the more than 200 species used commercially are harvested from natural habitats. This pertains, for example, to the numerous algae which are used as sources of agar. Several edible *Porphyra* species, however, are at present cultivated extensively in Japan and China. The genus *Porphyra* is one of the most studied algal groups (Dangeard, 1927; Drew, 1949, 1964; Kontmann, 1960; Kurooi, 1961, 1963a, b; Ogata, 1962; Iwasaki and Matsudaïra, 1963; Iwasaki, 1965; Austin and Pringle, 1969; Imada and co-authors, 1969, 1970a, b; Krishnamurthy, 1969a; Yabu, 1969a, b, 1970, 1971; Imada and Saito, 1971). Methods of cultivation have been developed for *Porphyra* species by several authors (Arasaki and co-authors, 1966; Yamada, 1959; Kurogi, 1963a, b; Kurogi and Akiyama, 1965; Kurogi and Yoshida, 1966; Suto, 1966; Kurogi and Sato, 1967; Kurogi and co-authors, 1967; Iversen, 1968; Okazaki, 1971); for details consult the next section.

Other red algae genera, such as *Gelidium*, *Gigartina*, *Gracilaria* and *Pterocladia*, which are important for industrial uses (mainly agar industry), have been intensively studied (Wood and Peddie, 1941; Stokke, 1966, 1967; Ohmi, 1958; Mitra-Kos, 1964; Yamamoto, 1969; Mathieson and Burns, 1971; Burns and Mathieson, 1972a, b; Ogata and co-authors, 1972); however, only a few species are at present cultivated on a commercial scale.

### Table 4-20—Continued

<table>
<thead>
<tr>
<th>Species</th>
<th>Culture Form</th>
<th>Species</th>
<th>Culture Form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. membranifolia</em></td>
<td>u</td>
<td><em>P. suborbiculata</em></td>
<td>h</td>
</tr>
<tr>
<td><em>P. nervosa</em></td>
<td>u</td>
<td><em>P. tenera</em></td>
<td>h</td>
</tr>
<tr>
<td><em>P. rubens</em></td>
<td>r</td>
<td><em>P. umbilicalis</em></td>
<td>h</td>
</tr>
<tr>
<td><em>Plumaria plumosa</em></td>
<td>m</td>
<td><em>P. vulgaris</em></td>
<td>h</td>
</tr>
<tr>
<td><em>Porphyra angusta</em></td>
<td>h</td>
<td><em>P. yezoensis</em></td>
<td>h</td>
</tr>
<tr>
<td><em>P. atraspurnurea</em></td>
<td>h, mp</td>
<td><em>Pterocladia capillacea</em></td>
<td>iu</td>
</tr>
<tr>
<td><em>P. columnina</em></td>
<td>h</td>
<td>(Syn: <em>P. pinnata</em>)</td>
<td></td>
</tr>
<tr>
<td><em>P. dentata</em></td>
<td>h</td>
<td><em>P. densa</em></td>
<td>iu</td>
</tr>
<tr>
<td><em>P. kuniedai</em></td>
<td>h</td>
<td><em>P. lucida</em></td>
<td>h</td>
</tr>
<tr>
<td><em>P. kantihana</em></td>
<td>h</td>
<td><em>P. nana</em></td>
<td>iu</td>
</tr>
<tr>
<td><em>P. laciniata</em></td>
<td>h</td>
<td><em>P. tenuis</em></td>
<td>iu</td>
</tr>
<tr>
<td><em>P. leucosticta</em></td>
<td>h</td>
<td><em>Rhodoglossum hemisphaericum</em></td>
<td>iu</td>
</tr>
<tr>
<td><em>P. ochotensis</em></td>
<td>h</td>
<td><em>R. japonicum</em></td>
<td>iu</td>
</tr>
<tr>
<td><em>P. okamurai</em></td>
<td>h</td>
<td><em>Rhodymenia indica</em></td>
<td>mp</td>
</tr>
<tr>
<td><em>P. onoi</em></td>
<td>h</td>
<td><em>R. palmata</em></td>
<td>h, af, mp</td>
</tr>
<tr>
<td><em>P. perforata</em></td>
<td>h</td>
<td><em>Sarcocysta montagneana</em></td>
<td></td>
</tr>
<tr>
<td><em>P. pseudo-linearis</em></td>
<td>h</td>
<td><em>Soliera chordalis</em></td>
<td>ma</td>
</tr>
<tr>
<td><em>P. purpurea</em></td>
<td>h</td>
<td><em>Sphaerococcus cartilagineus</em></td>
<td>mp</td>
</tr>
<tr>
<td>(Syn: <em>P. umbilicalis</em></td>
<td></td>
<td><em>Suhria vittata</em></td>
<td>h, iu</td>
</tr>
<tr>
<td>f. laciniata)</td>
<td></td>
<td><em>Turnerella mertensiana</em></td>
<td>iu</td>
</tr>
</tbody>
</table>

CULTIVATION 493
Genus *Porphyra*

The genus *Porphyra* comprises three subgenera, viz. Euporphya, Diplodera and Diploderma (Krishnamurthy, 1972). According to Imada and co-authors (1971, p. 81), *Porphyra* species are now the basis for 'the most important coastal marine industry' in Japan. In China and Japan, the most frequently cultivated species are *Porphyra tenera*, *P. angustata*, *P. kumiedai* and *P. yeozonis* (Kurogi, 1961, 1963a, b). In the USA, the principal species utilized as food on the Pacific coast is *Porphyra perforata* (Tseng, 1947). In the British Isles, three species are harvested for food ('laverbread'): *Porphyra leucosticta*, *P. purpurea* and *P. umbilicalis* (Hampson, 1957; Conway, 1964a, b, c). *Porphyra* species are also cultivated along the southern coast of Korea (Kurogi, 1963a). The plants are generally harvested between October and February. The development of new culture techniques, using refrigerated collectors (see below), has enabled harvesting of good quality *Porphyra* to be extended even to March and April (Okazaki, 1971).

*Porphyra* species have been cultivated in Japan since 1870 (Okazaki, 1971). Studies on their life history have been published by Drew (1949, 1954), Kurogi (1953, 1963a, b), Graves (1955), Tseng and Chang (1955), Hollenberg (1958), Kornmann (1960, 1961b, 1970), Iwasaki (1961), Conway (1964a, b, c), Krishnamurthy (1969b) and Chen and co-authors (1970). The first investigations in this field were made by Drew (1949, 1954) and by Kurogi (1953, 1963a, b).

Drew found that the leafy thallus of *Porphyra umbilicalis* produces carpospores sexually, which develop into a filamentous shell-boring Conchocelis-phase. The growing Conchocelis filaments form pink patches on mollusc shells. This pink colour on shells was attributed as early as 1892 to Conchocelis rosea (Batters quoted by Drew, 1954 and Louis, 1967). The Conchocelis produces conchospores asexually, which develop into the leafy thallus, thus completing the cycle.

The growth of Conchocelis of *Porphyra linearis* within a piece of mussel shell is illustrated in Fig. 4-41. The Conchocelis is able to grow also free in culture dishes (Fig. 4-42A). Fig. 4-42 shows Conchocelis, conchospores and sporelings of *Porphyra leucosticta* obtained in culture (Kornmann, 1961b). In culture, the Conchocelis can be maintained, apparently indefinitely, through vegetative propagation and monosporcs (Iwasaki, 1961; Iwasaki and Matsuda, 1963; Conway, 1967; Krishnamurthy, 1969b) and this is probably the case in nature (Chen and co-authors, 1970).

Formation of neutral spores by the leafy thallus was found in *Porphyra umbilicalis* (Conway, 1964c), *P. tenera* (Tseng and Chang, 1955) and *P. leucosticta* (Kornmann, 1961b), but not in *P. linearis* and *P. miniata* (Chen and co-authors, 1970). According to Chen and co-authors (1970) perennation of leafy thalli through neutral spores probably occurs. Small leafy thalli develop directly from vegetative cells of Conchocelis in the species *Porphyra cuneiformis* (Krishnamurthy, 1969b).

The physical and nutritional factors which control the life cycle of *Porphyra* species have been investigated by many authors (Ogata, 1965a, b, c, 1966a, b, 1962; Iwasaki, 1961, 1963; Iwasaki and Matsuda, 1963; Kurogi and Sato, 1967; Kurogi and co-authors, 1967; Chen and co-authors, 1970; Furukawa, 1970). The conditions under which the various stages of the life history of *Porphyra miniata* occur are reported in Table 4-21. Release of conchospores in this species...
CULTIVATION

CULTIVATION takes place only at low temperatures (between 3° and 7° C). According to present knowledge on life histories of Bangia and Porphyra species, the formation of reproductive structures in the Conchocelis is induced by a photoperiodic, phytochrome-mediated system (HOSHAW and WEST, 1971).

OGATA (1962) tested the capacity of Conchocelis for growth in different media and found that phosphorus is indispensable for its development. IWASAKI (1965) studied the influence of different vitamin B₁₂ analogues, purines and pyrimidines and of plant hormones on the development of Conchocelis of Porphyra tenera. Maximum growth of Conchocelis was obtained with 0.2 μg ml⁻¹ of kinetin, 0.02 μg ml⁻¹ of indole-acetic acid and 0.4 μg ml⁻¹ of gibberellic acid. Maturation of Conchocelis is accelerated by a treatment with 50 to 100 μg ml⁻¹ of β-indol-potassium acetate (FURUKAWA, 1970). Additional studies of hormone effects are needed to elucidate the agents controlling reproduction and morphogenesis in Porphyra species.

The discoveries made by DREW (1949, 1954) and by Kurogi (1953, 1963a) are of great interest to both scientists and farmers. The modern farming methods developed for Porphyra species are based on laboratory cultures of the Conchocelis phase (FURUKAWA, 1970). Traditional methods of cultivating Porphyra species employed vertical collectors, the so-called 'Hibi' (YAMADA, 1959). A hibi consists mainly of bundles of tree branches (2–6 m long), set up diagonally to the bottom of a sandy sea area, and of blinds of split bamboo bound together. They were used for

![Image showing growth of Porphyra linearis Conchocelis phase](image-url)
attracting settling spores. Since 1930, horizontal collectors have been introduced (Suto, 1966). They consist of nets (mesh size about $15 \times 15$ cm) made from coconut palm, hemp palm, or from synthetic plastic fibres. The nets (40 m long, 1.2 m wide) are fixed by bamboo supports (Yamada, 1959). Floating of the nets is regulated by adjusting the length of the fixing ropes. Since 1953, the collection of spores is performed under artificial conditions (Okazaki, 1971). *Conchoecis* are cultivated in tanks, where disinfected oyster shells are used as spore collectors. The oyster shells are then hung on the nets when favourable culture conditions prevail.

The recent discovery that the *Porphyra* thalli may survive more than a year at $-20^\circ$C and grow again if returned to sea water (Suto, 1966; Okazaki, 1971) has permitted a further improvement of culture methods. When the *Porphyra* thalli

---

**Fig. 4-42:** *Porphyra* leucostica. *Conchoecis* phase and sporelings of the monostromatic thallus. a: Fertile filament of the *Conchoecis* phase grown free in a culture dish; b: release of its conchospores 4 hrs later; c: mass of freshly released conchospores; d: young *Porphyra*-fronds developed from conchospores. (After Kornmann, 1961; reproduced by permission of Biologische Anstalt Helgoland.)
Table 4-21

*Porphyra miniata.* Conditions under which the various life-cycle stages occur (After Chen and co-authors, 1970; modified; reproduced by permission of National Research Council of Canada from the *Canadian Journal of Botany, 48,* 385–389, 1970)

<table>
<thead>
<tr>
<th>Life-cycle stage</th>
<th>Temperature (°C)</th>
<th>Photoperiod (hrs)</th>
<th>Illuminance (lux)</th>
<th>Duration</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Release of α- and β-speses</td>
<td>5-15</td>
<td>8-16</td>
<td>200-5000</td>
<td>1 week</td>
<td>Release from field specimens</td>
</tr>
<tr>
<td>Germination of α-spores</td>
<td>5-15</td>
<td>8-16</td>
<td>200-5000</td>
<td>1 week</td>
<td>Most spores germinated within 7-10 days</td>
</tr>
<tr>
<td>Development of Conchocelis filaments</td>
<td>5-10</td>
<td>8-16</td>
<td>200-5000</td>
<td>4-5 weeks</td>
<td>Monosporangia developed soon after filament formation</td>
</tr>
<tr>
<td>Formation of conchosporangia</td>
<td>13-15</td>
<td>10-16</td>
<td>200-400</td>
<td>10-12 weeks</td>
<td>Sporangia 2 months after α-spospore germination</td>
</tr>
<tr>
<td>Discharge of conchospores and germination</td>
<td>3-7</td>
<td>8</td>
<td>300-1500</td>
<td>6 months</td>
<td>No release under other temperatures or photoperiods; most α-sporangia discharged during third and fourth month</td>
</tr>
<tr>
<td>Growth of leafy thallus</td>
<td>5-15</td>
<td>8-16</td>
<td>300-6000</td>
<td>4 months</td>
<td>Best growth after induction at low temperature followed by incubation at 13°C; aeration (air or 0.6% CO₂ in air) improved growth considerably</td>
</tr>
<tr>
<td>Formation of α- and β-sporangia</td>
<td>13</td>
<td>10</td>
<td>1000-2000</td>
<td>4-5 weeks</td>
<td>Spores usually formed after thalli reached lengths of 30-50 cm</td>
</tr>
</tbody>
</table>
4. CULTIVATION OF PLANTS (S. BONOTTO)

reach lengths of 2 to 3 cm on the collectors, they are refrigerated at \(-20^\circ\) C in polyethylene bags, after their moisture content has been reduced to 20 to 30%. Cultures of *Porphyra* are then started simply by returning the collectors to sea water of normal temperature. This technique has spread rapidly; in 1970, about 50% of the *Porphyra* produced were harvested from previously refrigerated nets (OKAZAKI, 1971). The refrigerated-net method is very profitable: it assures a good crop and permits the harvesting season to be prolonged.

**Genus Gracilaria**

*Gracilaria* species are important as a source of extractives. They are used for agar production in Australia, Japan, New Zealand, South Africa and USA, and are exploited also in other countries (WOOD and PEDDIE, 1941; HUMM, 1944; BONEY, 1965; LEVRING and co-authors, 1969). According to SIMONETTI and co-authors (1970), *Gracilaria confervoides*, which grows abundantly in the Northern Adriatic Sea, (PIGNATTI, 1962) is exploited for agar in Italy. Several reports exist on the ecology of some *Gracilaria* species and on their growth in nature (MINO, 1949; PILLSBURY, 1960; SAWADA and co-authors, 1955a, b; STOKKE, 1956, 1957; OHMI, 1958; SAWADA, 1958; JONES, 1959a, b; PIGNATTI, 1962; MITRAKOS, 1964; YAMAMOTO, 1969; KIM, 1970).

Although many investigators have studied different aspects of the biology of *Gracilaria*, details on its life cycle have become known only recently (OGATA and co-authors, 1972). These authors have obtained the complete life cycle of *Gracilaria verrucosa* in culture. Uni-algal cultures have been achieved by adding to the medium 5 ppm of germanium dioxide, which suppresses diatom growth (LEWIN, 1966b).

OGATA and co-authors (1972) found that *Gracilaria verrucosa* has a typical 'Polysiphonia-type' of life cycle and, by separating individual gametophytes, were able to demonstrate the necessity of fertilization. These findings may have significant practical consequences for improving the culture techniques employed in commercial *Gracilaria* production. In Japan, *Gracilaria* species is mainly cultivated by suspending its branches in twisted ropes in zones where the sea water is rich in nutrients.

(6) Multicellular Algae: Conclusions

Research cultivation has elucidated the life history of *Saccorhiza bulbosa* (SAUVAGEAU, 1915), 'an epoch-making discovery' (KORNMAN, 1970, p. 40); of species of the red edible alga genus *Porphyra* (DREW, 1949, 1964; KUROGI, 1953, 1963a); and of several other seaweeds of commercial importance (HILTON and MCLEAN, 1971; HOSHIW and WEST, 1971; CHEN and MCLACHLAN, 1972; OGATA and co-authors, 1972). Many results obtained in basic research have helped to build the fundament for large-scale commercial cultivation of marine algae.

Marine algae are useful for man in many ways: they are used as human and animal food, in medicine, in terrestrial farming projects (manure), and provide raw materials for several industries (for details consult LEVRING and co-authors, 1969). Seaweeds have been used as food since ancient times, especially in Japan and China. Their importance as food is expected to increase in the future. Terrestrial
ecosystems alone cannot meet the increasing nutritional demands of the growing human population:

‘If we consider that at the present the photosynthetic activity in oceans is providing about $120 \times 10^9$ tons of organic carbon per year and terrestrial ecosystems only $20 \times 10^9$, it is obvious that the great hope for food is from the oceans, although the present technologies make only 0.1% of that available as food for man’ (STIRN, 1971, p. 14).

Additional efforts must be made in the future to increase the harvest from the sea. Increased production of multicellular algae for economic ends requires: (i) improvements of cultivation techniques; (ii) extension of seaweed cultures to a larger portion of the suitable sea areas; (iii) reduction of plant loss due to diseases (Chapter 8), predation and other factors. Marine pollution (KECKES and BERNHARD, 1970; ZATTERA and BERNHARD, 1970; STIRN, 1971; Volume V) will progressively interfere with life in oceans and coastal waters, and seriously impede aquaculture on our shores, unless regulations are issued and effectively controlled.

(7) Sea Grasses

The marine flowering angiosperms are commonly known as sea grasses or eel grasses (PHILLIPS, 1960; DEN HARTOG, 1964). They have the remarkable capability to flower and pollinate underwater. All belong to the order Naiadales (Helobiaceae) which contains 5 families (McCONNAUGHEY, 1970): Hydrocharitaceae (Enhalus, Halophila, Thalassia), Posidoniaceae (Posidonia), Ruppia (Ruppiaceae), Zannichelliaceae (Althenia, Amphibolis, Cymodocea, Halodule, Syringodium, Zannichelia), and Zosteraceae (Phyllospadix, Zostera).

Most sea grasses inhabit tropical areas; but the genera Phyllospadix and Zostera are typical of temperate zones. Ecologically, Zostera, Phyllospadix, Cymodocea and Thalassia are perhaps the best known and the most important genera. Marine grasses support animal life, stabilize the bottom, favour sediment deposition and contribute to local organic production. Some sea grasses (e.g. Thalassia testudinum) may also be used as animal fodder (BAUERSFELD and co-authors, 1969). The potential harvest of the turtle grass T. testudinum is very large: the area off the coast of Florida, between Tarpon Springs and Apalachee Bay, alone can produce 11,200,000 tons of dry leaves (BAUERSFELD and co-authors, 1969).

The eel grass Zostera and the surf grass Phyllospadix have been studied by SETCHELL (1929), DEN HARTOG (1964), MACFARLANE (1964), BONEY (1965), CERMA and CAPPELLO (1965), OGATA and MATSU (1965a, b), DAWSON (1968), GESSNER (1968), OGATA (1968). Zostera marina and Z. nana usually grow in shallow lagoons and bays; but Z. marina may occur also at depths down to 50 m. The importance of the genus Zostera in nature was dramatically demonstrated in 1931-1932, when a severe disease, caused by the parasitic slime mould Labyrinthula macrocystis (YOUNG, 1943), destroyed the eel-grass beds on the Atlantic coasts of America and Europe (Chapter 8). This ecological disaster considerably reduced the number of animals (invertebrates, fishes, birds) depending directly or indirectly on the eel grass. In some areas the effects of the eel-grass disaster were still apparent 20 to 30 years later (McCONNAUGHEY, 1970).
Harvesting of *Zostera* on a commercial basis occurred from 1929 to 1932, until the eel grass became unavailable because of the disease; later on, from 1945 to 1960, the annual crop in the Maritime Provinces (Canada) reached 750 tons per year (MacFarlane, 1964). In spite of its great ecological importance and of the *Zostera* demand for industrial uses, commercial cultivation is not at present performed. However, laboratory culture of *Zostera* for ecological research was successfully obtained by North and his group (Kelp Habitat Improvement Project, 1970, p. 48).

### (8) Mangroves

In addition to wholly submerged marine angiosperms, there is a large group of marine plants which are only partially covered at high tide. They are collectively known as mangroves, but do not represent a taxonomically defined group: There are about 30 species of mangroves which belong to several different plant families. A classification of mangroves has been presented by McConnaughey (1970).

Mangroves are found in quiet lagoons and estuaries. They are provided with prop roots which hold the plant body above the water. Mangrove plants often form impenetrable thickets in humid tropical areas. They play an important role in stabilizing the bottom, in holding sediments and in adding detritus to their substrate, thus facilitating the extension of coastal land areas. From an ecological point of view, mangroves are particularly interesting, because they support the life of numerous other plants and animals (e.g. Glebch, 1958; Dawson, 1966; McConnaughey, 1970). The algae associated with mangroves belong mainly to the Rhodophyceae (genera *Bostrychia*, *Caloglossa*, *Catenella* and *Murrayella*). On mud, species of *Caulerpa*, *Cladophoropsis* or *Vaucleria* may occur (Dawson, 1966).

In view of their ecological importance, more attention should be paid to mangroves and their habitats—including the associated multicellular algae and planktonic organisms (Tundisi and Teixeira, 1968; Tundisi and Tundisi, 1968). The habitat-forming role of mangroves qualifies them for controlled protection. Although mangrove plants have been used in the past for tannin production, they are not cultivated commercially.

### (9) The Salt-marsh Plants

Salt-marsh plants do not represent a particular taxon. They comprise mainly terrestrial plants rooted within tidal reach. The main species of this flora belong to the genera *Limonium*, *Puccinellia*, *Salicornia*, *Spartina* and *Spergularia* (e.g. Dawson, 1966). These halophytes are often subjected to partial inundation and are only rarely submerged for brief periods. Salt marshes usually contain numerous channels, where several marine algae (*Enteromorpha*, *Percuraria*, *Rhizoclonium*, *Ulota*, *Ulva*) may be found. In North Atlantic and Baltic areas, modified forms of *Ascomythum*, *Fucus* and *Pelvetia* have been found which live free on the marsh or embedded in the mud (Dawson, 1966).

Members of the low marsh flora, such as *Salicornia* have, in the past, attracted attention for economic reasons. *Salicornia* species have been used to produce soda (p. 471). Some salt-marsh plants have attained importance because they protect
the coasts and facilitate gain of new land from the sea; large numbers of young plants are cultivated in the laboratory and later planted in suitable areas. _Puccinellia_ species have been cultivated for ecological studies on salt and submergence tolerances, conducted under artificial conditions (von Weixl and Dreyling, 1970).

(10) Spermatophyta: Conclusions

In spite of the ecological importance of these plants (sediment stabilization, coast protection, gain of new land, habitat-forming qualities for other plants and for a large variety of animals), only few attempts have been made to make representatives available for research cultivation. The present economic value of most of these plants is low. No cases of large-scale commercial cultivation have come to the reviewer's attention.

**Literature Cited (Chapter 4)**


LITERATURE CITED


LITERATURE CITED


4. CULTIVATION OF PLANTS


LITERATURE CITED


IVERSEN, E. S. (1968). Forming the Edge of the Sea, Fishing News (Books), London.


4. CULTIVATION OF PLANTS


Kylin, H. (1917). Über den Einfluss der Wasserstoff und Konzentration auf einen Meeres-


Lau, E. P. (1934). Retention of dichromate by glassware after exposure to potassium dichro-


Lersten, N. R. and Voth, P. F. (1960). Control of zooid discharge and other responses in the


16, 45-56.


Levin, J. C. (1966a). Physiological studies of the boron requirement of the diatom, Cylindro-

Levin, J. C. (1966b). Silicon metabolism in diatoms. V. Germanium dioxide, a specific in-
hibitor of diatom growth. Phycologia, 6, 1-2.


Phycologia, 6, 211-217.


Levin, J. and Hellgeurst, J. A. (1970). Heterotrophic nutrition of the marine pennated diatom,

LITERATURE CITED


LEWIS, E. J. (1962). Studies on the proteins, peptides and free amino acid contents in some species of brown algae from the south-eastern coast of India. J. gen.


LITERATURE CITED


4. CULTIVATION OF PLANTS


LITERATURE CITED


LITERATURE CITED

4. CULTIVATION OF PLANTS


LITERATURE CITED


### AUTHOR INDEX

Numbers in italics refer to those pages on which the Author's work is stated in full.

<table>
<thead>
<tr>
<th>Author</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aaronson, S.</td>
<td>347, 356, 370, 406, 407, 430, 446, 501, 515</td>
</tr>
<tr>
<td>Abbott, B. C.</td>
<td>420, 521</td>
</tr>
<tr>
<td>Abdel-Fattah, E.</td>
<td>36, 268</td>
</tr>
<tr>
<td>Abe, T.</td>
<td>141, 268, 287</td>
</tr>
<tr>
<td>Abraham, F. S. H.</td>
<td>188, 268</td>
</tr>
<tr>
<td>Ackman, R. G.</td>
<td>371, 501</td>
</tr>
<tr>
<td>Adair, F. W.</td>
<td>331, 356</td>
</tr>
<tr>
<td>Adams, I.</td>
<td>367, 521</td>
</tr>
<tr>
<td>Adams, M. N. E.</td>
<td>388, 392, 456, 459, 523</td>
</tr>
<tr>
<td>Addison, R. F.</td>
<td>371, 501</td>
</tr>
<tr>
<td>Aderberg, E. A.</td>
<td>326, 363</td>
</tr>
<tr>
<td>Adron, J. W.</td>
<td>263, 267, 268</td>
</tr>
<tr>
<td>Ahearn, D. G.</td>
<td>347–351, 356, 358, 361, 362</td>
</tr>
<tr>
<td>Akiyama, K.</td>
<td>493, 516</td>
</tr>
<tr>
<td>Aksuvo-Bebrasse, J.</td>
<td>368, 523</td>
</tr>
<tr>
<td>Albrecht, D.</td>
<td>19, 180, 268</td>
</tr>
<tr>
<td>Alder, M. G.</td>
<td>160, 268</td>
</tr>
<tr>
<td>Alderdice, D. F.</td>
<td>40, 254, 256, 257, 258, 269</td>
</tr>
<tr>
<td>Alderman, D. J.</td>
<td>337, 358, 359</td>
</tr>
<tr>
<td>Aldrich, D. V.</td>
<td>416, 418, 438, 450, 501</td>
</tr>
<tr>
<td>Aker, A. A.</td>
<td>253, 269, 370, 380, 413, 420, 501</td>
</tr>
<tr>
<td>Alexander, M.</td>
<td>333, 356</td>
</tr>
<tr>
<td>Alogus, S.</td>
<td>392, 444, 501</td>
</tr>
<tr>
<td>Alley, W. C.</td>
<td>79, 206, 269, 294</td>
</tr>
<tr>
<td>Allen, E. C.</td>
<td>323, 365</td>
</tr>
<tr>
<td>Allen, E. J.</td>
<td>370, 379, 382, 388, 407, 421, 426, 427, 432, 501</td>
</tr>
<tr>
<td>Allen, M. B.</td>
<td>450, 501</td>
</tr>
<tr>
<td>Allen, P. M.</td>
<td>128, 287</td>
</tr>
<tr>
<td>Altman, P. L.</td>
<td>198, 269</td>
</tr>
<tr>
<td>Amant, P. P. St.</td>
<td>125, 269</td>
</tr>
<tr>
<td>Ambuhl, H.</td>
<td>203, 269</td>
</tr>
<tr>
<td>Ames, L. L. Jr.</td>
<td>164, 167, 269, 287</td>
</tr>
<tr>
<td>Amsterdam, D.</td>
<td>452, 521</td>
</tr>
<tr>
<td>Anastasiou, C. J.</td>
<td>343, 356</td>
</tr>
<tr>
<td>Anderson, A. H.</td>
<td>263, 269</td>
</tr>
<tr>
<td>Anderson, G.</td>
<td>472, 501</td>
</tr>
<tr>
<td>Anderson, D.</td>
<td>126, 269</td>
</tr>
<tr>
<td>Anderson, E. K.</td>
<td>477, 489, 501</td>
</tr>
<tr>
<td>Anderson, J. I. W.</td>
<td>315, 356</td>
</tr>
<tr>
<td>Anderson, J. W.</td>
<td>78, 269</td>
</tr>
<tr>
<td>Anderson, P. A.</td>
<td>217, 269</td>
</tr>
<tr>
<td>Anderson, W.</td>
<td>470, 501</td>
</tr>
<tr>
<td>Ando, S.</td>
<td>493, 513</td>
</tr>
<tr>
<td>Anonymous, 110, 160, 269</td>
<td></td>
</tr>
<tr>
<td>Ansell, A. D.</td>
<td>375, 426, 457, 459, 502</td>
</tr>
<tr>
<td>Anthony, R.</td>
<td>301, 269</td>
</tr>
<tr>
<td>Antia, N. J.</td>
<td>98, 132, 133, 269, 381, 416, 418, 429, 460, 494, 502, 504, 505, 507</td>
</tr>
<tr>
<td>Aquarium Water Chemistry Manual, 33, 269</td>
<td></td>
</tr>
<tr>
<td>Arasaki, S.</td>
<td>493, 502</td>
</tr>
<tr>
<td>Ardré, F.</td>
<td>472, 502</td>
</tr>
<tr>
<td>Arthur, J. W.</td>
<td>158, 269</td>
</tr>
<tr>
<td>Arx, J. A. von</td>
<td>338, 356</td>
</tr>
<tr>
<td>Ashton, J. B.</td>
<td>155, 270</td>
</tr>
<tr>
<td>Atkins, W. R. G.</td>
<td>108, 269</td>
</tr>
<tr>
<td>Atkinson, J. W.</td>
<td>158, 269</td>
</tr>
<tr>
<td>Atz, J. W.</td>
<td>19, 24, 42, 97, 98, 147, 166, 269</td>
</tr>
<tr>
<td>Aubert, J.</td>
<td>26, 27, 269</td>
</tr>
<tr>
<td>Aubert, M.</td>
<td>26, 27, 269</td>
</tr>
<tr>
<td>Austin, A. P.</td>
<td>493, 502</td>
</tr>
<tr>
<td>Avault, J. W., Jr.</td>
<td>10, 16</td>
</tr>
<tr>
<td>Azam, F.</td>
<td>98, 269</td>
</tr>
<tr>
<td>Baalen, C. van</td>
<td>206, 207, 269, 276, 479, 480, 508</td>
</tr>
<tr>
<td>Baam, R. B.</td>
<td>27, 270</td>
</tr>
<tr>
<td>Baars, J. K.</td>
<td>189, 270</td>
</tr>
<tr>
<td>Baatz, I.</td>
<td>443, 502</td>
</tr>
<tr>
<td>Bachrach, H.</td>
<td>463, 454, 502</td>
</tr>
<tr>
<td>Backhaus, D.</td>
<td>32</td>
</tr>
<tr>
<td>Bader, R. G.</td>
<td>140, 270</td>
</tr>
<tr>
<td>Bahl, K. N.</td>
<td>85, 270</td>
</tr>
<tr>
<td>Bailey, P. S.</td>
<td>155, 270</td>
</tr>
<tr>
<td>Bailey, W. A.</td>
<td>441, 506</td>
</tr>
<tr>
<td>Baker, H.</td>
<td>370, 373, 386, 388, 446, 452, 501, 512, 521</td>
</tr>
<tr>
<td>Baku, G. I.</td>
<td>467, 502</td>
</tr>
<tr>
<td>Balakrishnan, S.</td>
<td>125–125, 270</td>
</tr>
<tr>
<td>Baldwin, F.</td>
<td>85, 270</td>
</tr>
<tr>
<td>Balinsky, J. B.</td>
<td>85, 270</td>
</tr>
<tr>
<td>Balloy, M.</td>
<td>46, 270</td>
</tr>
<tr>
<td>Baly, E. C. C.</td>
<td>128, 270</td>
</tr>
<tr>
<td>Banerjee, R. K.</td>
<td>62, 282</td>
</tr>
<tr>
<td>Bank, H.</td>
<td>419, 509</td>
</tr>
<tr>
<td>Barber, R. T.</td>
<td>140, 270</td>
</tr>
<tr>
<td>Bardach, J. E.</td>
<td>10, 16, 62–64, 66–70, 270</td>
</tr>
<tr>
<td>Bardsley, D. M.</td>
<td>128, 270</td>
</tr>
<tr>
<td>Baren, C. P.</td>
<td>440, 446, 509</td>
</tr>
<tr>
<td>Barisco, E. S.</td>
<td>337, 356</td>
</tr>
<tr>
<td>Barker, H. A.</td>
<td>409, 421, 422, 432, 447, 454, 502</td>
</tr>
<tr>
<td>Barnabé, C.</td>
<td>265, 270</td>
</tr>
<tr>
<td>Barnes, H.</td>
<td>80, 93, 97, 146, 270</td>
</tr>
</tbody>
</table>
AUTHOR INDEX

533

Brown, C. W., 105, 272
Bryant, M., 89, 90, 290
Bryson, V., 217, 272
Buck, J. D., 27, 272, 347, 357
Buegel, R. G., 471, 504
Bulman, M., 92, 272, 462, 504
Bullen, J. J., 451, 506
Bulnheim, H.-P., 10, 17, 69, 284
Bunt, J. S., 391, 415, 504
Burkhardt, B., 155, 157, 158, 287
Burkholder, P., 387, 504
Burkholder, P. R., 303, 307, 309, 357
Burlew, J. S., 396, 504
Burnett, W. E., 129, 272
Burns, R. L., 493, 504, 513
Burns, R. W., 107, 272
Burrows, A., 449, 454, 503
Burrows, R. E., 62, 81, 90, 91, 98, 128, 162, 188, 273, 296
Butler, S. D., 310, 332, 357
Busby, W. P., 374, 399, 411, 431, 504, 516, 517
Butcher, R. W., 378, 379, 445, 504
Butkevitch, V. S., 333, 357
Butler, E. I., 84, 273
Butler, M. R., 381, 528
Butelefield, C. T., 128, 273
Buttolph, L., 161, 273
Buzzati-Traverso, A. A., 10, 16
Byrne, P., 338, 359

Cahn, R. D., 315, 357
Campbell, J. W., 80, 83, 85, 98, 273
Campion-Alsumard, T., Le, 338, 360
Cannefax, G. R., 206, 273
Caperon, J., 402, 504
Capello, M., 499, 505
Canaves, A., 468, 471, 504
Canazzone, M. M., 158, 273
Carley, C. L., 25, 20, 297, 381, 528
Carlson, C. W., 423, 521
Carlucci, A. F., 26-27, 95, 96, 98, 102, 273, 276, 280, 315, 332, 357, 358, 369, 417-421, 426, 504, 516, 523
Carnes, W. C., 261, 273
Carpenter, E. J., 401, 402, 410, 465, 504
Carpenter, J. H., 145, 273
Carr, N. G., 197, 198, 273
Carr, T. E. F., 470, 505
Carrit, D. E., 145, 146, 279
Carver, C. E., Jr., 149, 273, 282
Casey, R. P., 388, 505
Cassee, V., 381, 418, 434, 510
Castelao-Branco, R., 347, 349, 364
Castrabbert, A. O., 155, 275
Castro, W. E., 236, 237, 293, 300
Cechel, W. H., 156, 286

Cerma, E., 499, 505
Challen, S. B., 470, 471, 505
Chalopin, M. C., 471, 504
Chang, T. J., 494, 527
Chapman, A. R. O., 224, 226, 273
Chapman, G., 78, 273
Chapman, G. B., 334, 364
Chapman, V. J., 460, 472, 484, 491, 505
Chau, H., 368, 505
Chaudhuri, T. K., 475, 503
Chen, C., 412, 516
Chen, L. C.-M., 473, 494, 497, 498, 505
Chen, P. K., 334, 364
Cheng, J. Y., 416, 418, 429, 464, 602
Cheng, T. H., 71, 273, 468, 471, 472, 484, 485, 487, 505
Chenouwer, H. H., 62, 90, 273, 296
Chin, E., 46, 274
Chipman, W. A., 103, 274
Chirife, J., 489, 505
Cholodny, N., 323, 357
Chorney, V., 374, 517
Chorn, R., 338, 358
Christensen, E., 155, 278
Chu, S. P., 372, 381, 382, 410, 421, 426, 432, 434, 505
Chuo, T., 128, 274
Churchland, L. M., 343, 356
Clancey, V. J., 26, 168, 295
Clark, J. B., 38, 274
Clark, L. B., 375, 400, 519
Clark, R. L., 38, 274
Clark, W. J., 378, 463, 505
Clarner, J. F., Jr., 8, 17
Claudio, F., 469, 505
Clauss, H., 367, 510
Chrlenk, J. R., 463, 516
Chrlenk, K. A., 489, 505
Clesker, N. L., 164, 274
Clifton, C. E., 102, 274
Cobet, A. B., 331, 364
Cohen, J. M., 402, 505
Coint, L., 166, 274
Colard, J., 470, 503
Coler, R. A., 382, 505
Coler, E. H., 350, 364
Collier, A., 422, 529
Colliver, A. W., 21, 25, 274
Colwell, R. R., 303, 334, 357, 364
Comes, B. D., 126, 162, 188, 273
Conn, J. E., 313, 365
Conners, D. N., 30, 31, 35, 36, 283
Connover, R. J., 33, 84, 212-214, 274, 331, 357
Conway, E., 494, 505
AUTHOR INDEX 537

HALL, R. P., 405, 510
HALLDAL, P., 207, 279, 289, 444, 445, 480, 510
HALLISON, S. V., 468, 510
HAMILTON, R. D., 96, 102, 279, 280, 315, 318, 358, 426, 510
HAMMEN, C. S., 83, 280, 286
HAMPSHIRE, M. A., 494, 510
HANES, N. H. B., 20, 26, 280
HANLEY, F. D., 280
HANKS, J. E., 456, 517
HANNERZ, L., 201, 280
HANSEN, S. F., 112, 280
HARADA, T., 72, 162, 280, 283
HARDER, W., 149, 278
HARDY, A. C., 79, 280
HART, R., 484, 510
HARTMAN, G. F., 203, 280
HARTMAN, R. A., 484, 510
HARTOG, C. DEN, 499, 510
HASEOAWA, Y., 468, 473, 483–485, 487, 511, 524
HASELTINE, A. W., 39–41, 276
HASELTINE, T. R., 128, 280
HASKINS, C. P., 407, 512
HASTINGS, W. H., 400, 445, 465, 511, 526
HATTORI, A., 81, 280
HAVENSCHILD, C., 31, 35, 58, 202, 280
HAUG, A., 484, 511
HAWLEY, J. E., 22, 23, 291
HAXO, F. T., 71, 289, 422, 477, 485, 490, 511, 519
HAYNES, H., 161, 273
HEAD, R. N., 83, 84, 274
HEDIGER, H., 42, 280
HEFFERMAN, W. P., 315, 356
HEITZ, J. A., 154, 280
HEIT, G., 140, 280
HEIMBERG, S., 230, 279
HELLEBUST, J. A., 409, 415, 416, 429, 463, 484, 506, 511, 516
HELMERS, E. N., 124, 281
HEMELRIJK, L. VAN, 480, 524
HEMENS, J., 132, 281
HEMINGWAY, J. C., 470, 471, 505
HENNEUSE, P. R., 332, 357
HERALD, E. S., 42, 162, 163, 275
HERBERT, D., 3, 4, 17, 90, 211, 213, 216, 220, 276, 281
HERBERT, D. W., 38, 285
HERITAGE, G. D., 256, 258–261, 272
HERMERT, D. VAN, 140, 292
HERN, D. H., 160, 290
HERNANDEZ, T., 85, 274
HERICK, C. C., 160, 284
HERR, K., 206, 281
HERTZ, M. R., 349, 359
HERVEY, A., 419, 424, 523
HERVEY, R. J., 372, 511
HETZLER, W. F. JR., 40, 41, 281
HEUSER, G. F., 423, 521
HICKS, D. B., 206, 281
HIDAKA, T., 305, 306, 359
HIDU, H., 404, 511
HIESY, W. M., 446, 511
HIGGS, D. A., 256, 272
HILL, G. R., 160, 268
HILL, W. F., JR., 373, 377, 450, 455, 506
HILTON, R. L., JR., 473, 498, 511
HINTON, S., 41, 166, 281
HIRAYAMA, K., 97, 171, 173–176, 181, 281
HIRSCHFELD, D. S., 140, 270
HJELM, K. K., 203, 281
HOAGLAND, D. R., 446, 506
HOAR, W. S., 46, 286
HOATHER, R. C., 154, 161, 281
HOBBIE, J. E., 475, 511
HOEK, C. VAN DEN, 378, 511
HOLLADAY, L. L., 161, 286
HOLLEMBERO, G. J., 494, 511
HOLM-HANSEN, O., 98, 269, 276, 373, 375, 388, 443, 458, 464, 466, 512, 526
HONIG, C., 23, 97, 282
HOOD, D. W., 140, 270, 415, 512
HOOGENHOUT, H., 397, 399, 400, 512
HOOFER, S. N., 371, 501
HOOVER, R. L., 222, 278
HOPPE, H. A., 467–469, 471, 472, 480–483, 489–491, 498, 516
AUTHOR INDEX

HOFSTETTER, H., 21, 282
HOSHAW, R. W., 473, 495, 498, 512
HOSKIN, C. M., 203, 289
HOTCHKISS, M., 26, 298, 381, 528
HOUET, G., 85, 277
HOWELL, J. A., 401, 432, 512, 523
HOSKING, T. H., 46, 108, 272
HOUWELL, J. W., 473, 477, 512
HUANG, C.-Y., 62, 285
HUANG, C. L., 156, 282
HUBER, L., 128, 282
HUDSON, J. P., 446, 512
HUCKSTEEDT, G., 31, 32, 35, 36, 157, 191, 282
HUG, W., 356, 359
HUGHES, J. C., 412, 512
HUQUE, K., 401, 432, 512
HUMPHREY, R. E., 9, 10, 17, 48–50, 57, 199, 282, 296, 388, 524
HUNTER, S. H., 330, 362, 368, 373, 386, 387, 388, 407, 411, 412, 423, 446, 452, 512, 527
HUTTON, W. E., 329, 339
HUYNH, H., 468, 572
HWANG, S., 345, 359, 464, 512
ICHIMURA, T., 64, 299
IIMAMA, O., 475, 493, 494, 513
IIMOTO, T., 440, 444, 447, 460, 529
ING, R. B., 369, 512
INGELSTAD, B., 313, 359
INGELS, R. S., 155, 277
IPPEL, A. T., 149, 282
ISENBERG, H. D., 413, 417, 454, 513
ISHIKAWA, Y., 64, 299
ISHII, K., 149, 282
ISHIO, F., 97, 98, 285
IVERSEN, S. E., 62, 282, 460, 472, 493, 513
IVES, K. J., 462, 513
IWANOTO, K., 482, 513
IWANOTO, K., 482, 513
IWASHI, H., 418, 420, 476, 493–495, 513
IWASE, K., 149, 289
JACOB, H.-E., 109–111, 282
JACOBSEN, H., 421, 513
JACOBSON, A. R., 155, 282
JAHN, T. L., 416, 523
JAMISON, D., 472, 513
JANNASCH, H. W., 212, 297, 301, 302, 315, 317, 318, 335, 348, 353–361
JANOWSKI, M., 475, 503
JANSON, S. O., 206, 282
JATZKE, P., 231, 232, 282
JAWED, M., 82, 84, 282
JEBRIM, D., 191, 242–244, 282
JENG, D., 141, 178, 282
JENSEN, A., 218, 219, 282, 375, 377, 468, 513, 519
JERLOV, N. G., 161, 282
JHONSON, V. G., 441, 443, 445, 446, 513
JORANSSON, B., 118, 131, 133, 134, 157, 191, 282
JOHANNESSON, B., 128, 292
JOHANSSON, H., 93, 279
JOHANSSON, M. W., 21, 80, 296, 432, 526
JOHNSON, W. H., 123, 128, 282
JOHNSTONE, R., 93, 282, 370, 380, 420, 425, 429, 514
JOHNSTONE, R. I., 380, 514
JONES, G. E., 313–315, 331, 333, 358, 360, 364
JONES, G. L., 127, 282
JONES, J. J., 472, 509
JONES, L. G., 477, 484, 520
JONES, R. F., 400, 401, 410, 411, 445, 446, 453, 514, 518
JONES, W. E., 473, 477–479, 498, 514, 525
JORGENSEN, P. H., 86, 283
JUKES, T. H., 423, 512
JUNE, H. D., 73, 76, 272
JUST, E. E., 420, 514
KACHMAR, J. F., 128, 292
KACHWALLA, N., 347, 357
KADOTA, H., 307, 308, 360
KAKAMATSU, T., 128, 289
KARIMOTO, D., 334, 360, 420, 514
KALLE, K., 21, 29, 35, 97, 98, 145, 283, 415, 433, 448, 514
KALLENBERG, H., 203, 283
KAMIMURA, M., 162, 283
KANAZAWA, A., 420, 468, 514
KANJO, J. L., 470, 509
KARLSON, P., 79, 283
KASHIWADA, K., 420, 514
KATZ, M., 91, 275
# Author Index

<table>
<thead>
<tr>
<th>Author</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otoe, H.</td>
<td>329, 361</td>
</tr>
<tr>
<td>Otona, E.</td>
<td>154, 290</td>
</tr>
<tr>
<td>Overbeck, J.</td>
<td>93, 268</td>
</tr>
<tr>
<td>Overholt, M. N.</td>
<td>472, 509</td>
</tr>
<tr>
<td>Owens, J. S.</td>
<td>191, 289</td>
</tr>
<tr>
<td>Oyama, Y.</td>
<td>149, 289</td>
</tr>
<tr>
<td>Ozone Chemistry and Technology</td>
<td>155, 290</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Author</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paasche, E.</td>
<td>238, 290, 382, 400, 409, 445, 448, 454, 508, 521</td>
</tr>
<tr>
<td>Pace, R. D.</td>
<td>223–225, 290</td>
</tr>
<tr>
<td>Packer, E. L.</td>
<td>384, 452, 521, 523</td>
</tr>
<tr>
<td>Padeh, E.</td>
<td>379, 521</td>
</tr>
<tr>
<td>Paffenholzer, G. A.</td>
<td>231–234, 235, 290</td>
</tr>
<tr>
<td>Paerati, B. B.</td>
<td>62, 282</td>
</tr>
<tr>
<td>Paik, A. T.</td>
<td>158, 269</td>
</tr>
<tr>
<td>Palermoni, N. J.</td>
<td>311, 363</td>
</tr>
<tr>
<td>Palmer, C. M.</td>
<td>404, 521</td>
</tr>
<tr>
<td>Parke, G. H.</td>
<td>158, 287</td>
</tr>
<tr>
<td>Park, K.</td>
<td>415, 512</td>
</tr>
<tr>
<td>Park, M.</td>
<td>367, 379, 521</td>
</tr>
<tr>
<td>Parker, B.</td>
<td>140, 290</td>
</tr>
<tr>
<td>Parkhurst, J. D.</td>
<td>136–139, 290</td>
</tr>
<tr>
<td>Parry, E. P.</td>
<td>160, 290</td>
</tr>
<tr>
<td>Parry, G.</td>
<td>89, 83, 290</td>
</tr>
<tr>
<td>Parsons, J. R.</td>
<td>73, 93, 95, 97, 146, 178, 290, 295, 460, 464, 466, 502, 517, 526</td>
</tr>
<tr>
<td>Pasero, J.</td>
<td>164, 275</td>
</tr>
<tr>
<td>Pastor, Z.</td>
<td>420, 521</td>
</tr>
<tr>
<td>Pasteur, M. L.</td>
<td>80, 101, 290</td>
</tr>
<tr>
<td>Paul, T. M.</td>
<td>470, 525</td>
</tr>
<tr>
<td>Pavese, A.</td>
<td>151–153, 189, 290</td>
</tr>
<tr>
<td>Pavoni, J. L.</td>
<td>107, 155, 156, 158, 289, 290, 462, 527</td>
</tr>
<tr>
<td>Payne, W. J.</td>
<td>306, 329, 362</td>
</tr>
<tr>
<td>Peach, E. A.</td>
<td>421, 432, 521</td>
</tr>
<tr>
<td>Peak, M. J.</td>
<td>206, 290</td>
</tr>
<tr>
<td>Peddie, H. M.</td>
<td>493, 498, 529</td>
</tr>
<tr>
<td>Pedersen, M.</td>
<td>475, 476, 521</td>
</tr>
<tr>
<td>Pechers, P. N.</td>
<td>149, 290</td>
</tr>
<tr>
<td>Perleb, H. T.</td>
<td>423, 521</td>
</tr>
<tr>
<td>Pelczar, M. J., Jr.</td>
<td>102, 290, 314, 362</td>
</tr>
<tr>
<td>Pellegrini, M.</td>
<td>488, 512</td>
</tr>
<tr>
<td>Pennelly, D. M.</td>
<td>468, 521</td>
</tr>
<tr>
<td>Perfiliev, B. V.</td>
<td>323, 324, 362</td>
</tr>
<tr>
<td>Perkins, D. D.</td>
<td>464, 521</td>
</tr>
<tr>
<td>Perlman, R. G.</td>
<td>156, 290</td>
</tr>
<tr>
<td>Perrier, J. P.</td>
<td>411, 515</td>
</tr>
<tr>
<td>Perry, E. S.</td>
<td>102, 290</td>
</tr>
<tr>
<td>Persone, G.</td>
<td>38, 208–210, 287, 295</td>
</tr>
<tr>
<td>Pesando, D.</td>
<td>27, 269</td>
</tr>
<tr>
<td>Pesch, G.</td>
<td>33, 35, 36, 300</td>
</tr>
</tbody>
</table>

---

<table>
<thead>
<tr>
<th>Author</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pescheck, E.</td>
<td>154, 290</td>
</tr>
<tr>
<td>Peter, H.</td>
<td>102, 274</td>
</tr>
<tr>
<td>Petersen, H. J.</td>
<td>337, 362</td>
</tr>
<tr>
<td>Petersen, J. A.</td>
<td>86, 283</td>
</tr>
<tr>
<td>Petersen, R.</td>
<td>446, 572</td>
</tr>
<tr>
<td>Peterson, R. J.</td>
<td>155, 158, 287</td>
</tr>
<tr>
<td>Pfau, J.</td>
<td>217, 220, 290, 294</td>
</tr>
<tr>
<td>Pfennig, N.</td>
<td>330, 363</td>
</tr>
<tr>
<td>Phillips, A.</td>
<td>220, 223, 290</td>
</tr>
<tr>
<td>Phillips, A. M.</td>
<td>89, 90, 290, 291</td>
</tr>
<tr>
<td>Phillips, C. R.</td>
<td>389, 522</td>
</tr>
<tr>
<td>Phillips, J. N.</td>
<td>217, 291</td>
</tr>
<tr>
<td>Phillips, J. N., Jr.</td>
<td>392, 519</td>
</tr>
<tr>
<td>Phillips, R. C.</td>
<td>499, 521</td>
</tr>
<tr>
<td>Pfeifer, P. J.</td>
<td>213, 216, 520, 281</td>
</tr>
<tr>
<td>Pickford, G. E.</td>
<td>80, 291</td>
</tr>
<tr>
<td>Pierce, S.</td>
<td>523, 277</td>
</tr>
<tr>
<td>Pignatti, S.</td>
<td>491, 498, 521, 525</td>
</tr>
<tr>
<td>Pillay, T. V. R.</td>
<td>10, 17</td>
</tr>
<tr>
<td>Fillis, R. W.</td>
<td>499, 521</td>
</tr>
<tr>
<td>Pires, W. O.</td>
<td>400, 522</td>
</tr>
<tr>
<td>Pironson, A.</td>
<td>397, 522</td>
</tr>
<tr>
<td>Pitelka, D. A.</td>
<td>387, 522</td>
</tr>
<tr>
<td>Plessis, Y. B.</td>
<td>191, 248, 251, 291</td>
</tr>
<tr>
<td>Pollinger, U.</td>
<td>381, 509</td>
</tr>
<tr>
<td>Poon, C. C.</td>
<td>189, 291</td>
</tr>
<tr>
<td>Porter, J. W.</td>
<td>380, 410, 513</td>
</tr>
<tr>
<td>Portner, D. M.</td>
<td>389, 522</td>
</tr>
<tr>
<td>Postgate, J. R.</td>
<td>329, 334, 362</td>
</tr>
<tr>
<td>Potts, W. T. W.</td>
<td>80, 83, 86, 291</td>
</tr>
<tr>
<td>Povolny, M.</td>
<td>470, 471, 522</td>
</tr>
<tr>
<td>Powell, E. O.</td>
<td>211, 213, 291</td>
</tr>
<tr>
<td>Prager, J.</td>
<td>392, 437, 515</td>
</tr>
<tr>
<td>Prakash, A.</td>
<td>371, 424, 503, 522</td>
</tr>
<tr>
<td>Pramer, D.</td>
<td>25–27, 273</td>
</tr>
<tr>
<td>Pratt, D.</td>
<td>461, 522</td>
</tr>
<tr>
<td>Pratt, D. B.</td>
<td>305, 364</td>
</tr>
<tr>
<td>Pratt, D. M.</td>
<td>27, 294</td>
</tr>
<tr>
<td>Pretorius, W. A.</td>
<td>128, 132, 291</td>
</tr>
<tr>
<td>Pringle, J. D.</td>
<td>493, 502</td>
</tr>
<tr>
<td>Privett, O. S.</td>
<td>154, 274</td>
</tr>
<tr>
<td>Prud'Homme van Reine, W. F.</td>
<td>378, 523</td>
</tr>
<tr>
<td>Puschel, S.</td>
<td>189, 293</td>
</tr>
<tr>
<td>Puseux-Dao, S.</td>
<td>368, 476, 523</td>
</tr>
<tr>
<td>Purday, C.</td>
<td>333, 529</td>
</tr>
<tr>
<td>Author Name</td>
<td>Page Numbers</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Purdom, M. E.</td>
<td>408, 524</td>
</tr>
<tr>
<td>Puvvmarkoeck, S. van</td>
<td>470, 503, 512</td>
</tr>
<tr>
<td>Quraishi, F. O.</td>
<td>373, 429, 444, 523</td>
</tr>
<tr>
<td>Rabbinowitz, E. J.</td>
<td>445, 523</td>
</tr>
<tr>
<td>Rabotnov, H. L.</td>
<td>369, 523</td>
</tr>
<tr>
<td>Rahal, M.</td>
<td>380, 416, 426, 440, 523</td>
</tr>
<tr>
<td>Rain, H.</td>
<td>146, 291</td>
</tr>
<tr>
<td>Rakestraw, N. W.</td>
<td>80, 271, 291</td>
</tr>
<tr>
<td>Randall, M. C.</td>
<td>164, 291</td>
</tr>
<tr>
<td>Randle, F. R.</td>
<td>44, 45, 121, 191, 300, 285</td>
</tr>
<tr>
<td>Ray, M.</td>
<td>380, 410, 416, 426, 440, 523</td>
</tr>
<tr>
<td>Ray, E.</td>
<td>218, 287</td>
</tr>
<tr>
<td>Raymont, J. E. G.</td>
<td>80, 297, 361, 426, 450, 451, 510, 523</td>
</tr>
<tr>
<td>Redding, E. M.</td>
<td>149, 291</td>
</tr>
<tr>
<td>Redfield, A. C.</td>
<td>21, 291, 392, 427, 463, 515</td>
</tr>
<tr>
<td>Reed, R. D.</td>
<td>102, 290</td>
</tr>
<tr>
<td>Reid, S. M.</td>
<td>420, 502</td>
</tr>
<tr>
<td>Reilly, H. C.</td>
<td>336, 362</td>
</tr>
<tr>
<td>Reinmann, B. E.</td>
<td>399, 431, 517</td>
</tr>
<tr>
<td>Reiner, G. S.</td>
<td>375, 523</td>
</tr>
<tr>
<td>Reitsch, W.</td>
<td>26, 289</td>
</tr>
<tr>
<td>Reinsen, C.</td>
<td>410, 466, 504</td>
</tr>
<tr>
<td>Renfr, G. Jr.</td>
<td>64, 291</td>
</tr>
<tr>
<td>Renn, C.</td>
<td>381, 528</td>
</tr>
<tr>
<td>Renn, C. E.</td>
<td>80, 271</td>
</tr>
<tr>
<td>Renward, H.</td>
<td>35, 277</td>
</tr>
<tr>
<td>Retzser, H. W.</td>
<td>381, 528</td>
</tr>
<tr>
<td>Reynolds, E. S.</td>
<td>344, 361</td>
</tr>
<tr>
<td>Reyniers, J. A.</td>
<td>5, 17</td>
</tr>
<tr>
<td>Rheinheimer, G.</td>
<td>21, 102, 103, 145, 279, 291</td>
</tr>
<tr>
<td>Rhodes, M.</td>
<td>329, 362</td>
</tr>
<tr>
<td>Ribbons, D. W.</td>
<td>373, 619</td>
</tr>
<tr>
<td>Ricci, J. E.</td>
<td>31, 291</td>
</tr>
<tr>
<td>Rice, A. L.</td>
<td>202, 241, 291</td>
</tr>
<tr>
<td>Richards, F. A.</td>
<td>21, 291</td>
</tr>
<tr>
<td>Richards, O. W.</td>
<td>372, 523</td>
</tr>
<tr>
<td>Richardson, D. E.</td>
<td>376, 507</td>
</tr>
<tr>
<td>Richardson, F. L.</td>
<td>390, 446, 504</td>
</tr>
<tr>
<td>Richman, S.</td>
<td>238, 291</td>
</tr>
<tr>
<td>Richter, G.</td>
<td>367, 510</td>
</tr>
<tr>
<td>Richter, O.</td>
<td>305, 362</td>
</tr>
<tr>
<td>Riedl, R.</td>
<td>201, 291, 292</td>
</tr>
<tr>
<td>Riley, G. A.</td>
<td>140, 141, 270, 292</td>
</tr>
<tr>
<td>Riley, J. D.</td>
<td>132, 294</td>
</tr>
<tr>
<td>Riley, J. P.</td>
<td>50, 93, 145, 248, 292, 414, 441, 523</td>
</tr>
<tr>
<td>Rincke, G.</td>
<td>126, 292</td>
</tr>
<tr>
<td>Robber, J. A.</td>
<td>189, 278</td>
</tr>
<tr>
<td>Robbins, W. J.</td>
<td>375, 419, 424, 523</td>
</tr>
<tr>
<td>Robertson, E.</td>
<td>455, 578</td>
</tr>
<tr>
<td>Robinson, R. J.</td>
<td>92, 299</td>
</tr>
<tr>
<td>Rodgers, E. O.</td>
<td>89, 90, 290</td>
</tr>
<tr>
<td>Rodina, A. G.</td>
<td>327, 355, 362</td>
</tr>
<tr>
<td>Rodriguez, E.</td>
<td>440, 512</td>
</tr>
<tr>
<td>Roels, O.</td>
<td>419, 509</td>
</tr>
<tr>
<td>Roels, O. A.</td>
<td>408, 460, 517, 524</td>
</tr>
<tr>
<td>Roff, J.</td>
<td>126, 292</td>
</tr>
<tr>
<td>Rogers, J. N.</td>
<td>238, 240, 291, 408, 508</td>
</tr>
<tr>
<td>Rohlich, G. A.</td>
<td>165, 286</td>
</tr>
<tr>
<td>Rosen, H. M.</td>
<td>158, 292</td>
</tr>
<tr>
<td>Rosenfeld, W. D.</td>
<td>27, 292</td>
</tr>
<tr>
<td>Rosenthal, H.</td>
<td>155-161, 292</td>
</tr>
<tr>
<td>Roskamp, R. T.</td>
<td>93, 292</td>
</tr>
<tr>
<td>Ross, S. S.</td>
<td>351, 352, 362</td>
</tr>
<tr>
<td>Roth, F. J. Jr.</td>
<td>347-351, 356, 358, 361, 362</td>
</tr>
<tr>
<td>Roth, L.</td>
<td>414, 441, 523</td>
</tr>
<tr>
<td>Roubeke, G. A.</td>
<td>462, 505</td>
</tr>
<tr>
<td>Roux, E.</td>
<td>218, 287</td>
</tr>
<tr>
<td>Rubin, E.</td>
<td>141, 292</td>
</tr>
<tr>
<td>Rachhoft, C. C.</td>
<td>128, 292</td>
</tr>
<tr>
<td>Röb, F.</td>
<td>189, 292</td>
</tr>
<tr>
<td>Ruikina, E. A.</td>
<td>354, 360</td>
</tr>
<tr>
<td>Rystad, B.</td>
<td>218, 219, 232, 375, 377, 513</td>
</tr>
<tr>
<td>Sachs, W. B.</td>
<td>46, 191, 292</td>
</tr>
<tr>
<td>Sackett, W. M.</td>
<td>415, 506</td>
</tr>
<tr>
<td>Saeki, A.</td>
<td>23, 24, 46, 92, 97, 108, 124, 125, 167-173, 177, 179, 181, 292</td>
</tr>
<tr>
<td>Saio, T.</td>
<td>93, 292</td>
</tr>
<tr>
<td>Saito, Y.</td>
<td>98, 287, 292</td>
</tr>
<tr>
<td>Saito, Y.</td>
<td>475, 493, 494, 502, 513</td>
</tr>
<tr>
<td>Sakai, M.</td>
<td>162, 289, 305, 306, 359</td>
</tr>
<tr>
<td>Saks, N. M.</td>
<td>443, 524</td>
</tr>
<tr>
<td>Salimbenedi, T. A.</td>
<td>218, 287</td>
</tr>
<tr>
<td>Salser, B. R.</td>
<td>60, 61, 191, 193, 237, 287, 292</td>
</tr>
<tr>
<td>Sampson, D.</td>
<td>125, 277</td>
</tr>
<tr>
<td>Samuel, G. L.</td>
<td>58, 60, 291</td>
</tr>
<tr>
<td>Sandbonsuga, Y.</td>
<td>468, 473, 484, 485, 511, 524</td>
</tr>
<tr>
<td>Sanchez, J. A.</td>
<td>199, 298</td>
</tr>
<tr>
<td>Sanders, E.</td>
<td>141-143, 158, 169, 292, 293</td>
</tr>
<tr>
<td>Sanders, M.</td>
<td>412, 446, 512</td>
</tr>
<tr>
<td>Sandifer, P. A.</td>
<td>236, 237, 293, 300</td>
</tr>
<tr>
<td>Sandovai, H. K.</td>
<td>335, 362</td>
</tr>
<tr>
<td>Sandit, D. G.</td>
<td>46, 293</td>
</tr>
<tr>
<td>Sanzin, W. D.</td>
<td>128, 293</td>
</tr>
<tr>
<td>Safin, M. F.</td>
<td>238, 284</td>
</tr>
<tr>
<td>Sastry, A. N.</td>
<td>239, 240, 293</td>
</tr>
<tr>
<td>Satoh, S.</td>
<td>494, 516</td>
</tr>
</tbody>
</table>
AUTHOR INDEX

Smith, L. O., 155, 295
Smith, M., 487, 528
Smith, N. R., 314, 357
Smith, R. L., 452, 525
Smith, K. M., 473, 525
Smith, S. B., 147, 280
Smith, W. E. E., 26, 217, 277, 446, 508
Smith, W. L., 185, 295
Soli, G., 329, 363, 381-383, 525
Soltero, P. V., 461, 525
Soman, P. D., 470, 501
Sontheimer, H., 140, 280
Soppeland, L., 128, 285
Sorbolegos, P., 208-210, 295
Sorokin, C., 132, 296
Sorokin, Yu. I., 323, 326, 331, 363
South, G. R., 477, 478, 523
Southward, A. J., 78, 295
Southward, E. C., 78, 295
Sparrow, F. K., 337, 338, 341, 343-345, 363
Speier, H. L., 410, 453, 514
Spencer, H. E., 155, 158, 299
Spencer, H. T., 156, 107, 290
Spencer, R., 334, 363
Spencer, R. F., 475, 505
Spencer, T., 420, 525
Spira, Z., 416, 523
Spotnitz, A., 417, 513
Sproul, O. J., 107, 156, 272, 286
Sreenivasan, A., 329, 363
Srin, R. F., 46, 47, 93, 178, 222, 223, 244, 276, 295
Staaland, H., 206, 295
Stagg, C. H., 140, 298
Stander, G. J., 132, 281
Standfast, A. F. R., 128, 299
Stanier, R. Y., 311, 326, 328, 363
Stanley, H. I., 132, 278
Starkey, R. L., 329, 364
Starr, M. P., 335, 364
Starr, R. C., 371, 525
Sterbins, M. E., 419, 424, 523
Stedham, M. A., 350, 364
Steffmann Nielsen, E., 320, 364, 373, 381, 415, 445, 514, 555, 526
Steen, J. R., 10, 17
Stendadro, B., 469, 505
Stephan, D. G., 140, 298
Stephenson, W. M., 470, 471, 526
Stephens, G. C., 77, 78, 295, 410, 426, 431, 475, 519
Stephens, G. S., 78, 299
Stephens, K., 441, 443, 445, 446, 460, 502, 513, 517
Stewart, G. J., 331, 364
Stewart, M. J., 129, 295
Stien, J., 498, 499, 526
Stokes, J. L., 381, 528
Stoke, K., 493, 498, 526
Storkstad, E. L. R., 423, 512
Stolt, H., 335, 364
Storey, P. R., 154, 286
Storosch, H. A., 429, 476, 526
Stout, R., 325, 361
Stowell, E. F., 23, 25, 166, 295
Strammer, G., 283, 286, 295
Strunce, C., 81, 295
Stubb, A., 93, 295
Stumm, W., 154, 160, 165, 295, 296, 300
Suhow, N. N., 33, 296
Suhikado, S., 350, 351, 364
Sulkin, S. D., 33, 296
Sullivan, P., 157, 276
Sulzer, R., 154, 160, 296
Sundene, O., 485, 526
Svomaalainen, H., 347, 364
Sutcliffe, W. H., 140, 270, 296
Sutskirch, N., 90, 283
Sutherland, D. B., 254, 256, 258-261, 269, 272
Suto, S., 493, 496, 526
Sutter, R. P., 375, 526
Sutton, A., 470, 505, 526
Suzuki, H., 419, 528
Suzuki, N., 162, 283
Suzuki, T., 329, 361
Svetdru, H. U., 21, 80, 296, 432, 526
Svoboda, A., 201, 202, 296
Sweers, S., 153, 189, 290
Swift, E., 439, 451, 453, 454, 526
Sykes, G., 102, 104, 105, 107, 161, 296
Sykes, J. E., 499, 502
Szidar, L., 213, 289
Taek, K. T., 132, 294
Taing, N., 84, 299
Takahashi, H., 123, 289
Takahashi, T., 128, 296
Takano, H., 389, 411, 439, 526
Tam, L. Q., 319, 557
WALKER, T., 381, 428, 509
WALLACE, G. T., 140, 141, 143, 298
WALLEN, D. G., 443, 528
WALLIS, C., 140, 298
WALNE, P. R., 383, 460, 528
WANGERSKY, P., 21, 98, 108, 109, 140, 292, 298, 387, 528
WANNER, H. V., 160, 298
WARBURG, O., 367, 415, 441, 528
WARD, C. N., 191, 298
WARD, W. W., 231, 229, 298
WARING, W. S., 373, 528
WAREN, C. E., 263-265, 272, 274, 298
WAREN, K. S., 87, 88, 298
WASSERWIRTSCHAFTSVERWALTUNG, 91, 298
WATABE, N., 448, 528
WATANABE, A., 464, 528
WATSON, S. W., 96, 298
WATTENBERG, H., 92, 298, 408, 528
WEBB, K. L., 78, 282, 470, 511
WEBBER, H. H., 9, 17
WEBER, W. J., Jr., 139, 288
WEGMANN, K., 400, 528
WEIHE, K. VON, 252, 253, 298, 501, 528
WEINBERGER, L. W., 140, 298
WEINSTECK, J. J., 141, 292
WEIYS, J., 154, 298
WEISSEFELLNER, H., 413, 417, 454, 513
WEISSMAN, B. J., 368, 505
WECH, P. S., 183, 298
WERBKAN, C. H., 373, 528
WERNER, D., 400, 528
WEINMÜLLER, K., 217, 220, 290, 294
WERZ, G., 367, 510
WEST, J. A., 473, 496, 498, 512
WEYLAND, H., 394, 365
WHEELER, R. S., 62, 298
WHIPPLE, G. C., 25, 298, 464, 528
WHISENAND, A., 381, 428, 509
WHITFIELD, M., 21, 109, 298
WHITFORD, L. A., 203, 298
WICKERHAM, L. J., 349, 361, 365
WIDEMANN, V. E., 383, 492, 525
WIDEMANN, E., 42, 191, 298
WIBSNER, W., 413, 528
WILDER, C. G., 26, 298
WILBUR, K. M., 448, 528
WILDOOSE, P. B., 471, 504
WILHELM, A., 165, 271
WILKEN, T. O., 355, 360
WILLIAMS, P., 388, 426, 502
WILLIAMS, P. A., 98, 276, 315, 356, 357
WILLIAMS, W. C., 99, 298
WILLIAMSON, D. L., 202, 241, 291
WILSON, D. F., 140, 141, 143, 298
WILSON, D. P., 21, 41, 108, 166, 299, 370, 529
WILSON, K. W., 207, 299
WILSON, T. R. S., 148, 288
WILSON, W. B., 367, 422, 438, 450, 501, 529
WINDLICH, S., 351, 352, 360
WONGRICH, H., 186, 299
WOGGARDENSKY, M. S., 80, 299
WIRTH, H. E., 92, 299
WISSEY, B., 393, 529
WITHROW, A. P., 198, 299
WITHROW, R. E., 198, 299
WOKER, H., 87, 88, 90, 299
WOLF, K., 92, 299
WOLFE, R. S., 333, 361
WOLTERS, C., 42, 162, 281
WOOD, E. J. F., 465, 493, 498, 529
WOOD, F., 370, 371, 529
WOOD, H. C., 469, 525
WOOD, J. D., 80, 85, 299
WOOD, P. C., 161, 162, 299
WOODRUFF, B., 222, 278
WOODS, D. L., 470, 471, 503
WOODWARD, R. L., 462, 505
WOOLRIDGE, W. R., 128, 299
WOZEL, J. L., 460, 524
WRIGHT, R. T., 322, 369
WURTMANN, K., 81, 87, 88, 90, 123, 128, 203, 276, 299
WULF, F., 206, 277
WURMLER, R., 110, 299
WURTZ, A. G., 382, 389, 529
WYCKHOFF, B. M., 164, 276
WYNN, J., 158, 286
YABU, H., 493, 529
YACOWITZ, H., 423, 521
YAMADA, N., 473, 487, 529
YAMAMADA, Y., 482, 493, 495, 496, 529
YAMAMOTO, H., 493, 498, 529
YAMAMOTO, T., 94, 299
YANASE, R., 440, 444, 447, 529
YANOFSKY, S., 335, 361
YAO, K. M., 112, 299
YENTSCH, C. S., 381, 466, 518, 529
YOSHIDA, T., 493, 494, 516
YOSHIDA, Y., 319-321, 332, 360
YOSHIO, I., 90, 283
YOUNG, E., 499, 529
YU, M. Y., 79, 80, 299
ZABOR, J. W., 80, 271
ZAHASKY, A. C., 370, 501
ZAHN, P. A., 371, 384, 392, 437, 518, 529
ZAHN, M., 206, 300
ZAROOGHIAN, G. E., 33, 35, 36, 300
ZATTERRA, A., 373, 377, 380, 413, 499, 502, 529
ZEHENDER, F., 88, 166, 299, 300
ZENITANI, B., 468, 527
Zenz, D., 158, 300
Ziegelmeier, E., 203, 204, 300
Zielinski, P. B., 236, 237, 293, 300
Zillioux, E. J., 142-144, 233-236, 300
Zimmermann, P., 203, 300
ZUCKER, W., 371, 516
ZUILEKOM, J. T. VAN, 355, 360
TAXONOMIC INDEX

Acanthopelis japonica, 491
Acanthophora apicifera, 491
Acartia chzmi, 83-85, 233
A. tonsa, 233
Acetabularia, 373, 380, 429, 505, 514, 524, 527
A. crenulatula, 463
A. mediterranea, 367, 368, 383, 418, 420, 463, 473, 475, 503, 510, 516, 518, 519, 521, 526
A. peniculata, 523
A. sp., 383, 441
Actinobacter, 305, 329
A. crenulata, 447
Actinomycetes, 279, 328, 334
Aequipecten iradians, 491
Aequoreo aequorea, 82
Aerobacter, 468, 477, 485, 525
A. esridenta, 477, 478, 483
A. fislulosa, 483
A. marginata, 483
A. ochotensis, 483
A. yezoensis, 483
algae, blue-green, 81, 206, 279, 280, 286, 291, 300, 308, 361, 362, 366, 372, 480, 510, 528
algae, brown, 328, 468, 470, 471, 477, 480, 482-484, 503, 517, 519, 521, 525
algae, green, 488, 478, 480, 481, 498, 513, 516, 628
Algalum, 502
Alsidium helminthochorton, 469, 491
Alternaria, 338
Alythinia, 499
Alophronia crouchi, 12, 337, 338, 356, 359
Amoeba sp., 469
Amphibia (amphibians), 85, 270, 288
Amphibolus, 499
Amphidinium, 410, 411, 450, 518
A. carteri (carterae), 95, 96, 133, 410, 412-414, 418, 433, 439, 504
A. klebsii, 433
A. rhynchocephalum (rhynchocephalus), 410, 418
A. sp., 445, 450
Amphipoda, 82, 83, 199, 275
Amphipora, 402
A. sp., 447, 457
Amphora coffeiformis, 419
A. hystrix, 133
A. perpusilla, 433, 435
A. sp., 444, 447
Anabaena cylindrica, 280
Ancylostoma medusae, 469, 481
Anomia marina, 133
Anchovella sp., 338
Anemonia sulcata, 78, 293
Anglespermus, 472, 499, 500
Anneliden (annelides), 13, 280, 521
Anoplopoma fimbria, 69
Anthurium (anthozoa), 78, 293
Antithamniun, 527
Arctium aculeatum, 275
Arphysia, 44
appendicularians, 233
Archeas, 516
Arenicola marina, 295
Artemisia salina, 239, 240, 298
Arthrobacter, 329, 363
A. sp., 335
arthropods, 280
Arthrotamnus bifidus, 483
A. kurilensis, 483
Ascomycetes, 12, 337, 338, 343-346, 359, 361
Aspergillum, 470, 471, 500
A. nodosum, 472, 483
Asparagus sandfordiana, 491
Aspergillus, 338
Astasia, 522
Asteroidea formosa, 512
A. japonica, 411, 414, 437, 451, 514
A. notata, 278
Athorhodaceae, 330
Athorhodaceae, 337, 341
Bacillariophyta, 133
bacilli, 307
Bacillus subtilis, 107, 111, 162
B. subtilis savamura, 162
Bacteriostearum halymenllm, 370
Bangia, 495, 506
barnacles—see cirripedes
baso, 270
Bathophora oerdstedii, 523
Bedellobria, 334
B. bacteriovorus, 335, 364
Beckerella sp., 491
Beggioa, 133, 357
B. sp., 332
beggioas, 331
Beneckea, 326
Beroe gracilis, 229
B. ovata, 231
B. sp., 231
Biddulphia, 274
B. aurita, 408, 517
B. mobiliensis, 408
B. sinensis, 84
Biomphalaria sudanica, 79
birds, 16, 56, 350, 467, 499
bivalves, 41, 116, 286, 360
bluegill sunfish, 85, 278, 297
Bolitopsis, 229
Bostrychia, 500
B. radicans, 491
B. radiicans, 491
Bougainvillea muscus, 206
B. sp., 33, 297
Brachiostoma submariina, 418, 444
Brachionus plicatilis, 214
brachiopoda, 286
Brachydanio rerio, 80, 299
brachyurans, 239, 240, 293
bream, 172, 176
Brevoria tyrannus, 40
Bryopsis halymenllm, 473, 512
Bryozoa (bryozoa), 13, 41, 243, 244, 246, 282
Cachonina niei, 459
Calanus, 273, 274
C. helgolandicus, 83, 84, 95, 231, 238, 274, 290, 291
C. sp., 82
Calliopius sp., 82
Calliphora sp., 5
Caloglossa, 500
C. odhnia, 491
C. leprieurii, 491
Campylophora crassa, 491
C. hypnaeoides (Syn: Ceramium hypnaeoides), 491
Candida, 348, 350, 363
C. albicans, 350, 351, 353, 355, 361
C. humicola, 355
C. lypotylica, 356
C. marinra, 347, 364
C. parapsilosis, 355
C. pseudotropicalis, 355
C. sp. 353
C. suecica, 347, 361
C. tropicalis, 355, 356, 359
C. zeylanoides, 352
Cancer irroratus, 239, 240
Carangidae, 238
Carassius auratus, 90
C. carassius, 90, 129
Cardisoma guanum, 83, 278
Cardium edule, 288
carp, 90, 122, 129, 167, 177, 283, 292
Carpopeltis affinis, 491
C. flabelata, 491
Carteria, 408
C. sp. 224, 445, 446
Catenella, 500
C. impudica, 491
C. nipae, 491
catfish, 64, 177, 275, 279, 288, 295
Cauderpa, 500
Celtivibrio, 327
Centroceras clavulatum, 208
eelaphopodis, 80, 83
Ceramium, 337
C. aduncum, 491
C. boydenii, 491
C. cintricatum, 491
C. ciatum, 491
C. codii, 491
<table>
<thead>
<tr>
<th>Taxonomic Name</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. crassum</td>
<td>491</td>
</tr>
<tr>
<td>C. fastigiatum</td>
<td>491</td>
</tr>
<tr>
<td>C. fimbriatum</td>
<td>491</td>
</tr>
<tr>
<td>C. gracillimum</td>
<td>491</td>
</tr>
<tr>
<td>C. kondoi</td>
<td>491</td>
</tr>
<tr>
<td>C. nakamurai</td>
<td>491</td>
</tr>
<tr>
<td>C. paniculatum</td>
<td>491</td>
</tr>
<tr>
<td>C. paniculatum</td>
<td>491</td>
</tr>
<tr>
<td>C. rubrum</td>
<td>491</td>
</tr>
<tr>
<td>C. tenerrimum</td>
<td>491</td>
</tr>
<tr>
<td>C. tenuissimum</td>
<td>491</td>
</tr>
<tr>
<td>Ceratium sp.</td>
<td>421</td>
</tr>
<tr>
<td>Ceraosporopha</td>
<td>344</td>
</tr>
<tr>
<td>Chaeloceros</td>
<td>462, 463</td>
</tr>
<tr>
<td>C. calcitrana</td>
<td>440, 444</td>
</tr>
<tr>
<td>C. decipiem</td>
<td>422</td>
</tr>
<tr>
<td>C. dydimus</td>
<td>370, 381, 382</td>
</tr>
<tr>
<td>C. gracilis</td>
<td>411</td>
</tr>
<tr>
<td>C. sp.</td>
<td>370, 446, 448</td>
</tr>
<tr>
<td>Chaetomorpha</td>
<td>473, 510</td>
</tr>
<tr>
<td>Chironomids</td>
<td>262</td>
</tr>
<tr>
<td>Chlamydomonas</td>
<td>413</td>
</tr>
<tr>
<td>Chlamydomonas</td>
<td>276, 408</td>
</tr>
<tr>
<td>C. palla</td>
<td>132, 133</td>
</tr>
<tr>
<td>Chlorella</td>
<td>370, 447, 448</td>
</tr>
<tr>
<td>C. ovalis</td>
<td>370, 444</td>
</tr>
<tr>
<td>C. pyrenoidosa</td>
<td>415</td>
</tr>
<tr>
<td>C. sp.</td>
<td>370, 447, 456, 457</td>
</tr>
<tr>
<td>C. stigmatophora</td>
<td>396, 448, 458</td>
</tr>
<tr>
<td>Chlorobium</td>
<td>331</td>
</tr>
<tr>
<td>C. limicola</td>
<td>330</td>
</tr>
<tr>
<td>C. thiosulfatophilum</td>
<td>330</td>
</tr>
<tr>
<td>Chlorococcus</td>
<td>132, 417</td>
</tr>
<tr>
<td>C. sp.</td>
<td>447</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>380, 426, 480, 508, 511</td>
</tr>
<tr>
<td>Chlorophyta (chlorophytes)</td>
<td>133, 382, 384, 385, 404, 414, 416-418, 420, 429, 433, 450, 452, 463, 509</td>
</tr>
<tr>
<td>Chinoeora quinquicirrha</td>
<td>231</td>
</tr>
<tr>
<td>Chrysomonads</td>
<td>95, 214, 378, 400, 407, 409-411, 413, 416-418, 433, 436, 449, 518, 522</td>
</tr>
<tr>
<td>Chrysohasys major</td>
<td>172, 173, 175</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td>416, 504</td>
</tr>
<tr>
<td>Chrysophyta (chrysophytes)</td>
<td>133, 403, 404, 411, 429, 433, 454, 463</td>
</tr>
<tr>
<td>Chytridionymates</td>
<td>12, 337, 338</td>
</tr>
<tr>
<td>Chytridium</td>
<td>337</td>
</tr>
<tr>
<td>Chytrids</td>
<td>357</td>
</tr>
<tr>
<td>Ciliata (ciliates)</td>
<td>215, 217, 220, 233, 297</td>
</tr>
<tr>
<td>Cirripedes (barnacles)</td>
<td>182, 246, 250, 296, 378, 523</td>
</tr>
<tr>
<td>Cladocora</td>
<td>518</td>
</tr>
<tr>
<td>Cladophora</td>
<td>473, 508</td>
</tr>
<tr>
<td>C. subhriana</td>
<td>473, 608</td>
</tr>
<tr>
<td>Cladophoropsis</td>
<td>500</td>
</tr>
<tr>
<td>Clams</td>
<td>10, 17, 46, 47, 57, 155, 244, 511</td>
</tr>
<tr>
<td>Clostridium</td>
<td>327</td>
</tr>
<tr>
<td>C. botulinum</td>
<td>158</td>
</tr>
<tr>
<td>C. pecfringens</td>
<td>155</td>
</tr>
<tr>
<td>Clario marinus</td>
<td>252</td>
</tr>
<tr>
<td>Clytia johnstonii</td>
<td>279</td>
</tr>
<tr>
<td>Cnidaria (enidarians)</td>
<td>13, 76, 199</td>
</tr>
<tr>
<td>Coccolithineae</td>
<td>528</td>
</tr>
<tr>
<td>Coccolithophorids</td>
<td>44-417, 449, 453, 454, 513, 519, 521, 526</td>
</tr>
<tr>
<td>Coccolithus huxleyi</td>
<td>290, 369, 400, 409, 413, 414, 416, 417, 419, 448, 449, 519, 521, 526, 528</td>
</tr>
<tr>
<td>C. pelagicus</td>
<td>521</td>
</tr>
<tr>
<td>Cocconeis, sp.</td>
<td>415</td>
</tr>
<tr>
<td>Codiotum</td>
<td>515</td>
</tr>
<tr>
<td>Codium dichtotomum</td>
<td>481</td>
</tr>
<tr>
<td>C. diversicatum</td>
<td>481</td>
</tr>
<tr>
<td>C. fragile</td>
<td>481</td>
</tr>
<tr>
<td>C. intricatum</td>
<td>481</td>
</tr>
<tr>
<td>C. lindenbergii</td>
<td>481</td>
</tr>
<tr>
<td>C. muelleri</td>
<td>481</td>
</tr>
<tr>
<td>C. sp.</td>
<td>481</td>
</tr>
<tr>
<td>C. tenue</td>
<td>481</td>
</tr>
<tr>
<td>C. tomentosum</td>
<td>481</td>
</tr>
<tr>
<td>Coelacanths</td>
<td>291</td>
</tr>
<tr>
<td>Coelenterata (coelenterates)</td>
<td>293, 297</td>
</tr>
<tr>
<td>Copepods</td>
<td>78, 82, 84, 35, 104, 142, 144, 199, 208, 231, 233-235, 237-239, 300, 456</td>
</tr>
<tr>
<td>Corallina officinalis</td>
<td>469, 491</td>
</tr>
<tr>
<td>Corals</td>
<td>176, 268, 326, 487, 502, 518</td>
</tr>
<tr>
<td>Cordyphora caspia</td>
<td>199</td>
</tr>
<tr>
<td>Cororogonus laveretns</td>
<td>276</td>
</tr>
<tr>
<td>Corolliospora</td>
<td>344</td>
</tr>
<tr>
<td>Coccinodiscus astromorphus</td>
<td>408, 409</td>
</tr>
<tr>
<td>Costaria costata</td>
<td>483, 524</td>
</tr>
<tr>
<td>Oreb, fiddler</td>
<td>287</td>
</tr>
</tbody>
</table>
crab, hermit, 97
crabs, 33, 46, 47, 239, 278, 293, 296
Crassostrea virginica, 33, 83, 157, 162, 280, 460
crayfish, 527
Cricophara, 449
C. carterae, 414
C. elongata, 439, 453, 454, 526
C. sp., 417
Crustacea (crustaceans), 9, 13, 14, 16, 60, 77, 83, 92, 132, 208, 236, 237, 289, 290, 291, 293, 296, 328, 462, 527
Cryptococcus albidus, 352
C. laurentii, 353
C. laurentii, 353
C. luteolus, 353
C. wce?am, 351
C. neoformans, 351
cryptomonads, 269, 409, 412, 413, 415, 418, 433, 449, 450, 502
Cryptomonas ovata, 418, 445
C. sp., 385, 396, 448
Cryptophyceae, 416
Cryptophyta (cryptophytes), 133, 433
Crypturalolithus hyalinus, 521
Ctenophora (ctenophorea), 13, 231, 232, 279, 298
Cyanea capillata, 231
Cyanophyllus, 483
C. polypodioides (Syn: D. menziesii), 483
Cyprinodon macularius, 132
decapods, 80, 104, 237, 241, 286, 291, 293
TAXONOMIC INDEX

Echinodermata (echinoderms), 13, 33
Ecklonia, 468
E. bicyclis, 483
E. cava, 483
E. kurome, 483
E. latifolia, 483
E. maxima, 483
Eckloniopsis sp., 483
Ectecocarpus confervoides, 475
E. fasciculatus, 476, 521
E. sp., 487
Ectrogella, 337
E. perforans, 337
eels, 64–70, 189, 171, 177, 187, 297
Egregia menziesii, 483
Elygia biclyclis, 483
Erinaria hederi, 133
E. sp., 483
E. intestinalis, 481, 505
E. linze, 481
E. prolifera, 481
E. sp., 132
Escherichia coli, 25–27, 132, 155, 162, 273, 297, 335, 361
E. luciuf, 90
Euwheuma, 470
E. arnakuweni, 491
E. cruabefme, 481
E. cuproideum, 491
E. cule, 491
E. gelatinosa (Syn: E. gelatinosa), 491
E. horridum (Syn: Orithia Wa), 491
E. isoforme, 491
E. muricatum (Syn: E. denticulatum, E. spinosum, Fucus muricatum), 470, 491
E. okamurai, 491
E. serra, 491
E. striatum, 491
Euglena, 512, 523
E. gracilis, 423
E. sp., 419
Euglenophyta, 372
Eunicidae, 283
Eupagurus bernhardus, 231
Euphausia pacifica, 82, 283
euphausiidae, 82
Euplates vanass, 233
Euporphyra, 494
Euryachasmidium tumefaciens, 337
Eutreptia, 462
Entereptella sp., 450
Exuvariaella, 450, 462
E. baltica, 450
E. sp., 420, 421
Fistularia villosa, 238
Flavobacterium, 334
Foraminifera (foraminifer), 206, 277, 516
Fragilaria pinnata, 133
Fucaceae, 470, 471, 518
Fucales, 482
Fucus, 471, 481, 500
F. ceranoides, 515
F. edentatus, 518
F. gardneri, 469
F. serratus, 483
F. sp., 480
F. spiralis, 479, 519
F. vesiculosa, 472, 483, 519
F. heteroclitus, 132, 167
Fungi actiniformis var. palavensis, 268
fungi, 10–12, 15, 18, 132, 279, 286, 291, 300, 328, 337–345, 347, 356, 368–365, 455, 471, 525
Fungi, Higher, 344
Fungi imperfecti, 337, 343
Fungi, Lower, 337, 328, 340, 347
Fuscocellaria fastigiata, 491
Fusarium, 338
Galathea squamifera, 231
Gallionella, 333
G. ferruginea, 333, 361
G. sp., 324, 333
gammarids, 203
Gammarae duebeni, 17
G. locusta, 132
G. oceanicus, 469
gastropods, 44, 83, 279
Gelidiella acerosa (Syn: Gelidium rigidum), 491
Gelidiopsis rigida, 491
Gelidium, 469, 472, 484, 493, 529
G. amansii, 491
G. arborescens, 491
G. arbuscula, 491
G. attenuatum, 491
G. capillaceum, 491
G. cartilagineum, 491
G. corneum, 491
G. crispum, 491
G. decumbensum, 491
G. diversatum, 491
G. elegans, 491
G. incisuratum, 491
G. japonicum, 491
G. kintaroi, 491
G. kritiae, 491
G. linoidea, 491
G. linoideum, 491
G. longicatanum, 491
G. spinifera, 219
G. subcostatum, 492
G. subulatum, 492
G. suburtunculatum, 492
G. suburtunculatum, 492
G. tentaculum, 492
G. teedii, 492
G. tenax, 492
Glossoscolex giganteus, 283
G. grandis, 283
Gobio gobio, 88-90
Gobius flavescens, 132
goldfish, 167, 169
Gonyaulax, 424
G. attenuata, 450, 455, 506
G. platygaster, 459, 511
G. sp., 421
G. spinifera, 219
G. tamarense, 219, 522
gourami, blue, 80, 299
Gracilaria, 468, 469, 493, 498, 515, 520, 529
G. arcuata, 492
G. blodgettii, 492
G. burma-perspicua, 492
G. caudata, 492
G. chorda, 492
G. compressa, 492
gar, 492, 498, 519, 621, 525, 526, 529
G. cornea, 492
G. coronopifolia, 492
grosea, 492
denticulata, 492
dura, 492
edulis, 492
eucheumoides, 492
G. gigas, 492
G. incurvata, 492
G. multiraria (Syn: G. lacinulata, G. foliifera), 492
G. punctata, 492
G. purpurascens, 492
G. salicornia, 492
G. sublittoralis, 492
tenioides, 492
textorii, 492
G. verrucosa, 492, 498, 514, 520, 524, 526
G. chorda, 492
G. rhodeticum, 492
G. vermiculophylla, 492
Gramineae, 298, 528
grasses, edl, 499, 500, 520
glasses, surf, 499
glasses, marine, 13, 467, 472, 499, 510, 521
glasses, surf, 499
glasses, turtle, 499, 502
G. filicina, 492
G. imbricata, 492
gudgeons, 88
G. kasumai, 492
gulls, 69
G. mambilosa, 492, 504, 518
G. neptiius, 492
G. pacifica, 492
G. pistillata, 492
G. pteropus, 492
G. stellata (Syn: G. mammilosa), 492, 504, 518
G. teedii, 492
G. tentaculum, 520
Gloeiopeltis complanata, 492
G. furcata, 492
G. tenax, 492
G. tentaculum, 520
Gloieopeltis complanata, 492
G. furcata, 492
G. tenax, 492
Glossoscolex giganteus, 283

Cymnodominium breve, 157, 271, 416, 418, 420, 438, 450, 501, 521, 529
TAXONOMIC INDEX

G. simplex, 418
G. sp., 437, 446
G. splendens, 381, 382, 410, 418, 420, 423, 432, 433, 438
G. veneficum, 79
Cymnogongrus flabelliformis, 492
G. griffithiæae, 492
G. jvanicus, 492
G. pinnaulata, 492
Gyrodinium, 411, 463, 527, 627
G. californiacum, 381, 410, 432, 439
G. coh.ii, 383, 392, 416, 418, 440, 446, 509, 523
G. reapkndens, 381, 383, 410
C. RP., 437
G. splendena, 410
G. zcncalenum, 492
Hacmatococcu pluwialis, 607, 518
Holicystia, 515
H. ovalia, 473
H. prvula, 473, 508
Haliphiloro8, 337, 341
Halodræle, 499, 510
Halophila, 499
Halosawion glandiforme, 492
halophyts, 500
Halosphræteriaceae, 337
Halymenia durvilliae, 492
Haptophycem, 503
Helobieae, 499
helm~nths, 16
HemLqelmw sp., 380, 436
H. virescens, 132, 133, 269, 409, 418
Hepaticarum, 502
I~erring, 207, 299
Heterochordaria abietir~a, 469, 483
Heterothrix sp., 133
Heterotricha, 297
Himanthalia, 471
H. elongata (Syn: H. lorea), 472, 483
Hizikia fusiforme, 483
Homarus americanus, 175
H. gammarus, 231
Hormosira banksii, 483
hydras, 276
Hydrocharitaceae, 499
Hydrocloathrus clathratus (Syn: H. cancellatus), 483
hydroids, 33, 202, 207, 235, 272, 279
Hymenomenas carterae, 417
H. (Syracosphaera) elongata, 418
H. sp., 409, 410, 416, 417, 449
Hypona boergensis, 482
H. cenomyce, 492
H. cervicornis, 492
H. charoides, 492
H. chordaceæa, 492
H. cornuta, 492
H. divaricata, 492
H. esperi, 492
H. flabelliformis, 492
H. humulosa, 492
H. japonica, 492
H. musciformis, 492
H. nudifica, 492
H. nidulana, 492
H. pannosa, 492
H. saidana, 492
H. spicifera, 492
H. valentiae, 492
H. variabilis, 492
Italurus punctatus, 177
Idotea baltica, 132, 469
insecta, 155, 471
Irida cornucopis, 492
I. edulis, 492
I. flaccida (Syn: I. laminaroides), 492
Isochrysis galbana, 133, 215, 378, 384, 394, 396, 403, 418, 446–449, 453, 458, 504, 514, 518
I. sp., 96
isopods, 83, 155, 275, 280
jellyfish, 82, 84, 298
Katdiniurn dosaisulcum, 437
kelp, 15, 71, 273, 289, 484, 487, 489, 490, 500, 501, 505, 519, 520
killifish, 167
Kjellmaniella gyrata (Syn: Laminaria gyrata), 483
Labyrintherkz, 12, 324, 529
L. fomomum, 337, 342, 360, 362
L. macrocystis, 499
Lactobacillus, 419
L. leichmaunii, 419
Lagenidium, 337, 341
L. callinectes, 337, 338
L. coccinoides, 338
lamellibranchs, 83
Laminaria, 467–472, 477, 484, 485, 487, 511
L. angustata, 469, 483
L. cichorioides, 483
L. clonostomi, 472, 510
L. diabolica, 483
L. digitata, 472, 483, 510, 611, 526
L. fragilis, 483
L. hyperborea (Syn: L. cloustonii), 483, 485–487, 511, 514, 517
L. japonica, 472, 483, 487
L. longipes, 483
L. longissima, 483
L. ochroleuca, 484
L. religiosa, 483
L. rodriquezii, 484
L. saccharina, 472, 473, 483, 610
L. sp., 96
Laucca, 512
L. botryoides, 409
L. obtusa, 492
L. papillosa, 492
L. perforata, 492
L. pinnaeifida, 492
laver, 502, 513, 515
laver, purple, 490
Leishmania tarentolae, 389
Lemma perpusilla, 187
Lepomis macrochirus, 85, 297
Leptocylindrus sp., 467
Leptolegia, 337
Lessonia sp., 483
L. variegata, 483
Lyoidora farinosa, 492
L. decussata, 492
Limonium, 500
Lithothamnion calcareum, 492
L. crasum, 492
Littorina obtusata, 518
lobster, American, 175
lobsters, 57
lobster, spiny, 275
Lulworthia, 344
Lymnaea stagnalis, 83
L. stagnalis jugularis, 271
Macona balbica, 519
Macrobrachium, 300
M. rosenbergii, 237
Macrocyclus, 289, 470, 477, 484, 485, 489, 490, 514, 520
M. angustifolia, 489
M. integrifolia, 224, 226, 483, 489, 528
M. pyrifera, 469, 471, 483, 489, 490, 505, 506, 519, 525, 527
mammals, marine, 18, 77, 419, 467
mangroves, 13, 467, 472, 500, 509, 527
Marionina predictella, 483
Mastocarpus klenzianus, 492
medusae, 231, 232, 272
Meganathus norvegica, 83–85
M. borisborer, 422
M. mammoleae, 409, 418, 511
M. sp., 409, 437, 447
menhaden, 40, 281
Menidia, 522
Mercenaria mercenaria, 244, 460, 611
Merkistotheca papulea, 492
Mesogloia craeae, 483
M. de&pha, 483
M. japonica, 362
Metaculina andrewsi, 297
Latimeria chalumnae, 29
Metazoa (metazoans), 16, 606
M. sp., 96
M. carbonatiphila, 392
M. wbelli, 347, 364
M. obtusa, 492
M. prolifeva, 175
M. perpusilla, 167
M. macroura, 157
Microcyathinae, 518
M. pul-ple, 490
M. crbplaces, 285
M. minnows, 279
Mixophyceae, 420
M. sp., 231
Modiolus demissus, 83
M. angustifolia, 224, 226, 483, 489, 528
M. pyrifera, 469, 471, 483, 489, 490, 505, 506, 519, 525, 527
mammals, marine, 18, 77, 419, 467
Phaeodactylum, 502, 523
Phaeophyceae, 482, 525
Pheratima hawayana, 283
P. sp., 327
Phyllodictyon, 337
Phyllodictyon sp., 54
Phyllomyces, 523
Phycocyanin, 45, 502
Phycomyces, 337
Phycomycetes, 337-341, 344, 345, 350-358, 361-364
Porphyridium aerigineum, 400, 445, 512
P. sp., 133, 413, 413
Poridonia, 499
Posidonia, 499
Posidoniaceae, 510
Prasinoclados marinus, 133
Prasinophyceae, 502
Prasiola japonica, 48
P. sp., 237
Proboscispectyla sp., 82
Prorocentrum, 462
P. gracilis, 421
P. micans, 219, 385, 409, 418, 421, 444, 457, 514
P. sp., 433
P. triangularium, 456
Prosobranchs, 275
Protodictyos sp., 162
Protista, 507, 512
Protococcus sp., 447
Protopodia, 363
Pseudomonas, 303, 309, 326, 328, 357, 362, 363
P. aeruginosa, 381
P. piscicida, 272
P. perfectomarinus, 362
P. sp., 335
Pterocladia, 469, 493
P. capillacea (Syn: P. pinnata), 493
P. densa, 493
P. lucida, 493
P. nana, 493
P. tenuis, 493
P. ovii, 475, 493
P. perforata, 403, 494, 511
P. pseudocrassa, 516
P. pseudo-linearis, 493
P. purpurea (Syn: P. umbilicalis f. lacinia), 493, 494
P. sp., 327
P. subcircularis, 483
P. tenera, 473, 476, 493–495, 502, 513, 515, 527, 529
P. umbilicalis, 473, 493, 494, 505, 507, 516
P. vulgaris, 493
P. yezoensis, 493, 494
Porphyridium aerigineum, 400, 445, 512
P. sp., 133, 413, 413
Puccinellia, 298, 500, 501, 528
Pygaiella istoridis, 476
Pyramimonas inconstantis, 418
P. reticulata, 420, 463, 502
P. sp., 414, 448
Pyrophlyta, 133
Pythium, 341
Rhodomonas, 522
Rhodomonas sp., 283
Rhithropanopeus harrissi, 33
Rhzidium, 337
Rhizoclonium, 500
Rhizomycetes, 337
Rhizopodyctium, 337
Rhizosolenia, 358
Rhodoglossum hemiepiphyllum, 493
R. japonicum, 493
Rhodomonadophycaceae, 493
Rhodopseudomonas, 330
R. palustris, 362
Rhodopseudopirillum, 330
R. palustris, 330
R. rubrum, 330
Rhodotorula, 348, 349, 351
R. glutinis, 352, 353
R. infirma-miniata, 351
R. minuta, 272
R. mucilaginosa, 353, 355
R. rubra, 352, 353
Rhodymenia, 468
R. indica, 493
R. palmata, 468, 489, 524
Rouch, 86
Rotifer (rotifers), 13, 208, 212-214, 455
Ruppia, 499
Ruppiaeeae, 499
Rutulis rutilis, 88-90
sablefish, 69
Saccharomyces bisporus, 362
S. cerevisiae, 351-355
S. melis, 362
S. rouxi, 352
S. sake, 162
Saccorhiza, 471
S. bulbosus, 15, 473, 498, 524
Sagittaria seta, 84, 85, 229
Salicornia, 471, 500
Salmo gairdneri(i), 85, 86, 88-91, 199, 265, 271, 275, 277, 285
S. solar, 265, 283
S. trutta, 283
salmon, 90, 92, 126, 188, 265, 272, 273, 283, 295
salmon, chinook, 91, 98
salmon, Pacific, 254
salmon, sockeye, 272
salmonids, 99, 179, 273, 285, 296
Saprolegnia, 271
S. parasitica, 359
Sarcina lutea, 312
Sarcodina montagneana, 493
Saradinella fimbricornis, 238
Sargassum, 467, 459, 484, 487
S. equiforme, 483
S. echinocarpum, 483
S. enurea, 483
S. furfuraceum, 483
S. granuliferum, 483
S. hemiphyllum, 483
S. horneri, 483
S. tintofoie, 483
S. micracanthum, 483
S. rotileum, 489
S. natans (Syn: S. taeceiferum), 483
S. nigrofum, 483
S. polycystum, 483
S. serratifolium, 483
S. siliculosum, 483
S. sp., 483
S. tennerrimum, 483
S. thumbergii, 483
S. vulgare, 483
S. wrightii, 483, 527
scallop, bay, 293
scallop, 10
Scenedesmus, 207
Schizochytrium, 340, 341, 345
S. aggregatum, 340
scuplin, 272, 274
scypharid, 241
Scytophysa sp., 483
sea grasses—see Grasses, marine
sea lettuce, 475
sea otters, 490
sea urchins, 175, 490, 520
seaweeds, brown, 468, 471, 472
seaweeds, green, 468, 472
seaweeds, red, 468, 471, 472, 509
Sedentaria, 500
Serranidae, 238
S. marinus rubra, 27, 28
Shigella sp., 163
shrimp, brine, 58, 298
shrimp, brown, 60
shrimps, 60, 61, 64, 178, 272, 287, 298–300
shrimp, white, 60
Sipunculidae, 276
Skeletonema, 462, 514
S. costatum, 95, 98, 219, 294, 369, 380, 385,
389, 400, 410, 412, 413, 422, 424, 426, 433,
438, 444, 446, 454, 460, 506, 507, 514
S. sp., 191, 408, 409, 414, 430
S. tropicum, 512
snails, 79, 83, 84, 100, 271, 276
Soliera chordalis, 493
Sparidae, 238
Sparrina, 500
Spargularia, 500
Spermatophyta, 472, 500
Sphaerococcus cartilagineus, 493
Sphaeroma hookeri, 132
Sphaerotiulus, 132
S. natans, 292
Splanchnidium rugosum, 483
sponges, 175
Sporocytophaga, 327
Squallis acanthi, 278
S. cephalus, 90
squid, 44
Sphymlococcus aureus, 27, 161, 162, 514
S. falcatus, 162
Stephanopera gracilis, 450, 453
Stephanopyxis costata (Syn: Skeletonema
costatum), 410
Stichochrysis immobiliis, 417
Stichoeoneus, 372, 385, 410, 450
S. bacillaris, 508, 529
S. cylindricus, 418, 432
S. sp., 418, 434, 448, 453, 517
Stolephorus zollingeri, 238
Streptococcus, 162
Streptomyces, 334
Strongylocentrotus franciscanus, 490
S. purpuratus, 175
Sphyria viatica, 493
Symbiodinium gen. nov., 508
S. microaerificum sp., 508
Synechococcus, 133
Syracosphaera, 450
S. carterii (carterae), 412, 413, 432, 448
S. elongata, 433
S. sp., 410, 416, 417, 449
Syndromium, 499
Tapes decussatus, 155
teleosts, 85, 277, 278, 297, 299
Temora longicornis, 231, 233
Tetrahymena, 281
T. pyriformis, 203
Tetraselmis maculata, 133
T. sp., 396, 445, 448, 518
T. striata, 133, 381
T. tetratheline, 396
Thalassia, 499
T. testudinum, 499, 502
Thalassionema nitzschioides, 219
Thalassiosira decipiens, 451
T. furcata, 412, 508
T. gracilis, 421, 432, 501
T. pseudonana, 219
T. rotula, 370, 447, 524
T. sp., 191
Thiococcus Ferrooxidans, 333
Thiothixiens, 331
Thiorhodaceae, 330
Thiovulum majus, 331, 361
Thraustochytrium, 337, 340, 341, 345, 358
T. aureum, 340
T. rosae, 340
T. sp., 340, 363
Thysanoessa longipes, 82
Tilapia mossambica, 92, 285
Tinca tinca, 90
Tynocladia sp., 483
Tintinnida (tintinnids), 214, 278
Tintinnopsis beroidea, 215
Torpedospora, 344
T. candida, 353
T. famata, 342
T. maris, 347, 364
T. pulcherrima, 353
T. rosea, 347, 364
Trichogaster tetracerus, 80, 290
Trichosporon cutaneum, 350, 351, 355
T. pullulans, 350, 351
trout, 90, 126, 269, 272, 280, 283, 294, 295, 299
trout, brook, 167, 291
trout, rainbow, 85, 86, 88, 107, 199, 265, 271,
275, 277, 279, 285, 286
Tubifex sp., 155
tunicates, 235
Turbellaria, 13
Turbinaria coenoides, 527
T. ornata, 483
T. sp., 483
turbot, 269
Turnerella mortensiana, 493
Tursiops truncatus, 175

Uca pugilator, 297
Ulva, 473, 480, 482, 500, 508, 522
U. fasciata, 481
U. tincta, 132, 468, 473, 475, 481, 482, 508, 518
U. latissima, 421, 481
U. nematoida, 481
U. penniformis, 481
U. pertusa, 481, 482
U. reticulata, 481
U. taeniata, 482
U. thuretii, 482

Ulotrix, 500
Undaria, 467, 468, 472, 477, 484, 485, 487, 488
U. peterseniana, 483, 487, 488
U. pinnatifida, 483, 487, 488, 524
U. undarioides, 483, 487, 488
Uropsora, 515
U. wormskjoldii, 473

Vaucheria, 500
Vibrio, 326, 328, 334
V. sp., 305
Vicia faba, 157, 216
Volvocales, 398

whitefish, 276
Wittia anomala, 162
worms, 10, 78, 521
Xanthophyta, 133

yeasts, 12, 81, 162, 338, 342, 347–364, 369, 384, 468, 527

Zannichellia, 499
Zannichelliaceae, 499
zebra fish, 80, 299
Zostera, 484, 487, 499, 500
Z. marina, 499, 524
Z. nana, 499
Zosteraceae, 499
SUBJECT INDEX

Accumulation, 218-223
Acidification of culture water, 172, 173, 176
Activated carbon (charcoal), adsorption, 134-140, 425
filter, 134
pilot plant, 136-139
Activated sludge, 127-129
process, 127-129
systems, 128
Aeration, 145-153, 316, 392
advantages of, 147
artificial, 145
definition, 145
disadvantages of, 147
dynamics of, 148-153
gas exchange, 152
gas requirements, 145, 146
gas-transfer rate, 148
natural, 145
pretreatment of air, 147, 148
solubility of gases, 148
theory of, 148-153
Aerators, 183-190
air strippers, 184, 187, 188
bubble, 189
cascaders, 145, 184, 186
dripplers, 145, 184
ejectors, 189
normal pressure, 183, 184
pressurized, 183
splashers, 68, 145, 184, 187
sprayers, 145, 184, 190
sprinklers, 145, 184
submersed, 183
surface, 183, 184, 186, 189
wave makers, 145, 184, 209, 228, 229
Aerobic cultivation, bacteria, 316
Agar, 378, 379, 384, 469
marine, 309
Agar-digesting bacteria, 328
Agar plates, 312, 313, 378, 384
Agar shake tubes, 313
Agar slants, 313, 349, 378
Agnotobiotic cultures, definition, 5
Agnotospecific cultures, definition, 5
Air, contamination, 105
disinfection, 147
filtration, 105, 106, 147, 148
reduction of CO₂ content, 147, 148
sterilization, 105, 106, 147
washing, 147
water-vapour saturation, 147, 148
Air bubbles, ascent height, 152
contact time, 150, 152
diameter, 149, 150, 152, 153
motion, 149
shape, 149
size, 149
surface, 149, 150
velocity, 149, 150, 152, 153
volume, 149
Air dispersion, horizontal flow, 151, 153
Air distributors, 185
Airlifts, 60, 61, 115, 141, 142, 183, 190, 191, 208, 209, 222, 224-228, 236-238, 242-244, 319
capacities of, 192
circulator, 191, 193
operational principle, 190, 191
typical examples, 190, 191
water-transport efficiency, 191
Air-shear units, 145, 188
Air sterilizers, 105
Air stones, 185, 186
Air stripping—see Foam separation
Air supersaturation, 146
Air-water interphase, 150, 151
Algae—see Plants
Algal water treatment (Algal filters), 129-134, 159
'Algifert', 470
Alginates, 470, 489
Alkalinity, 173, 176
Amino acids, 80, 477
utilization by plants, 410
Ammonia, 80, 81
accumulation, 88, 99
biological consequences, 88, 99
permissible levels, 99
biological effects, 90
clubbing of gill filaments, 91
concentration in blood, 85, 86
counteraction of poisoning, 92
damage to blood components, 91
damage to liver parenchyma, 91
decrease in blood-cell number, 90
decrease in blood oxygen, 90
decrease in physical performance, 90
Ammonia—continued
decrease in disease resistance, 90, 92
determination, 92, 93
exchange, 88
excretion rates, 82—86, 90
exophthalmos (popeye), 92
gill-surface diminution, 90
hyperexcitability, 90
hyperplasia, 91
importance of pH, 92
inflammation, 91
in situ concentration, 81
lethal limits, 89, 90
microbial decomposition, 124—126
NH₃, 81, 98
permeability, 92
NH₄⁺, 81
oxidation, 124, 168—171
amination, 167
pollution, 88
proportion, NH₃ to NH₄, 88, 87
reduction in erythrocyte number, 91
reduction in gas exchange, 90
standard concentration, 179
stress, precursor of bacterial disease, 91
structural modifications, 91
sublethal limits, 90
terminology, 81
toxicity, 87—90
uptake by phytoplankters, 132, 133
utilization by plants, 408—410
Ammonification, 94
Ammonotelism, 92
Anaerobic cultivation, bacteria, 316
Animal load, 166
Antibiotic mixes, 384
Apparatus, for intertidal organisms, 248—253
for measuring copepod feeding rates, 238
for planktoners, 226, 244
for seaweeds, 228, 477, 478, 480
for sessile plankton feeders, 244
for substrate dwellers, 245
Aquaculture, 9, 46, 47, 87
definition, 2
goals, 6, 7
pilot farm, water-quality monitoring, 178
sewage-treatment system, 9, 10
' Aqua Marine', 29
Aquaria, basic types, 59
Artificial microcosms, 47
Artificial sea water, 29—37, 307, 350, 431—441, 475, 476
mixer systems, 36
recipes, 29—37
Aseptic sampling, bacteria, 315
Ascospore discharge, 343, 344
Aspirators, 184, 187, 188
Autoclaving, 314, 388, 389
Automatic feeders, 68, 259—264, 267, 268
Autotrophic bacteria, 331
Auxins, 420, 477
Auxotrophy, 416
Axenic cultures, 5, 380, 384, 388, 389, 404
definition, 4
fungi, 341
Axenicity, 5
Axenobiont, definition, 5
Axen, 5
'Baby Bio', 470
Backwashing, 39, 46, 119, 126
Bacteria, agar-digesting, 328
autotrophs, 331
carbon—nitrogen sources, 303
carbon sources, 303
cellulose decomposers, 327
chemo-autotrophs, 305, 331—334
chitin-digesting, 328
cultivation of, 301—335
filter-bed, 19, 20
green, 330, 331
growth requirements, 303—312
heterotrophs, 326—329
hodid medium, 304
hydrogen, 334
hydrocarbon decomposers, 328, 329
hydrostatic-pressure requirements, 312
importance for culture systems, 19, 20
in sea water, 25
iron, 333
'Knallgas', 334
light requirements, 311
lipid decomposers, 328, 329
manganese, 333
mineral requirements, 304—306
nitrifiers, 332, 333
nitrogen-fixing, 305
non-sulphur, 330
organic nutrients, 303
photosynthetic, 305, 329—331
pH requirements, 308
physiological types, 326—335
proteolytic forms, 327
purple, 330
role in cultivation, 80
sea-water requirements, 306—308
particulate-matter requirements, 307, 308
Bacteria—continued
'sulphate-reducing', 334
sulphur, 330
temperature requirements, 312
vitamins, 303
Bacteria-free cultures, 380, 381
definition, 6
Bacteriocins, 335
Bacteriophages, 26
importance in cultures, 334, 335
Bacteriostatic filters, 315
Baiting, fungi, 339
Batch cultures, 210, 400
definition, 3
serially renewed, definition, 4
Biochemical oxygen demand (BOD), 108–112
Biochemical potential technique, 320, 322
Biohumus, 470
Biological water treatment, 122–134, 185
algal water treatment, 129–134
microbial water treatment, 123–129
'Bio Sea', 34
Biphasic culture, 420, 421
BOD—see Biochemical oxygen demand
Borel, utilization by plants, 413, 414
Borosilicate glass, 374
Boveri dishes, 58
Breeding, definition, 2
Bromine, utilization by plants, 413
Bubble cup, 244
Bubble pump, 243
'Büsum Meersalz', 29
Buffer capacity, 109, 171
Buffers, 314, 451–453
Calcium, requirement by bacteria, 305
utilization by plants, 412, 413
Capillary priming, 246, 248
Captive sea water, 19, 22, 24, 25, 77
Carbon, utilization by plants, 414–417
Carbon contactor, 134–137
Carbon dioxide, equilibrium, 148
generator, 148
requirement by bacteria, 306
Carbon dioxide system, 109
Carbon/nitrogen sources, bacteria, 303
Carbon sources, bacteria, 303
Carboys, 395
Carrageenans, 470
Carrying capacity, 166–182
assessment of, 167–178
Cartridge filters, 239
Cascaders, 145, 184, 186
Cellulose-decomposing bacteria, 327
Centrifugation, 113, 315
CFU—see Colony-forming units
Charcoal—see Activated-carbon
Chelating agents, 407, 408
Chemically defined culture media, 6
Chemical oxygen demand (COD), 108, 112, 321
Chemo-autotrophic bacteria, 305, 331–334
Chemo-organotrophy, 415
Chemostat, 211–216, 317–319, 322, 355, 400–403
advantages, 213
construction, 213
disadvantages, 122
dynamics, 213
Chitin, 328
Chitin-digesting bacteria, 328
Chloride, 107
Chlorination, 107
Chlorine, biological effects, 155
utilization by plants, 413
Chromium, utilization by plants, 413
Classification of culture systems, 3
Cleaning, of glassware, 372, 373
Climate deterioration technique, 219
Clone cultures, 378
Closed sea-water systems, 42–46, 477
Cobalt, utilization by plants, 413
COD—see Chemical oxygen demand
Collection of organisms, 183, 185, 371
Collectors, 487
Colonial-forming units (CFU), 25–28
Commercial cultivation, 9
definition, 2
goals, 6, 7
Commercial importance, of brown algae, 482–484
of green algae, 480, 481
of red algae, 490–493
Compressors, 188
'Conditioning', 79
Constant-level siphon, 191, 193–195, 246
'Contacting', 155, 189
Contaminants, 79, 95
Continuous cultures, 4, 400–404
apparatus, 401
definition, 3
light thermostat, 217
Continuous culture devices, 210–217
Continuous culture systems, 216, 317, 403
Control, dissolved gases, 206
environmental gradients, 206, 480
light, 197–199, 479, 480
salinity, 200, 201
Control—continued
  temperature, 198–200, 479, 480
  water movement, 201–205
Copper, utilization by plants, 413
Cultivation, animals, 13
  basic prerequisite, 19
  chemical contamination, 15
  commercial, 6, 7, 9
  definition, 2
  diseases of animals, 10
  diseases of plants, 15
  ecological implications, 16
  fungi, 337–366
  history, 1, 2
  in situ, 41
  microcosms, 14
  micro-organisms, 11, 301–356
  multispecies cultures, 14
  plants, 12, 367–501
  research, 6, 7, 9
  goals, 6, 7
  team interaction, 46
  technology, 11, 19
  terminology, 2–6
  water-quality management, 11, 19
Culture Centre of Algae and Protozoa, Cambridge, 371
Culture Collection of Algae, Bloomington, 371
Culture Collection of Algae, Tokyo, 371
Culture devices, continuous, 210–217
  in situ, 69–77
  large, 58–69
  medium-sized, 58–68
  small, 57, 58
Culture flask with conical bottom, 374
Culture media, bacteria, 308, 309, 313
  basic, 6
  chemically-defined, 6
  diagnostic, 314
  disinfection, 102–105
  fungi, 340, 348–351
  holeric, 6, 304, 310
  mereric, 6, 308, 310
  multicellular algae, 473–477
  oligic, 6, 308, 310
  oligomereric, 6
  plants, 420–441
  polymotic, 6
  preparation, 387–392
  sterilization, 102–105, 387–392
  subculturing, 387–392
  terminology, 6
unicellular algae, 387–392
Cultures, bacteria-free, definition, 6
  bottom frames, 71
  cages, 69, 72, 74
  continuous, 4, 400–404
  definition, 3
  floating frames, 71
  floats, 69, 72
  gnotobiotic, definition, 5
  gnotospecific, definition, 5
  hanging frames, 72
  line, 71
  monospecific, 5
  definition, 4
  non-axenic, definition, 5
  oyster frames, 72
  pens, 69
  plastic bags, 69, 70, 73, 76, 77
  pure, definition, 6
  pounds, 69
  racks, 69, 71
  rafts, 69, 71
  serial, definition, 4
  synspecific, 5
  definition, 5
  dispecific, 5
  polyspecific, 5
  tetraspecific, 5
  trienspecific, 5
  synxenic, 5
  definition, 5
  dixenic, 5
  monoxenic, 5
  polyxenic, 5
  trixenic, 5
Culture systems, animal load, 166
  carrying capacity, 166–182
  classification, 3, 37–39
  closed, 37, 38, 42–46, 101
  dimensioning of components, 166, 181, 182
  flow-through, 39
  heterogeneous, 3, 4
  homogeneous, 3, 4
  ladder, 38, 39
  mixed, 3
  multi-container, 39
  multipurpose environmental (MES), 38, 47–57
  open, 37, 41, 101
  recirculation, 42–46
  running water, 37
  semi-open, 37, 38, 42, 101
  standards for design, 167, 175, 179, 181
  still water, 37
Culture tank, for ctenophores and medusae, 231-232
Culture-tank cleaner, 195, 196
Culture-water quality, assessment, 107-112
Culture-water reuse, as function of metabolism, 179-181
Culture-water treatment, 100-166
  biological, 122-134
  algal, 129-134
  microbial, 123-129
  definition, 100
mechanical, 112-122, 165
  diatomaceous-earth filters, 120, 121
  disposable cartridge filters, 121
  other filters, 122
  rapid sand filters, 119, 120
  sand-gravel filters, 114-119
perspectives, 166
physico-chemical, 134-165
  activated carbon adsorption, 134-140
  aeration, 145-153
  coagulation, 164
  electrolytic sewage purification, 165
  flocculation, 164
  foam separation, 140-144
  ion-exchange, 164
  oxygenation, 153, 154
  ozonation, 154-161
  ultra-violet irradiation, 161-164
post-treatment, 100, 101, 165
  pretreatment, 100, 101, 165
  principal types, 130, 101
  reconditioning treatment, 100, 101, 165
Culture-wet film, 373
  12C uptake, algal yields, 486
Dechlorination, tap water, 201
Deep tank, 373, 375, 443
DE filters—see Diatomaceous-earth filters
  Demand feeders, 261, 262, 284-286
  Denitrification, 81, 123, 127, 170, 353
Diphosphorization of culture water, 171
  'Desicote', 374
Detergents, 373
Diagnostic media, 314
Dialysis bag, 377
Dialysis cultures, 215, 219, 375, 377
Dialysis flasks, 375, 377
Diatomaceous-earth filters, 120-122
  cleaning, 120
  pressure filters, 120, 121
  vacuum filters, 120, 121
Difeo, 317
  'Differential poisoning' method, 382
'Diffusers'—see Dispersers
Dilution technique, isolation of algae, 379
Disfuser, 188
Discharge lamps, 197
  emission spectra, 198
Disinfection, 101-107
  air, 105, 106, 147
  antibiotics, 104, 105
  application of chemicals, 104
  application of heat, 103
  chlorination, 104
  definition, 101
  equipment, 107
  glassware, 107
  irradiation, 103
  microfiltration, 102
  ozonation, 104
sea water, 162
Dispersers, 145, 149, 160
  Disposable cartridge filters, 121
Dissolved gases, control of, 206
Dissolved organics, 77, 78
Domestication, definition, 2
Dosemeter, 214, 402
Double cuvette, 227, 229
Double cylinder, 222, 230
Double-cylinder recirculator, 208-210
Drainage in ponds, 64-66
Drippers, 145, 184
Dry mixes, 387
Dry-weight technique, algal yields, 465, 466
Earth extract—see Soil extract
Ecological diagnosis, 9
Ecological potential, 8, 9
Ecosystems, controlled models, 319-322
Ectocrines, 79, 80, 98
EDTA, 407, 408, 419, 432, 436-439, 441
Efficiency, of oxygen transfer, 151, 152
Eh, plants, 451-454
Ejector aerators, 189
Electrode potential—see Redox potential
Electrolysis method, algal purification, 382
Electron acceptors, 310, 311
Elements, required by unicellular algae, 406
Elutriation, 221
Embayment cultures, plants, 460-462
Emission spectra, 442
  fluorescent lamps, 197
  tungsten lamps, 197
Enclosures—see Culture enclosures
End products of nitrogen metabolism, 80-100
Enriched sea-water agar medium, 384
Enrichment cultures, 338, 341, 343, 344
Enrichment of sea water, 421-429
Enrichment technique, bacteria, 320
Environmental control tanks, 260
Environmental factors, control of, 196–207
Environmental gradients, control of, 205–207
Equipment, sterilization, 107
Equipment for cultivation, 182–268
accumulation, 218–223
aerators, 183–190
aerlifts, 60, 61, 115, 141, 142, 183, 190, 191, 208, 209, 222, 224–228, 236–238, 242–244, 310
apparatus for intertidal organisms, 246–253
apparatus for plankters, 227–244
apparatus for sessile plankton feeders, 244
apparatus for substrate dwellers, 245
collection of organisms, 183, 185
constant-level siphon, 191, 193–196
continuous culture devices, 210–217
culture tank cleaner, 195, 196
dialysis cultures, 218
experimental tanks, 253–259
float valve, 191, 193, 194
flow-direction reverser, 196
for controlling environmental factors, 196–207
holding tanks, 253–259
in situ sporophyte system, 224–226
isolation, 218–223
overflow sieves, 183, 186
phytoplankton harvester, 222–224
recirculation devices, 207–209
spray apparatus for algae, 226
Erdachreiber, 473
Erlenmeyer flasks, 58, 373
Eutrophication, 9
Excretion, ammonotelic, 80
guanotelic, 80
ureotelic, 80
uricotelic, 80
Experimental tanks, 253–259, 261
External free amino acids (FAA), 77, 78
External metabolites—see Ectocrines

Farming of marine organisms, definition, 2
Feeders, 58
automatic, 259–264, 267, 268
demand (self-), 281, 262, 264–266
mobile, 260
stationary, 260, 261
Feeding points, in ponds, 68
Feeding rate of copepods, determination, 238
Fermentor, 213
Fernbach flasks, 373
Fertilizers, 459–462, 470, 487
Fibre-glass tanks, 375, 399, 458, 489
Filterable substance (FS), 108
Filter-bed bacteria, 19, 20
oxygen consumption, 174, 175
Filter drums, 122
Filterite cartridge filters, 40
"Filterpressen-Schichtenfilter", 122
Filters, asbestos, 389
bacteriological, 389
cartridge, 239
cleaning, 118, 119
depth of layers, 118
diatomaceous earth, 120–122
diatomaceous glass, 389
disposable, 389
cartridge, 121
filter masses, 114
fritted-glass, 389
glass-wool, 235
hydrosol, 389
mechanical, 113
membrane, 389
mixed plant–animal, 132–134
other, 122
rapid sand, 119, 120
sand–gravel, 114–119
size of filter surface area, 118
surface area of filter bed, 172
trickling, 125, 126
turnover rates, 118
Filter-settler, 235, 236
Filtration, capacity, 171, 174, 175
efficiency, 114, 116, 171
filter material, 114, 116
filter pressure, 115, 116
filter resistance, 115
grain shape, 117
grain size, 117
maintenance, 118
packing of filter layers, 118
rate, 115, 116
sea water, 315
secondary reduction of interstices, 117
speed, 115
velocity, 172
Fish pound, 69, 70, 75
Float valve, 191, 193, 194, 245
Florence flask, 380
Flow-direction reverser, 196
Fluorescent lamps, 442–445
'Cool White', 198, 442
'Daylight', 198, 442
emission spectra, 197, 198, 442
plant light, 442
'Warm White', 198, 442, 445
'Fluvarium', 207
Foamate, 141
Foam collector, 142, 143, 158
Foaming, 140, 143
Foam separation (fractionation), 140–144, 159, 233
Foam separator, 141–143
Foam tower, 142, 143, 158–160, 233–235
Fouling, 39
FS—see Filterable substance
Fungi, baiting, 339
cultivation of, 337–356
incubation of, 342–344
isolation of, 338–344
pathogens, 350, 365
pH requirements, 352, 353
plating, 339–342, 344
salinity requirements, 351, 352, 354
sustenance, 344, 345
thermal requirements, 351
trapping, 343
vitamin requirements, 351

Gas-bubble disease, 147
Gas–liquid mixer, 189
Gas requirements of aquatic organisms, 145, 146
Gas saturation, 145, 146
Gas solubility, 145, 146
Gelatin, 313
'Gelbstoffe', 98
Gelling agent, 313
Gibberellins, 420
Glass columns, 375
Glassware, sterilization, 107
Gnotobiotic cultures, definition, 5
Gnotospecific cultures, definition, 5
Gradients, control of, 205–207, 480
Grain size, 172
Gravity tank, 42
Green bacteria, 330, 331
Greenhouses, 68
Growing tank, for clams, 244
Growth chambers, 394, 401, 446
Growth measurements, bacteria, 316
Growth–promoting substances, 27, 381, 423
Growth requirements, bacteria, 303–312
Guainine, 80

Haemoctyrometer, 465
Hanging-drop culture, 68, 373
Harvester, phytoplankton, 222–224
Harvesting of plants, 462–466
centrifugation, 462–464
coagulation, 464
filtration, 462, 463
flocculation, 462
flotation, 462, 463
ion exchange, 462
sedimentation, 462
Header tank, 42
Helgoland, multipurpose environmental system, 50–53
'Hibi', 482, 495
History of cultivation, 1, 2
Heterogeneous culture systems, 3, 4
single-phase, 3, 4
multi-phase packed towers, 3
two-phase, 4
Heterotrophic bacteria, 326–329
Holding tanks, 253–259
Holidic culture media, 304, 310
definition, 6
Homogeneous culture systems, 3, 4
multi-stage, 3
single-stage, 3
Hydraulic shear disperser, 188
Hydrogen bacteria, 334
Hydrogen-decomposing bacteria, 328, 329
Hydrogen degradation, 329
Illumination—see also Light
adaptation to, 445
plants, 441–445
Immobilization, of protozoans, 222, 223
Incandescent lamps, 444
emission spectra, 197
Incubation, fungi, 342–344, 348, 350, 361, 353–355
Inka system, 189
Inoculation, 373
In situ cultivation, 41
In situ incubator, 324–326
In situ culture plants, 460
In situ kreisel, 291, 232
In situ sporohyte system, 223–226
In situ techniques for bacteria, 322–326
incubator, 324–326
peloscope, 323
submerged-slide, 323
'Instant Ocean', 29, 33, 428
composition of, 34
Intermittent siphoning, 248
Inverted microscope chambers, 454
iodine number, 137
Ion exchange, 164
resins, 164
usefulness in cultivation, 164
Ion-pairing, 108, 109
Iron, requirement by bacteria, 306
utilization by plants, 414, 415
<table>
<thead>
<tr>
<th>SUBJECT INDEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron bacteria, 333</td>
</tr>
<tr>
<td>Irradiation, ultra-violet, 315</td>
</tr>
<tr>
<td>Isolation, 218–223</td>
</tr>
<tr>
<td>of fungi, 338–344, 348–355</td>
</tr>
<tr>
<td>of plants, 377–380</td>
</tr>
<tr>
<td>Jet aerator, 188</td>
</tr>
<tr>
<td>Kinetins, 420</td>
</tr>
<tr>
<td>'Knallgas' bacteria, 334</td>
</tr>
<tr>
<td>Kreisel, for seston-feeding plankters, 228, 229</td>
</tr>
<tr>
<td>in situ, 231, 232</td>
</tr>
<tr>
<td>plankton, 225–228</td>
</tr>
<tr>
<td>Laboratory streams, 203–205</td>
</tr>
<tr>
<td>Ladder system, 38, 39</td>
</tr>
<tr>
<td>Lamps, emission spectra, 442</td>
</tr>
<tr>
<td>special, 442</td>
</tr>
<tr>
<td>Levy haemocytometer, 464</td>
</tr>
<tr>
<td>Lexan®, 394</td>
</tr>
<tr>
<td>Life-endangering substances, 79–100</td>
</tr>
<tr>
<td>ammonia, 80, 81</td>
</tr>
<tr>
<td>nitrate, 81</td>
</tr>
<tr>
<td>nitrite, 81</td>
</tr>
<tr>
<td>organic substances, 97</td>
</tr>
<tr>
<td>other inorganic substances, 97</td>
</tr>
<tr>
<td>Life-supporting substances, 76–79, 98</td>
</tr>
<tr>
<td>Light—see also Illumination</td>
</tr>
<tr>
<td>control of, 197–199, 479, 480</td>
</tr>
<tr>
<td>requirements by bacteria, 311</td>
</tr>
<tr>
<td>requirements by plants, 441–445</td>
</tr>
<tr>
<td>Light quality, 443, 444</td>
</tr>
<tr>
<td>Light sources, 441–445</td>
</tr>
<tr>
<td>Light–temperature gradient plates, 206, 207</td>
</tr>
<tr>
<td>Light thermostat, 217</td>
</tr>
<tr>
<td>Limniculture, definition, 2</td>
</tr>
<tr>
<td>Lipid-decomposing bacteria, 328, 329</td>
</tr>
<tr>
<td>Lipids, 328</td>
</tr>
<tr>
<td>Liquid media, 313, 314</td>
</tr>
<tr>
<td>Lyophilization, 317</td>
</tr>
<tr>
<td>Macronutrients, plants, 405</td>
</tr>
<tr>
<td>Magnesium, requirement by bacteria, 305</td>
</tr>
<tr>
<td>utilization by plants, 412, 413</td>
</tr>
<tr>
<td>Magnetic stirrer, 403</td>
</tr>
<tr>
<td>Maintenance, definition, 2</td>
</tr>
<tr>
<td>of bacteria cultures, 316, 317</td>
</tr>
<tr>
<td>Manganese, utilization by plants, 413</td>
</tr>
<tr>
<td>Manganese bacteria, 333</td>
</tr>
<tr>
<td>Mangroves, 500</td>
</tr>
<tr>
<td>Mariculture, 46</td>
</tr>
<tr>
<td>definition, 2</td>
</tr>
<tr>
<td>Marine agar, 309</td>
</tr>
<tr>
<td>Marine Biological Laboratory Plymouth (England), sea-water system, 42</td>
</tr>
<tr>
<td>Marine Culture Laboratory, Granite Canyon (USA), sea-water system, 39, 40</td>
</tr>
<tr>
<td>Marine Laboratory Aberdeen (Scotland), sea-water system, 43–45</td>
</tr>
<tr>
<td>'Marinure', 470</td>
</tr>
<tr>
<td>Mass culture of plants, 392–398, 457, 459, 490</td>
</tr>
<tr>
<td>'Maxicrop', 470</td>
</tr>
<tr>
<td>Mechanical filter, capacity of, 116</td>
</tr>
<tr>
<td>Mechanical filtration, 113</td>
</tr>
<tr>
<td>Mechanical water treatment, 112–122</td>
</tr>
<tr>
<td>Medium 2216, see also Culture media</td>
</tr>
<tr>
<td>Membrane filters, 316, 333, 375, 389</td>
</tr>
<tr>
<td>MES—see Multipurpose environmental systems</td>
</tr>
<tr>
<td>Metabolic byproducts, 180–182</td>
</tr>
<tr>
<td>Metabolite concentration, in culture systems, 179–181</td>
</tr>
<tr>
<td>Metabolites, 79, 98</td>
</tr>
<tr>
<td>Metal mix, 437, 439, 440</td>
</tr>
<tr>
<td>'Meteor'-plankton-cuvette, 229, 230</td>
</tr>
<tr>
<td>Methaemoglobin, 100</td>
</tr>
<tr>
<td>Methaemoglobinemia, 100</td>
</tr>
<tr>
<td>Micro-aquarium, 245, 246</td>
</tr>
<tr>
<td>Microbial filtration, 123–129</td>
</tr>
<tr>
<td>in activated sludge, 127–129</td>
</tr>
<tr>
<td>mechanical filters, 124–127</td>
</tr>
<tr>
<td>Microbial water treatment, 123–129</td>
</tr>
<tr>
<td>Microcosms, controlled models, 319–322</td>
</tr>
<tr>
<td>Micronutrients, plants, 406</td>
</tr>
<tr>
<td>Micro-organisms, cultivation of, 301–365</td>
</tr>
<tr>
<td>Migration-tube system, 220, 221</td>
</tr>
<tr>
<td>Millipore filters, 378</td>
</tr>
<tr>
<td>Mineral requirements, bacteria, 304–308</td>
</tr>
<tr>
<td>Minerals, requirements of plants, 405–414</td>
</tr>
<tr>
<td>Miquel's solution, 426, 427, 432</td>
</tr>
<tr>
<td>Mixed culture systems, 3</td>
</tr>
<tr>
<td>Mixer for preparing artificial sea water, 36</td>
</tr>
<tr>
<td>Mixotrophy, 415</td>
</tr>
<tr>
<td>Molybdenum, utilization by plants, 413</td>
</tr>
<tr>
<td>Monitoring, of culture-water quality, 178</td>
</tr>
<tr>
<td>Monobiont, definition, 4</td>
</tr>
<tr>
<td>Monospecific culture, 5</td>
</tr>
<tr>
<td>definition, 4</td>
</tr>
<tr>
<td>Multicellular plants, cultivation of, 467–501</td>
</tr>
<tr>
<td>food, for animals, 468, 469</td>
</tr>
<tr>
<td>for man, 467, 468</td>
</tr>
<tr>
<td>importance, for economy, 467–472</td>
</tr>
<tr>
<td>for science, 467</td>
</tr>
<tr>
<td>industrial uses, 471</td>
</tr>
<tr>
<td>medicine, 469, 479</td>
</tr>
<tr>
<td>pharmacological applications, 470</td>
</tr>
<tr>
<td>use for manure, 470, 471</td>
</tr>
<tr>
<td>Multipurpose environmental systems (MES), 38, 47–57</td>
</tr>
</tbody>
</table>
Nanaimo, fish pound, 69
National Collection of Marine Bacteria (NCMB), 317
Neuman–Kolowicz cell, 464
NH₃ poisoning, biological consequences, 86–92
counteraction, 92, 99
importance of pH, 92
susceptibility to, 92
Nitrate, 93–97, 99, 100
accumulation, 97
determination, 97
permissible levels, 99
standard concentration, 179
uptake by phytoplankters, 132, 133
utilization by plants, 408–410
Nitrate production, 94–96
Nitrate-reducing potential, bacteria, 322
Nitrification, 81, 94, 127, 168, 172, 332
Nitrifying bacteria, 322, 332, 333
Nitrite, 93–97, 99, 100
accumulation, 97
determination, 97
permissible levels, 99
standard concentration, 179
uptake by phytoplankters, 132, 133
utilization by plants, 408–410
Nitrite production, 94–96
Nitrogen, excretion, 80
fixation, bacteria, 305
metabolism, end products, 80–100
recycling, 80, 81
requirement by bacteria, 304, 305
sources, utilization by plants, 408–410
Nitrogenous substances, 80
oxidation of, 94–96
non-axenic cultures, definition, 5
Non-nutrient media, 6
Non-sulphur bacteria, 330
Nutrient media, 6
Nutritional requirements of unicellular algae, 405–420

OCF—see Oxygen consumption of filter-bed micro-organisms
OD—see Optical density
Oil degradation, 329
Oligo culture media, 308
definition, 6
Oligomeridic culture media, definition, 6
OM—see Oxidizable matter
Open sea-water systems, 37–41
Optical density, algal yields, 465
Orifice dispensers, 188
O-ring seal, 257
Osmotic requirements, bacteria, 307
Overflow sieves, 183, 186
Oxidizable matter (OM), 108
Oxygen, diffusion, 151
efficiency of transfer, 151, 152
electron acceptor, 310
rate of diffusion, 151
Oxgenation, 153, 154
Oxgenation tablets, 154
Oxygen consumption, of filter-bed bacteria, 174, 175
of filter-bed micro-organisms (OCF), 108, 112
Oxygen production, algal yields, 466
Oxygen saturation, 145, 146
Oxygen solubility, 145, 146
Ozonation, as water pretreatment, 157
biological effects, 154–157
dosage, 154, 156
efficiency, 155
use in sea-water systems, 104, 158, 159
use in waste-water treatment, 158
Ozonator, 158
Ozone, absorber, 159
characteristics, 154, 155
chemical reactions, 155
decomposition, 154
disinfection capacity, 157
effectiveness for virus inactivation, 156
functional groups oxidized, 155
generator, 158, 159
injection, 158
methods for determination, 160, 161
production, 158
radiomimetic effects, 157
solubility, 154
toxicity, 156
Ozonides, 155
Paddle splasher, 66, 69, 70, 187
Palmer counting slide, 464, 465
Particulate matter, requirements by bacteria, 307, 308
Pasteur pipette, 313
Peloscope, 323
Percentage of saturation, 177
Percolation, 170, 173
Percolator, 167, 168
Perfusion chamber, 375, 376
Petri dish, 58
Petroff–Hausser bacteriological chamber, 464, 465
pH, adjustment of, 314
buffers, 314, 451–453
plants, 451–454
requirements by bacteria, 308
requirements, fungi, 352, 353
values, control, 108
importance in cultures, 108
Pheromones, 79
Phosphate, accumulation, 97
Phosphorus, requirement by bacteria, 304, 306
utilization by plants, 410, 411
Photoheterotrophy, 415
Photo-organotrophy, 416
Photosynthetic bacteria, 305, 329–331
Phototaxis, deletion of algae, 380
Phyico-chemical water treatment, 134–140
activated carbon adsorption, 134–140
aeration, 145–153
coagulation, 164
electrolytic sewage-purification, 165
treatment, 154–161
ultra-violet irradiation, 161–164
Phytoplankton harvesters, 222–224
Pigment determination, algal yields, 466
Plating, fungi, 339–342, 344–348
Plumbers, 200, 202
Polyethylene liner, 63
Polyethylene sheets, 461
Polymeric culture media, definition, 6
Pond cultures, plants, 460–462
Ponds, 58, 60–70, 76
aeration, 64
best sites, 62
cement-lined, 61, 62
circulation, 62, 65
directional water movement, 64
drainage, 64–66
farm design, 68, 70
feeding points, 68, 69
levee enforcement, 65
liners, 63, 64
pollination, 64
resting corner, 68, 70
seals, 63
sieves, 64, 65
soil compaction, 63, 64
soil sealing, 63
stability of bottom, 63
stability of levees, 63
water-quality management, 66, 67
water retention, 63
water supply, 64
vehicle access, 68
Pool cultures, plants, 454–460
Potassium, requirements by bacteria, 305, 306
Potassium, utilization by plants, 412
Pound, octagonal, 69, 76
Pour-plates, 313
Precoat, diatomaceous-earth filters, 120, 121
Pressure, bacteria cultures, 319
requirements by bacteria, 312
Propellers, 187
Protamine, 222
Protein foaming—see Foam separation
Proteolytic bacteria, 327
Protoplasmic oxidation, 155
Pumps, antifoam, 213
bubble, 243
defective, 147
induction-driven, 234
in ponds, 67
metering, 214, 216, 217
micro, 403, 404
peristaltic, 214–216, 318
pressure, 319
sea-water inert, 205
Steinzeug, 51
submersible, 183
timer-controlled, 246
vertical, 68, 70
Pure cultures, 380, 381
definition, 6
Purification, 380–387
Purple bacteria, 330
Putrefaction method, 390
Pyrex, 374
carboys, 375

Raceways, 58, 60, 61
Radiant energy, measurement of, 444
wavelength, 444

see also Light
Raft cultures, 487, 489, 490
Raising, definition, 2
Rapid sand filters, 119, 120
Rearing, definition, 2
Recipes for artificial sea water, 29-37
Recirculation devices, 207-209
Recirculation system, 42-46
for copepods, 234, 235
for crustacean larvae, 236, 237, 239-241
for sessile animals, 242-244
Recirculation tubes, 207
Recirculator, double, 208-210
single, 208
Redox dyes, 110
Redox potential, 109-111, 454
electrometric methods, 109
equipment for control, 109
indicator dyes, 109, 110
Reduction-oxygenation potential—see
Redox potential
Requirements of plants, amino acids, 410
ammonia, 408-410
boron, 413, 414
bromine, 413
calcium, 412, 413
carbon, 414-417
chlorine, 413
chromium, 413
cobalt, 413
copper, 413
iron, 414
macronutrients, 405
magnesium, 412, 413
manganese, 413
micronutrients, 405
minerals, 405-414
molybdenum, 413
nitrate, 408-410
nitrite, 408-410
vitamins, 417-420
zinc, 413
Research cultivation, 9
goals, 6-7
Resting corner in ponds, 68, 70
‘Rila Marine Mix’, 29, 428, 429
Rinsing procedures, 373
Roll tubes, 313
Rotators, 202, 203
Roto-compressor chamber, 223
Rotors, 187
for plankton, 231, 233
Roux flasks, 373
Running-water systems, 37
Salinity, control of, 200, 201
plants, 448-451
requirements, by fungi, 351, 352, 354
by bacteria, 307
Salt-marsh plants, 500, 501
Sammlung von Algenkulturen, Gottingen, 371
Sampling of bacteria, aseptic, 315
Sand-gravel filters, inside filters, 114, 115
outside filters, 114, 115
typical design, 114, 115, 117
Saran Box Barrier, 64, 65
Saran Screen, 64
Saran Sock Barrier, 64, 65
Saturation of culture system, 177
Sbirka Kultur Autotrofnich, Praha, 371
Schaukel, 201, 202
‘Seaborn’, 470
‘Seahorse’, 470
Sea salts, reconstituted, 427-431
Sea water, antibacterial activity, 27
antibiotic activity, 27
as culture medium, 20-37
bactericidal capacity, 27
captive, 19, 22, 24, 25, 77
cellular density, 21-23
chemical model, 21-23
cultivars, 22
growth-promoting substances, 27
major changes in cultures, 22, 23
natural, 21-29, 36, 37, 306, 353, 421-423,
425, 426, 428, 430, 432, 433
‘normal’, 307
requirement by bacteria, 306-308
standard, 425
storage, 24-29, 35, 37, 306
Sea-water intake, 39-41
Sea-water media, with inorganic enrichments,
428, 429
Sea-water system, closed, 477, 478, 480
SUBJECT INDEX

Seaweeds—see Multicellular plants
Sedgewick–Rafter cell, 464, 465
Sedimentation, 112, 113
 tanks, 113
Sediment dwellers, 245
Self-feeders—see Demand feeders
Semi-open sea-water systems, 37, 38, 42
Serial cultures, definition, 4
Serially renewed batch cultures, definition, 4
Sewage-treatment system, 9
Sox attractants, 79
Shakers, 201, 202, 373
Shaking table, 392
Silica gel, 31.3
Silicon, utilization by plants, 4
Silica gel, 31.3
Siphons, capillary-priming, 246
constant-level, 191, 193-195, 245
'SM-3', 470
Sodium, requirement by bacteria, 305, 306
 utilization by plants, 412, 413
Soil extract, 421-425, 473-475
Solid media, bacteria, 312, 313
Sparger, 188
Splashers, 68, 145, 184, 187
Spray apparatus, for algae, 224, 226
Sprayers, 145, 184, 190
Sprinklers, 145, 184
Standard sea water, 425
Standards for culture-system design, 167, 175, 179
Steinzeug Pumpen, 51
Sterility test, 384, 386
Sterilization, 101-107, 314, 315, 388, 389
 antibiotics, 104, 105
 application
 of chemicals, 104
 of heat, 103
 chlorination, 104
definition, 102
equipment, 107
glassware, 107
irradiation, 103
microfiltration, 102
 of air, 105, 106, 147
 of other culture media, 102-105
 of sea water, 102
 ozonation, 104
Still-water systems, 37
Stock improvement, definition, 2
Storage of sea water, 24-29, 36, 37
Submerged-slide technique, 323
Suction collector, 183-185
'Sulphate-reducing' bacteria, 334
Sulphur, requirement by bacteria, 304, 305
 utilization by plants, 410, 411
Sulphur bacteria, 330
Surface aerators, 183, 184, 186, 189
Sustenance, fungi, 344, 345
Synchronization, of cultures, 212, 396, 397, 399, 400
Synchronous cultures, unicellular algae, 396-400
Synspecific cultures, 5
definition, 5
Tank cultures, plants, 434-460
Tanks, deep, 373, 375, 443
 environmental control, 260
 experimental, 253-259, 261
 fibre-glass, 468, 499
 holding, 253-259
'Taneba', 482
Taumeltech, 229, 230
Temperature, control of, 198-200, 479, 480
 plants, 445-448
 requirements by bacteria, 312
Terminology of cultivation, 2-6
Test tubes, 373
'Tetra Marine', 60
Texel, multipurpose environmental system, 54-56
Thalasstotron tidal system, 478, 477, 479
Thermal gradient plates, 205-207
Thermal requirements, fungi, 351
Thioglycollate medium, 384
Tidal systems, 246-253
Total organic carbon (TOC), 108, 112
Trace elements, requirement by bacteria, 306
Trace metals, utilization by plants, 413, 414
Trapping, fungi, 343
Trickling filters, 125, 126
'Trident', 470
Tris, 432, 433, 437-440, 462, 463
'Triton Marine Salts', 29, 233
Tubes, use in bacterial cultures, 313
Tube settler, 113
Tungsten lamps, 441
 emission spectra, 197
 illumination characteristics, 197
 irradiance, 197
Turbidostat, 211, 215-217, 400
Tyndallization, definition, 102
Ultra-violet irradiation, 161-164, 315, 382, 383
 absorption, 161
 biological effects, 161
Ultra-violet irradiation—continued
   disinfective capacity, 162
   disinfective efficiency, 162, 163
   disinfective potential, 161
   effectiveness for virus inactivation, 161, 162
   effective wavelengths, 161
   germicidal lamp, 162
   oxidizing capacity, 161
   transmittance, 161
   ultra-violet discharge units, 162–164
   ultra-violet disinfection boxes, 162, 163
   ultra-violet lamps, 162, 163
Underwater growth station, 486
Uni-algal cultures, 378, 379
Unicellular algae, as food organisms, 464
   cultivation of, 367–466
Urea, 80, 98
   uptake by phytoplankters, 132, 133
   utilization by plants, 409, 410
Uric acid, 80
   utilization by plants, 409, 410
Utermohl technique, 465
   'Utility Seven Seas Marine Mix', 29, 33
Venturi disperser, 189
Viruses, ozone inactivation, 156
   ultra-violet inactivation, 161, 162
VISHNIAC's medium, 340
Vitamin assays, 418, 419, 425
   Vitamin Mix, 434–436, 440
   Vitamin requirements, fungi, 340, 351
   Vitamins, 370, 432
      bacteria, 393, 399
      plants, 417–420, 476, 477
   Vycor glass, 374
Washing technique, 382
   isolation of algae, 379
Waste production, in aquaculture farms, 177
   microbial oxidation, 110
Water–air interphase, 151, 152
Water movement, control of, 201–205
   piston-pump system, 201, 202
   rotators, 202, 203
   schaukel, 201, 202
   shaker, 201, 202
Water-quality assessment, 107–112
   quality management, 19
Wave makers, 145, 184, 209, 228, 229
Woods Hole, multipurpose environmental system, 48–50, 57
Yeasts, cultivation of, 348–356—see also Fungi
   Yield determination, of algae, 464–466
   'Zentriklones', 32, 113
Zinc, utilization by plants, 413