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REVIEW

The determination of nitrogen status in microalgae

Kevin J. Flynn

Dunstaffnage Marine Laboratory, PO Box 3, Oban, Argyll PA34 4AD, Scotland, United Kingdom

ABSTRACT: Present methods used for the determination of N-status in microalgae typically involve testing for the existence of gross metabolic changes which develop in response to N-stress. Such approaches have 2 problems. First, the experimental techniques may be inappropriate for the species present and may perturb the organisms, possibly creating artifacts. Second, these gross changes, such as changes in rates of CO_2-fixation and N-source uptake, may be affected by factors other than N-stress. There is a need to develop methods to detect metabolic changes which themselves trigger the genetic response to N-stress rather than to detect the products of that response. Such changes are likely to be in relative proportions of key metabolites of C and N metabolism, as in bacteria. It is suggested that only in the presence of excess NH_4^+ are the processes of cellular response to N-stress fully suppressed. As a consequence, microalgae throughout the oceans may show some symptoms of N-stress. The level of derepression of the N-stress responses which corresponds to growth-limitation, and hence is of ecological significance, needs to be determined.

INTRODUCTION

Understanding mechanisms which control production in the oceans is central to our wider understanding of marine ecology. Nutrient, usually nitrogen, limitation of phototrophic production is frequently suggested. In nature, nutrient limitation is invariably a rate limitation because nutrients are cycled between components of the food web; nitrogen may become limiting only when processes of regeneration are outstripped by demand (see Flynn 1989b). Results from tests for N-stress in microalgae, then, may be useful in attempting to explain ecology and productivity provided that the results and our interpretations of them are reliable.

The aims of this review are to consider the problems associated with methods used in past attempts to determine the N-status of microalgae and, with reference to recent developments, to look towards the future development of more sensitive and reliable methods. Readers who require further information on more general aspects of microalgal N-metabolism should consult Wheeler (1983) and Syrett (1988), and references therein.

Some of the examples given are not marine organisms; they are included because the biochemical basis for responses to nutrient stress are most likely similar while data for marine organisms are incomplete.

CAUSES OF NITROGEN STRESS; C, N, P INTERACTIONS

N-status is measured on a continuous scale from N-replete (zero stress), through N-sufficiency (enough stress to promote derepression of nitrate transport and assimilation processes, for example, but not enough to be growth limiting), down to N-deplete (maximum stress and no growth). The differences between the N-replete and N-sufficient phases may not be apparent by following growth rates in chemostat cultures. However, the N-replete cells may lay down more N in storage compounds, and the metabolic state of the cells may differ, with growth rate being limited by another factor (ultimately by genetic/cell cycle factors). By the criteria used in chemostat theory (single parameter variation), determining the level of stress which results in N-limitation of growth should be a simple process. However, in nature few parameters are near constant and because of the multi-variant conditions, which may stress the cells in different ways, simple models such as cell quota approaches (e.g. Droop 1974) are inadequate to describe growth in nature.

Stress may be caused by environmental factors (e.g. the nutrient is absent from the medium, or present at levels at which transport processes are rate limiting) or

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it may be a function of physiology (e.g. nutrient is in such a form that metabolic processes for conversion into other compounds are rate limiting). Physiological N-stress is an important concept which is rarely considered. It seems probable that the occurrence of physiologically induced stress forms the basis for the evolution of, for example, substrate preference for NH_4^+ over NO_3^- (but see below).

Interaction between environmental and physiological stress occurs at the level of nutrient transport, especially when 2 or more N-sources are present at different concentrations and require different metabolic processes for their incorporation. Although the rate of transport at the prevailing substrate concentration is more important than comparisons of the half-saturation coefficient K_s with substrate concentration, substrate concentration may still be a significant limiting factor at 3 to $5 \times K_s$. Because of the possible interference of metabolic and transport processes (Wheeler et al. 1982, Flynn & Butler 1986, Harrison et al. 1989), which raises further doubts about the reliability and usefulness of K_s values, we cannot be sure how important limitation is at the point of uptake in nature. Clearly, however, in an environment in which nutrients may be supplied in pulses, substrate concentrations will on occasion be rate limiting.

Illumination is subject to variation in both quantity and quality over both short and long time periods in nature. C or N status alone can only be described for cultures for which only one variable (photon flux density or nutrient concentration) is present; in nature the two are inextricably linked in C-N status. This interaction is complex because, for example, C and N are required for the synthesis of the photosynthetic apparatus, while the processes of inorganic C and N uptake need not be coupled. The importance of this interaction for N metabolism has long been known (e.g. MacIsaac & Dugdale 1972).

Amino acid synthesis is one of the first, and arguably the most important, points of interaction between N and C assimilations. The data in Fig. 1 show the

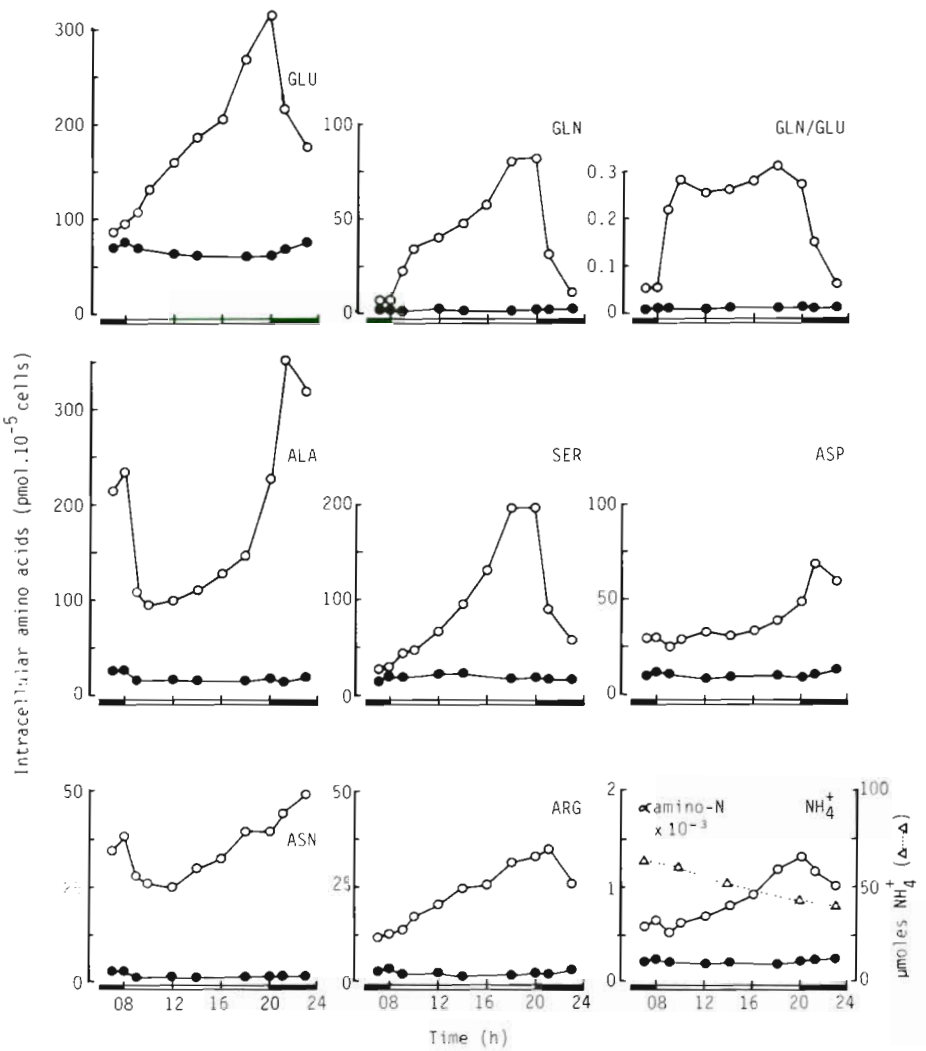

Fig. 1 *Dunaliella primolecta* Butcher. Changes in concentrations of intracellular amino acids during growth in a 12 h/12 h light-dark cycle (80 μmol m^{-2} s^{-1}). (○) Cells were growing in the presence of NH_4^+ (concentrations bottom right; Δ---Δ); or (●) had been resuspended in N-free medium and N-deprived for 2 d. Growth medium, methods of sampling and analysis as described by Flynn & Fielder (1989). Most cell division occurred between 22:00 and 06:00 h; typical cell densities of 1.5 to 3×10^4 ml^{-1}. Care was taken to prevent accidental illumination of the culture during sampling in the dark phase. GLU: glutamate; GLN: glutamine; ALA: alanine; SER: serine; ASP: aspartate; ASN: asparagine; ARG: arginine

changes in concentrations of intracellular free amino acids occurring in a chlorophyte during growth in a light-dark cycle. The onset of darkness results in a decrease in concentrations of many amino acids, probably as a consequence of continued protein synthesis in darkness (Cuhel et al. 1984) in the absence of significant new amino acid synthesis. The concentrations of amino acids in N-deprived cells are much lower and show less variation over the day. These data illustrate the significance of changes which occur during simple changes in illumination. They also show the potential problems in extrapolating results obtained, and in taking methods developed using continuously illuminated cultures into the real world. Unfortunately, most laboratory experiments have used cultures grown in continuous light both in an attempt to promote more rapid growth and to eliminate the significant fluctuations, such as those shown in Fig. 1, caused by changes in light and dark.

The effects of darkness or inadequate illumination can be divided into C-stress and energy-stress. There may be a specific requirement for photosynthesis to supply C-skeletons of some amino acids, whilst processes of ammonium incorporation into amino acids and the subsequent transamination processes require ATP and/or NAD(P)H. The preliminary processes of N_2-fixation or NO_3^- reduction, where applicable, make heavy demands for reductant which must be supplied either directly or indirectly from photosynthesis. Flynn & Gallon (1989) suggest that N_2-fixing non-heterocystous cyanobacteria are physiologically N-stressed.

Another problem stems from the use of measurements of C-fixation to estimate production (Flynn 1988a). The question of nutrient limited algal production often translates to a question of nutrient limitation of CO_2-fixation. The answers to these questions need not be the same if a heterotrophic potential is being realized. The ultimate function of a microalga is to reproduce and not to fix CO_2 for the benefit of the food chain. However, as rates of CO_2-fixation are of undoubted importance to the total ecosystem, the methods used for the determination of N-status may need to be chosen depending on the type of question being asked.

P-limitation in the oceans is usually considered less common than N-limitation (Goldman et al. 1979, Smith 1984, Smith et al. 1986). Even when levels of inorganic P are very low, P-stress may be reduced by the use of polyphosphate reserves maintained by many algae. The detection of alkaline phosphatase activity is often used to detect P-stress, yet by the time that this activity appears the cells may be suffering severe P-deprivation (see Flynn et al. 1986). Because of the role that P plays in cell energetics, P-stress could affect many reactions of C and N metabolism. Terry et al. (1985) report that P-deprivation severely restricts the ability to store N in non-protein forms, presumably affecting amino acid synthesis. Davies & Sleep (1989) review the interaction of P-stress with CO_2-fixation and N uptake, an important, though neglected, area of nutrient physiology.

PAST METHODS USED FOR THE DETECTION OF N-STRESS IN MICROALGAE

When N-stress is induced in laboratory cultures, many metabolic changes occur. There is not an immediate 'shutting down' of metabolic processes, rather a redirection of the cell's activity. Thus transport proteins for alternative N-sources (Syrett et al. 1986), and enzymes for the assimilation of those sources (such as nitrate reductase), may be synthesized or derepressed (Hipkin & Syrett 1977, Everest et al. 1986, Syrett & Peplinska 1988). Methods used for the detection of N-stress often rely on detecting the occurrence of such changes (Tables 1 and 2). Works in which different criteria for the determination of N-status have been compared include Vincent (1981), Chiaudani & Vighi (1982), Glibert & McCarthy (1984), Dortch et al. (1985) and Paasche & Erga (1988).

Methods requiring incubation of organisms usually involve the addition of the suspected limiting nutrient and monitoring any change in growth or metabolism (Table 1). Although increase in growth (Table 1a) appears the ideal parameter to monitor, the prolonged incubation required is to be avoided because of the risks associated with containment (stress and selective pressures for different organisms). Enhanced $^{14}CO_2$-fixation following 24 h incubation with a test nutrient (Table 1b) is an alternative to measuring growth rate changes; short-term incubations may produce misleading conclusions (Goldman & Dennett 1983). Measurements of protein synthesis, a direct function of growth, may offer a more attractive alternative but even this requires incubations exceeding 3 h (Lohrenz & Taylor 1987).

Tests of enhancement of dark $^{14}CO_2$ uptake following addition of, typically, NH_4^+ (Table 1c) only work well at extremes of high and low N-status, but the improved method comparing the enhancement in darkness and light ($V_D:V_L$; Table 1d) which can give information on the degree of stress is, not surprisingly, sensitive to changes in illumination during the incubation (Glibert et al. 1985). The alternatives, tests of N-source uptake rates and fate (Table 1e to i) offer little advantage except where specific information is required on the effects of perturbation with alternative N-sources. The use of ^{15}N analogues has its own problems. ^{15}N methods are not tracer studies and one may question what effects the addition of relatively high

Table 1. Some criteria used for the diagnosis of N-limitation of microalgal growth requiring incubations of samples. Organisms: LC, laboratory cultures or NP, natural populations; bac, Bacillariophyceae (Chrysophyta); chl, Chlorophyceae (Chlorophyta); cry, Cryptophyta; cya, Cyanophyta; pra, Prasinophyceae (Chlorophyta); pry, Prymnesiophyceae (Chrysophyta); pyr, Pyrrhophyta; var, various or unspecified. Sources are not exhaustive

Criterion	Organism	Comments	Source
(a) Enhanced growth on enrichment	NP (bac/var)	Incubations of days may cause artifacts critically reviewed by Hecky & Kilham (1988)	Menzel et al. (1963), Maestrini et al. (1986)
(b) Enhanced light C-fixation on enrichment	NP (var)	Incubation of 24 h may cause artifacts	Wafer et al. (1988)
(c) Enhanced dark C uptake on enrichment	LC (bac/chl/cya) NP (bac/cya/pyr)	Enhancement ratio depends on incubation period (typically 2 to 3 h), critical ratio ± enrichment = 2 for deprivation, 'all or nothing indicator' and response differs depending on growth-N & test-N. May not work with prokaryotes	Yentsch et al. (1977), Vincent (1981), Goldman & Dennett (1983, 1986), Glibert et al. (1985), Paasche & Erga (1988)
(d) $V_D : V_L$	LC (bac) NP (var)	Modified dark-C method comparing enhancement in dark and light. Gives degree of deprivation but sensitive to light changes during test	Goldman & Dennett (1983), Glibert et al. (1985)
(e) Enhanced NH_4^+ uptake rate or capacity	LC (bac/chl/cya/pyr) NP (cya/bac)	Luxury uptake may not indicate N-deprivation if cells grown on NO_3^- (see Horrigan & McCarthy 1982)	Vincent (1981), Dortch et al. (1982)
(f) V_{SAT}/V_{TRACE}	NP (var)	Comparison of uptake rates at different substrate concentrations; N-deprived if > 1	Glibert & McCarthy (1984)
(g) Degree of NO_3^- dependence	NP (var)	Compare rates of NO_3^- ± NH_4^+ uptake, if N-deprived NH_4^+ inhibition is less	Glibert & McCarthy (1984)
(h) Decreased L : D NO_3^- uptake	NP (bac/pyr)	If N-sufficient, NO_3^- uptake occurs in light, if N-deprived, uptake is less photoperiodic	Paasche & Erga (1988)
(i) Incorporation of NH_4^+ into macromolecules	LC (bac/pry) NP (var)	If N-deprived, incorporation is quicker. Level of illumination affects results because of effect on protein synthesis (TCA insol.)	Wheeler et al. (1982), Glibert & McCarthy (1984), Kanda et al. (1988)
(j) Periodicity of metabolism in L/D cycle	LC (bac)	Protein synthesis periodic when N-deprived Lipid synthesis periodic when P-deprived Nutrient uptake periodic when light limited	Terry et al. (1985)

concentrations of ^{15}N substrates (for which there may also be discrimination between ^{14}N and ^{15}N) have on cell metabolism. Collos (1987) discusses errors in analysis of ^{15}N data affecting, in particular, several studies in which the simultaneous uptakes of several N-sources have been investigated.

These enhancement tests make use of the changes in coupling of CO_2-fixation and N-source uptake, and differences in uptake of NH_4^+ and NO_3^-, which occur when algae are N-stressed. However, different groups of algae may use different metabolic pathways and response times and phasing of uptake and growth may also vary (Collos 1986, Glibert et al. 1986). Dortch et al. (1982) say that NO_3^- uptake by N-deplete cells cultured under continuous illumination is generally much slower than uptake of NH_4^+. This claim appears to contrast with the increased dependence on NO_3^- rather than NH_4^+ in N-deprived cells from a natural population reported by Glibert & McCarthy (1984). Such differences may be due to differences in the levels of C, N or P stress as well as any differences between species.

There are fluctuations in activities of key enzymes of N and C metabolism during both diurnal illumination changes and N-deprivation (Eppley et al. 1971, Wheeler et al. 1983). These are associated with periodic (e.g. diurnal) changes in gross metabolism (Terry et al. 1985, Rainbault & Mingazzini 1987; Table 1). It is likely that such fluctuations in metabolism, especially if they occur during incubations (see Glibert et al. 1985), will affect the reliability of methods used to determine N-status which require incubations. Clearly there are many inherent problems with any technique requiring the incubation of samples (Flynn 1989a).

Some criteria for the diagnosis of N-limitation which do not require incubation of organisms are listed in Table 2. The detection of low levels of inorganic N (DIN) does not mean that growth of a population of phytoplankton (Goldman et al. 1979, McCarthy &

Table 2. Some criteria used for the diagnosis of N-limitation of microalgal growth which do not require incubations of cell suspensions. Organisms as in Table 1. Comments in quotes are conclusions drawn by method developers. Sources are not exhaustive

Criterion	Organism	Comments	Source
(a) DIN	NP	Low concentration indicates N-limitation	Spoken arguments & numerous texts
(b) DIN/Chl	NP (var)	Low ratio indicates N- limitation	Furnas & Mitchel (1986)
(c) C/N	LC (bac/chl/pry) NP (bac/pyr/var)	High ratio indicates N-limitation Possible diurnal fluctuations (Lancelot & Billen 1985) and interference with detritus	Goldman et al. (1979), Goldman (1986), Paasche & Erga (1988)
(d) RNA/DNA protein/DNA Enzyme assays Internal DIN	LC (bac/chl/pry/pyr) NP (bac/pyr)	'High ratio indicative of high growth rate' 'High ratio indicative of high growth rate' 'Indicate source of N'; see text 'Indicate source of N'	Dortch et al. (1984, 1985)
(e) Amino-N/protein-N	LC (bac/chl/pry/pyr) NP (bac/pyr)	Low ratio indicates N-limitation, Will also fall on prolonged C-deprivation Important to use one methodology	Dortch et al. (1984, 1985), Clayton et al. (1988)
(f) Intracellular free amino acids	LC (bac/chl/cry/pry/pyr)	'Metabolic finger print' Too variable between species of similar N-status, and too complex to interpret	Admiraal et al. (1986), Martin-Jézéquel et al. (1988)
(g) GLN/GLU	LC (bac/chl/cya/pra/pyr)	Low ratio indicates N-stress – see Table 3	Flynn et al. (1989)

Goldman 1979, Flynn & Butler 1986, Flynn 1989b) or of macroalgae (Fujita et al. 1989) is N-limited. The organisms may have sufficient N-reserves to survive transient periods of DIN depletion. They may be able to use organic N-sources, or the use and regeneration of nutrients may be so closely coupled that concentrations of DIN in the bulk water column do not rise. Ratios of biological parameters against DIN (Table 2b) are likely to be of dubious value for the same reasons.

The classic biomarker is the C/N ratio (Table 2c). Values of C/N/P alter little with growth under different conditions of light and temperature (affecting growth rate), but significantly with changes in nutrient supply (Goldman 1986). Methods of cell collection should be chosen that do not cause cell rupture (Goldman & Dennett 1985) because the metabolic pool may contribute a significant proportion of C and N, and because of the nutritional value of prey to predators. The use of flow cytometry (Birkill 1987) to eliminate detritus, and to select particles according to size and shape (criteria possibly used for selection by predators), and pigment, would also be a useful extention.

Dortch et al. (1984, 1985) have suggested several indices of general physiological status (Table 2d) which could complement C/N/P data. The detection, or otherwise, of enzyme activities is, however, not a good approach. Problems range from uncertainties of optimal conditions in enzyme assays for different species, to the more complex problems of derepression stimuli. For example, nitrate reductase may be present in cells growing on nitrate and those which have been N-deprived, but not in cells growing on high concentrations of ammonium (Thacker & Syrett 1972, Rigano & Violante 1973, Syrett & Hipkin 1973; but see section below 'N-stress and gene regulation').

The problems associated with enhancement techniques (Table 1b, c, d), attempting to resolve the possibly uncoupled activities of inorganic C and N assimilation in conditions of high and low N-status, may be solved by a direct examination of the intracellular pool of metabolites. It is the presence or absence of products of organic synthesis, such as amino acids and α-keto acids, which are most likely to be of importance for the genetic regulation of biochemical processes, such as those stimulated when cells are N-deprived. The most simple index of the availability of intracellular free amino acids, a function of C-N status, is the ratio of amino-N/protein-N (Dortch et al. 1984, 1985; Table 2e). It is important that the methods used for amino-N and protein determinations are standardized because the fluctuations in results which may be caused by the use of different assays (Clayton et al. 1988) complicate comparisons of data (compare data and methods of Dortch et al. 1985 with Martin-Jézéquel et al. 1988). The value of amino-N/protein-N may be unaffected by N-source use so that the nutrient status of cells growing on organic-N, which may not stimulate CO_2-fixation (Flynn & Butler 1986), will also be indicated.

More specifically one could examine the composition of the amino acid pool, using it as a 'metabolic fingerprint' (Admiraal et al. 1986; Table 2f). The problem here is that interspecific differences appear to equal or exceed intraspecific differences caused during N-deprivation (Martin-Jézéquel et al. 1988) which would

complicate the interpretation of data obtained from field collections of mixed populations. Furthermore, significant changes in levels of individual amino acids can occur during a light-dark cycle (Fig. 1) when the availability of extracellular N does not alter.

Of the 20 or so amino acids commonly analysed, only a few are of universal importance in both quantity and quality. The most important of these are glutamate (GLU) and glutamine (GLN). GLU and GLN play central roles in the initial incorporation of intracellular ammonium and in the synthesis of other amino acids, nucleic acids and derivatives. The relative proportions of GLN and GLU appear of use in assessing C-N status (Tables 2g and 3).

GLN/GLU

The incorporation of ammonium occurs mainly either through the action of glutamic dehydrogenase (GDH) or glutamine synthetase + glutamine-oxoglutarate aminotransferase (GS-GOGAT). The latter is considered to be more important when the N-source is at low concentration because substrate affinity is higher, although energy demands are greater. Clayton & Ahmed (1986, 1988), Ahmed & Hellebust (1988) and Syrett & Peplinska (1988) provide information on the assay of these enzymes. Ahmed & Hellebust (1988) suggest that GS-GOGAT is the major entry point for the assimilation of intracellular NH_4^+ in microalgae. Experiments in which GLU decreases while GLN increases immediately following addition of NH_4^- to N-stressed cells (Turpin & Harrison 1978, Flynn et al. 1989, Flynn & Hipkin 1990) also suggest a key role for GS. By GS-GOGAT, the levels of GLN, GLU and 2-oxoglutarate (2OG) are closely coupled:

$$NH_4^+ + GLU \rightarrow GLN;\ GLN + 2OG \rightarrow 2GLU$$

The ratio GLN/GLU becomes high (> 0.5) when the supply of C (specifically 2OG) is rate limiting (Flynn et al. 1989) provided that reductant and ATP are non-limiting. This could occur either in darkness (but see below) or conceivably in light when levels of $CO_{2\,(aq)}$ become limiting (for coccolithophorids, for example, which cannot use HCO_3^-; Sikes & Wheeler 1982). GLN/GLU is low (< 0.1) when either environmental N-stress (absence of N-source from medium) or physiological N-stress (absence of adequate reductant for example) occurs resulting in a shortfall in intracellular NH_4^+. Short-term incubations (5 to 10 min) in darkness with NH_4^+ induce marked changes in GLN/GLU (Flynn et al. 1989) and could be used to help differentiate between environmental and physiological N-stress.

In the yeast *Candida nitratophila*, N-deprivation results in decreased GLN/GLU and C-deprivation causes increased GLN/GLU (Flynn & Hipkin 1990). In microalgae, however, photosynthesis may affect the response. When the diatom *Phaeodactylum tricornutum* is N-deprived in light the ratio falls, when N-deprived in darkness the ratio alters little and when darkened only (C-deprived), the ratio rises (Flynn et al. 1989). In the chlorophytes *Tetraselmis marina* (Flynn et al. 1989) and *Dunaliella primolecta* (Fig. 1), the ratio is again low when N-deprived but is also low when the cells are placed in darkness. The implication is that these species use different metabolic pathways for the synthesis of C-skeletons for amino acids and/or for the supply of reductant. Such differences may help to explain why results from the use of enhancement techniques on natural populations have sometimes given inconclusive results (e.g. Paasche & Erga 1988).

In the data of both Haberstroh & Ahmed (1986) and Flynn & Hipkin (1990), there are indications of the extra physiological stress imparted by the utilization of NO_3^- rather than NH_4^+, with levels of GLN/GLU and of concentrations of amino acids in general being slow to respond when N-deplete cells are re-fed with NO_3^-. Addition of NO_3^-, but not of NH_4^+, to N-deprived cells of *Chlorella fusca* stimulates O_2 evolution, the implication being that photogenerated electrons are diverted from CO_2 fixation to NO_3^- reduction (Thomas et al. 1976).

Table 3 gives values of GLN/GLU for a wide range of microalgae grown under different conditions and sampled, extracted and analysed using a range of techniques. The only inconsistent values are those for some of the diatoms tested by Admiraal et al. (1986), although the trend (higher values at higher N-status) is consistent. In contrast with the values of Admiraal et al. (1986), the values for *Skeletonema costatum* obtained by Haberstroh & Ahmed (1986) are in agreement with values given by Flynn et al. (1989). Differences may be due to the conditions of illumination at the time of sampling, as for the diatom *Phaeodactylum tricornutum* GLN/GLU is elevated when C-deprived (Table 3). The data of Rijstenbil & Sinke (1989) show GLN/GLU increasing with growth rate in N-limited chemostats of *S. costatum*. The data of Martin-Jézéquel et al. (1988) have not been included because it is not clear what the N-status of their cultures was; their data for amino-N/protein-N from cells reported to be growing in exponential or stationary phase are also not consistent with the reports of Dortch et al. (1984, 1985).

The data for N_2-fixing cyanobacteria (Table 3) gives an insight into the advantage of physically separating N_2-fixation and CO_2-fixation. In the non-heterocystous *Gloeothece* sp., the conflict of O_2 production during photosynthesis and its deactivation of nitrogenase, plus the heavy demand for reductant for N_2-fixation within one cell type, appear to conspire to give physiological

Table 3. Values of the mole ratio of intracellular glutamine/glutamate (GLN/GLU). Cultures have been grown to the N-status indicated unless otherwise stated; low N-status typically indicates a period of N-deprivation. Many of the studies were not performed specifically to measure GLN/GLU and the ratios have been calculated from the original data L: continuous illumination; C: chemostat-culture; LD: light-dark cycle

Species	GLN/GLU			Other conditions & comments	Source
	Low N-status	High N-status			
		NO_3^-	NH_4^+		
CYANOPHYTA					
Anabaena variabilis	0.10	–	–	0.50[a]; L; C[b,c]	Niven et al. (1987)
Gloeothece sp.	0.05	–	0.50[r]	0.10[a]; LD[d]	Flynn & Gallon (1989)
CHLOROPHYTA					
CHLOROPHYCEAE					
Dunaliella primolecta	0.10	–	0.45	LD[f]	Flynn (1990)
Nannochloris sp.	0.05	–	0.41	LD[f]	Flynn (1990)
Stichococcus bacillaris	0.10	0.5	1.75[e,r]	LD[f]	Flynn et al. (1989)
Stichococcus minor	0.03	–	0.54	LD[f]	Flynn (1990)
PRASINOPHYCEAE					
Mantoniella squamata	0.05	0.40	4.0[e,r]	LD[f]	Flynn et al. (1989)
Omega 48-23	0.06	–	0.48	LD[f]	Flynn (1990)
Tetraselmis marina	0.1	0.50/1.00[r]	–	L	Al-Amoudi & Flynn (1989)
CHRYSOPHYTA					
BACILLARIOPHYCEAE					
Biddulphia sinensis	0.47	0.91	–	LD[g,h,i]	Admiraal et al. (1986)
Chaetoceros decipiens	0.19	0.31	–	LD[g,h,i]	Admiraal et al. (1986)
Cosinodiscus granii	1.27	6.84	–	LD[g,h,i]	Admiraal et al. (1986)
Cyclotella criptica	1.28	2.36	–	LD[g,h,i]	Admiraal et al. (1986)
Ditylum brightwellii	0.13	1.35	–	LD[g,h,i]	Admiraal et al. (1986)
	0.10	–	0.80[j]	LD[f]; C	Rijstenbil et al. (1989)
Phaeodactylum tricornutum	–	0.35	–	L	Lu & Stephens (1984)
	0.1/0.8[k]	–	0.6/1.4[k]	L	Flynn et al. (1989)
Skeletonema costatum	0.86	1.11	–	LD[g,h,i]	Admiraal et al. (1986)
	0.11	0.24[r]	1.14[r]	L[i]	Haberstroh & Ahmed (1986)
Thalassiosira excentrica	0.16	1.00	–	LD[g,h,i]	Admiraal et al. (1986)
Thalassiosira pseudonana	0.13	0.55	–	L; C	Zehr et al. (1988)
EUSTIGAMATOPHYCEAE					
Ellipsoidion sp.	0.05	–	1.34	LD[f]	Flynn (1990)
Nannochloropsis oculata	0.01	0.50	1.50	L	Flynn (1990)
PRYMNESIOPHYCEAE					
Emiliania huxleyi	0.15	–	0.80	LD	Flynn (1990)
PYRRHOPHYTA					
Gymnodinium simplex	0.15	–	0.60[r]	L; C	Turpin & Harrison (1978)
Oxyrrhis marina	0.05	–	–	0.3[m]	Flynn & Fielder (1989)
YEAST					
Candida nitratophila	0.05	0.5	0.6/0.7[k]	0.3[n]	Flynn & Hipkin (1990)

[a] During N_2-fixation
[b] Nitrogenase activity most affected by osmotic shock – low N-status value is of shocked culture
[c] Heterocystous
[d] Non-heterocystous
[e] Re-fed in darkness (else in light)
[f] Sampled during light phase
[g] Sample time unknown, see text
[h] Cells grown on 'low initial' or 'high initial' NO_3^-
[i] Fluctuations in GLN greatest
[j] Recovery from N-limited growth due to osmotic shock reducing growth rate
[k] Darkness (algae) or C-deprived (yeast)
[m] Phagotrophic; ratio depends on N-status of prey
[n] NH_4^+ + amino-N
[r] Re-fed (spiked) with N-source after a period of N-deprivation

N-stress. This is relieved on addition of NH_4^+ when a typical response to N-pulsing of N-deprived cells is observed (Flynn & Gallon 1989).

N-STRESS AND GENE REGULATION

In the bacterium *Escherichia coli* the intracellular proportions of 2OG and GLN have been identified as having a key role in gene regulation of metabolic changes in response to N-stress (Magasanik 1988). On increased N-stress the low ratio GLN/2OG leads to a lack of signal transducer P_{II} because 2OG activates uridylylation of P_{II} to $P_{II}(UMP)_4$. The lack of P_{II} allows the phosphorylation of an effector which, through a feedback loop, eventually leads to activation of N-regulated promoters. The lack of P_{II} also promotes deadenylylation of $GS(AMP)_{12}$ to the active enzyme glutamine synthethase (GS). Phosphorylation plays an important role in this regulatory system so that P-stress could be expected to affect responses to N-stress. In *Rhizobium leguminosarum*, the isoenzymes of GS, GS_I and GS_{II}, are subject to post- and pretranscriptional control respectively (Rossi et al. 1989). GS_I is reversibly adenylylated as in *E. coli* and concentrations alter little. GS_{II}, however, is almost absent in cells grown on NH_4^+ but present at very high levels in cells grown on NO_3^- and GLU. By such mechanisms a cell has both coarse and fine controls over its N-metabolism. It may be concluded that only at the extreme of growth on excess NH_4^+ can cells be considered to be fully N-replete and that any conditions which result in the derepression/activation of other activities are stages of greater or lesser N-stress.

In comparison with our knowledge of bacterial systems, knowledge of eukaryote regulation of C and N metabolism is minimal. Nonetheless, the responses to N-stress, increased GS activity, increased ability to transport different N-sources and decreased GLN/2OG (if one accepts that if C is not limiting GLN/GLU gives an index of GLN/2OG), do appear similar. Akimova et al. (1976, 1977) suggest that in *Chlorella pyrenoidosa* deactivation of GS may be mediated by GLN and that ADP and AMP inhibit GS activity. Fig. 2 shows how GS activity and GLN/GLU varies in *Nannochloropsis oculata* grown to different N-status. The implication of this preliminary data, taken with existing knowledge of algal responses to N-stress and growth on different N-sources, is that (as in bacteria) only during growth with excess NH_4^+ (mid to high μM range) are cells NOT N-stressed to some extent. The increased energy requirement for the operation of GS-GOGAT as compared with GDH is available in N-stressed cells which, by definition, have an excess of C available for respiration.

In cells grown on NO_3^-, the responses to N-stress are already partly derepressed, hence the rapid uptake of

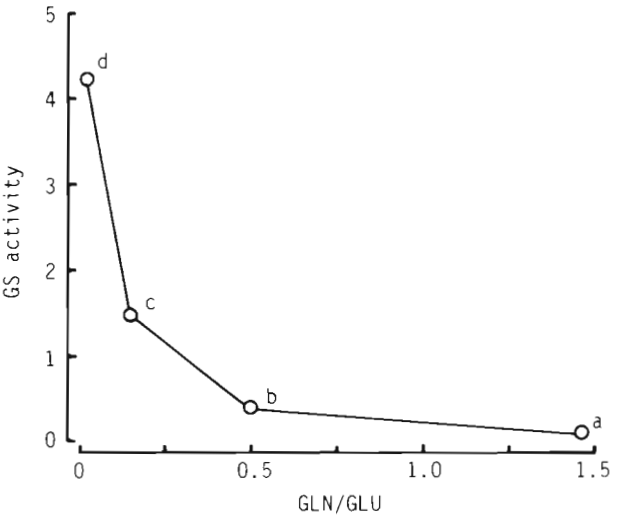

Fig. 2. Suggested relationship between glutamine synthetase (GS) activity and GLN/GLU. *Nannochloropsis oculata* Hibberd was sampled during growth in conditions described by Everest et al. (1986), under continuous illumination (10 μmol m^{-2} s^{-1}), in the presence of ammonium (a) or nitrate (b), during N-deprivation (c) and after 2 d N-deprivation (d). GS transferase activity (nmol glutamyl hydroxamate min^{-1} 10^{-6} cell) was assayed as described by Syrett & Peplinska (1988) and intracellular amino acids analysed as described by Flynn (1988b). Cell densities were typically 1 to 2×10^7 ml^{-1} Given a constant supply of C (i.e. constant illumination), GLN/GLU correlates with GLN/2-oxoglutarate and is an index of N-stress; N-stress increases right to left but does not become growth limiting until between nitrate grown (b) and (c). It is suggested that a similar regulatory system to that in *Escherichia coli* (Magasanik 1988), in which GLN/2-oxoglutarate regulates GS activity, may operate in microalgae (see text)

NH_4^+ by NO_3^--grown cells (e.g. Horrigan & McCarthy 1982). However, such stress is not often growth limiting and C/N ratios of NH_4^+ and NO_3^- grown cells are not significantly dissimilar. This concept of a sliding scale of N-stress, and more importantly of a sliding scale of the derepression of a cells response to N-stress, also helps to explain the incorporation of NO_3^- by cells grown in NH_4^+-limiting culture (Zehr et al. 1989). Likewise it may offer an explanation for the conflict between the 'inhibition' of NH_4^+ uptake by NO_3^- reported by Collos (1989) and Collos et al. (1989), using substrate concentrations of 50 μM, and the inhibition of NO_3^- uptake by NH_4^+ reported by, for example, Cresswell & Syrett (1979) who used substrate concentrations in the mM range. At high (unnatural) concentrations of NH_4^+, repression of nitrate transport and assimilatory processes becomes complete, whilst at lower concentrations progressive derepression results in an increased ability to use other N-sources. The substrate concentrations at which derepression becomes significant most probably varies between species and groups of microalgae.

CONCLUSIONS

Many studies of algal physiology have examined the effects of, and responses to, N-deprivation and refeeding. A major problem has been that there has been no independent measure of C-N status with which to compare cultures used in different studies, yet it is clear that C-N status of cells can vary significantly with conditions of illumination and quantity and quality of N-source. If we cannot manage to resolve these problems in cultures of known species composition and known nutrient history then how sure can we be of results from field studies? A problem common to most (all?) methods used at present to diagnose N-deprivation is that we measure a symptom of stress and attempt to relate it to growth limitation (the factor which we are usually actually interested in). Yet, often that test criterion may be affected by stress other than N-stress. If we only knew how the gene regulation of microalgal responses to N-stress functioned then we would undoubtedly have a better idea of the parameters that we should endeavour to measure when attempting to determine N-stress in natural populations.

The proportions of GLN/GLU appear to correlate well with C-N status (Table 3), at least giving an independent measure of C-N status in the light phase of growth with which to compare cultures grown under different conditions of photon flux density and substrate type and concentration. However, the proportions of these amino acids are unlikely to be used in gene regulation of the response to N-stress because GLN/GLU may fall either due to environmental N-stress or to darkness (Fig. 1). The ratio GLN/2OG may not fall significantly, if at all, under these conditions because levels of both 2OG and GLN would fall. The usefulness of GLN/2OG as a monitor of C-N status is unknown at present. There are, however, 2 potential problems; 2-oxoglutarate is extremely unstable in solution and, as it plays such a vital role in many biochemical reactions, the high turnover rate may also make reproducible sampling difficult. A second analysis would also be required, whereas at least measurements of GLN/GLU can be obtained using one simple and sensitive method (Flynn et al. 1989).

There is a clear need for more information on C-N biochemistry and gene regulation in microalgae (especially of groups other than chlorophytes and diatoms, such as prymnesiophytes and eukaryotic and prokaryotic picoplankters). With such data new methods for the study of N-stress, and of physiological ecology in general, can be developed. By the use of techniques from molecular biology or of short-term incubation methods (say of less than 10 min duration) in which changes in concentration of key metabolites (those likely to be sources of regulatory signals for gene regulation in specific groups of organisms) are measured in the presence or absence of enrichment, we may also be able to resolve the C-N status of components of microbial loop communities. It is essential that all studies be performed using cultures grown under conditions of light and dark simulating those in nature and consider such factors as cumulative stress, caused by P-stress for example, on an organism's response to N-stress.

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