C_{1} bacteria in the water column of Chesapeake Bay, USA. I. Distribution of sub-populations of O_{2}-tolerant, obligately anaerobic, methylotrophic methanogens that occur in microniches reduced by their bacterial consorts

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ABSTRACT: Sub-populations of O_{2}-tolerant, obligately anaerobic methanogens were enriched with monomethylamine (MMA) under 3 different regimens: Regimen 1, where oxygenated seawater was reduced by the aerobic bacterial consorts of the methanogens in sealed gas-permeable polycarbonate flasks that apparently prevented the accumulation of hydrogen sulphide while allowing the production of traces of ethane and ethylene; Regimen 2, like 1, but with bacterial reduction in glass serum bottles that permitted the accumulation of hydrogen sulphide formed by sulphate-reduction but not the C_{2} hydrocarbons; and Regimen 3, an anaerobic medium chemically reduced by sodium sulphide and cysteine that is used to culture methanogens from anoxic sediment. In Regimens 1 and 2, methanogenic bacterial consortia (MBC) were initiated by MMA-oxidizing bacteria that formed reduced microzones in which MMA could be cleaved by methylotrophic methanogens to form methane, which was apparently oxidized by aerobic methanotrophs almost as quickly as it was produced until dissolved oxygen was exhausted. The process was accelerated under Regimen 2, whose enrichments were used for the most probable number estimation of methanogenic particulates which showed that they accumulate in the pycnocline. These methanogenic particulates were quite sensitive to filter concentration. The distribution of the 3 sub-populations of methanogens is then shown at 10 stations along the density gradient of the estuary where water samples were obtained from the surface layer, pycnocline and bottom layer of water. The results were similar for each layer, where Regimen 2 consistently produced twice the number of methanogenic enrichments as obtained with Regimen 1 or 2. In contrast, there was a marked difference in the distribution of the methanogen populations down the density gradient of the estuary. Enrichment in Regimen 3, which was very successful up the estuary, decreased in effectiveness with increasing density. Enrichments in Regimen 2 were quite effective throughout the transect of stratified waters, while those in Regimen 1 had a narrower distribution. I conclude that O_{2}-tolerant methanogens that occur throughout the water column and peak in the pycnocline grow in fragile microniches that are reduced by bacteria that either consume oxygen, produce hydrogen sulphide, or both. A 'top-down' working hypothesis is presented that could explain the diversity, nature and distribution of the bacterial components of the methanogenic enrichments of the water column, and how the methanogens in anoxic sediments lacking aerobic bacterial consortes may be selected from them. I also postulate that the methanogens living in bacterially reduced microniches may be unique both physiologically and taxonomically, and may have redox potential (Eh) requirements less strict than methanogens from anoxic H_{2}S-rich sediments.

INTRODUCTION

A pronounced methane maximum in the pycnocline of the oxygenated upper ocean is usually 30 to 70\% supersaturated relative to the atmospheric equilibrium concentration (Swinnerton et al. 1969, Brooks & Sackett 1973, Williams & Bainbridge 1973, Seiler & Schmidt 1974) and is apparently due to \textit{in situ} production (Lamontagne et al. 1973, Scranton & Brewer 1977, Brooks et al. 1981, Burke et al. 1983). It has been postulated that the strictly anaerobic methanogens producing these methane maxima must occur in
the anoxic microniche of suspended particulates that accumulate in the pycnocline (Scranton & Brewer 1977, Burke et al. 1983, Sieburth 1983), but evidence for this has been lacking (Rudd & Taylor 1980). Jørgensen (1977) observed that anaerobic sulphate-reduction could occur in the presence of oxygen within reduced microniches, due to oxygen-consuming bacterial consortia. Recently, hydrogen sulphide has also been observed in the pycnocline of the oxygenated upper ocean by both indirect (Cutter & Krahnstoer 1988) and direct (Luther & Tsamakis 1989) chemical analysis. Sulphate-reducing bacteria which are oxygen-tolerant (Hardy & Hamilton 1981) have been cultured from oxygenated seawater (Sieburth 1987, 1988, Blasco et al. 1992). The presence of both dissolved hydrogen and fermentative hydrogen-producing bacteria in the open sea has been reported by Schropp et al. (1987a, b). Bacteria involved in both denitrification (Schropp & Schwarz 1983) and nitrogen fixation (Guerinot & Colwell 1985, Paerl & Prufert 1987) which require suboxia, are also present in oxygenated seawater. An obvious explanation for the presence of these anaerobic processes in the pycnocline is that the anaerobes involved can accumulate there to effective populations within reduced microniches.

The bacteria producing and utilizing single carbon compounds are for the most part obligate C1 bacteria that are unable to utilize C-C bonded organic matter. As a consequence, they differ physiologically from other heterotrophic bacteria (Anthony 1982, Large 1983, Murrell & Dalton 1992). A study to remedy the paucity of cultured C1 bacteria oxidizing methane used enrichment cultures inoculated with mud and water from ponds, rivers, streams and ditches (Whittenbury et al. 1970). This study led to an increased knowledge of this group (Whittenbury & Dalton 1981). S. W. Watson used his experience in the enrichment of ammonia-oxidizing (nitrifying) bacteria that also have distinctive and similar cytomembranes (Watson 1965, Watson et al. 1981) in an attempt to extend the terrestrial observations of the Warwick group into the marine environment in the 1970s. All attempts, however, were fruitless (S. W. Watson pers. comm.). The enrichment, isolation and characterization of *Methylomonas pelagica*, the first methane-oxidizing bacterium to be obtained from the open sea almost ended in failure too, when bacterivorous flagellates and ciliates grazed them down to non-visible populations by the time the enrichments were returned to shore (Sieburth et al. 1987). The addition of the protist inhibitor cycloheximide suppressed the bacterivorous protozoa, and allowed the pelicle of methane-oxidizing bacteria to become reestablished.

The physiological type of methanogen (Sowers et al. 1984) that could produce the well known peak of methane in the pycnocline required by the methane-oxidizers, has only been recognized in the last decade. A report of the ability of trimethylamine (TMA) and other N-methyl compounds to be used by Methanosarcina barkeri (Hippe et al. 1979) was the vanguard for those that would show that TMA could account for as much as 90% of the methane produced in a slurry of salt marsh soil (Oremland et al. 1982) or 35 to 61% of *in situ* methane production in an intertidal mud flat (King et al. 1983). The benchmark paper by King (1984) showed that the widespread osmolyte in marine organisms, the quaternary ammonium compound glycine betaine (GBT) (Yancey et al. 1982) and its C1 hydrolytic product TMA, but not C-C bonded organic matter, stimulated methanogenesis in the presence of sulphate reduction in marine sediments. TMA degrades to dimethylamine and MMA, which can also be used as substrates by the methylotrophic methanogens.

My introduction to the methylotrophic methanogens was also in 1984 when they paradoxically produced methane in semipermeable polycarbonate flasks of oxygenated seawater being used to enrich MMA-oxidizing bacteria in samples from stratified oceanic waters (Sieburth et al. 1993a). Follow-up observations on these methanogenic bacterial consortia (MBC) producing and oxidizing methan in polycarbonate flask enrichments in seawater from the stratified Chesapeake Bay, USA, (Sieburth 1987) also showed that similar MBC growing in methylotroph-reduced enrichments made in glass bottles were more reliable, usually contained hydrogen sulphide, and had much shorter incubation and transfer times. These MBC are initiated by methylamine-oxidizers similar to *Methyllophaga* spp. described by Janvier et al. (1985). The primary source of the methylamines in the water column that could initiate such enrichments *in situ* appears to be algae that support a high incidence of *MMA*-oxidizers in their physcosphere that accompany them into xenic culture (Sieburth & Keller 1988/89). Some of these enrichments contained the full complement of MBC and produced both methane and hydrogen sulphide (Sieburth 1988). Methane-cycling and sulphate-reducing bacteria that accompany algae in the upper ocean must be a major factor in sustaining primary production in the chlorophyll maximum of the pycnocline (Sieburth 1991).

The 3 trophic types of methylotrophic C1 bacteria are MMA-oxidizers, methane-producers, and methane-oxidizers. The C1 bacteria which work together in nature are classically studied separately, as illustrated in Sieburth (1991, Fig. 4.), as each group is selected by
trophic-specific media and by incubation under either aerobic or anaerobic conditions. The serendipitous coenrichment of all 3 trophic types of C1 bacteria with one substrate, monomethylamine (MMA), in 1 flask (Sieburth 1987, 1988), has allowed us to extend the knowledge of all these bacterial groups in one well known stratified marine ecosystem, Chesapeake Bay (Officer et al. 1984, Seliger et al. 1985, Sarabun et al. 1986, Malone 1991). In this paper I focus on the distribution of O2-tolerant, but obligately anaerobic, methylotrophic methanogens in the water column of Chesapeake Bay that fell into 3 sub-populations. Two distinctly different sub-populations required aerobic bacterial consortia to reduce microzones in the originally oxygenated seawater, but had very different H2S-tolerances. The third sub-population used sulfide and cysteine to reduce the anaerobic medium which excludes aerobic bacteria.

MATERIALS AND METHODS

1986 and 1987 sampling. In order to study the water column of Chesapeake Bay during seasonal stratification, but before anoxia occurred (Officer et al. 1984, Seliger et al. 1985, Malone 1991), the stations shown in Fig. 1 were occupied during May of both 1986 and 1987. Stns 904 and 858, above and below the Chesapeake Bay Bridge, respectively, were occupied between 4 and 9 May 1986 on cruise PROPHOT XVIII by the RV 'Cape Henlopen' (University of Delaware), the others were occupied between 9 and 12 May 1987 on cruise CORSAIR XII by the RV 'Cape Hatteras' (University of North Carolina). Continuously recording CTD profilers were used to obtain vertical profiles of the water column for the following properties: density (\(\sigma_t\)), salinity (\(\%\)), temperature (\(^\circ\)C), and oxygen (mg l\(^{-1}\)). Water samples were then collected in 5 l Niskin bottles attached to the CTD rosette at selected depths above, within and below the pycnocline for the most probable number (MPN) estimation of methanogen populations. Station locations shown in Fig. 1 are also listed by latitude and longitude down the density gradient of the estuary in Table 1.

1986 enumeration of MBC. Methylotrophic, oxygen-tolerant MBC were enumerated by a MPN procedure to determine their vertical distribution. For each depth, unfiltered seawater, filtered seawater, or the particles collected on 3.0 \(\mu\)m filters were used to inoculate a decimal-dilution series of the sample in triplicate, in the glass serum bottles of Regimen 2 which were then stoppered, crimped, incubated at 22°C, and analyzed 3 times during incubation for headspace methane by routine gas chromatography (King et al. 1983). The volumes of seawater used in 1986 were 1.0, 10 and 100 ml while the volumes used in 1987 were 5, 50 and 500 ml. The larger seawater samples in appropriately-sized serum bottles were supplemented by the addition of MMA (0.1 \% wt/vol as substrate, a dilute mineral/vitamin mix (Sieburth et al. 1987) and 0.0001 \% (wt/vol) resazurin as a redox indicator. The appropriate 100 ml enrichments for MPN estimations in 1987 were used instead for the comparison of regimens in the following section. The small volume samples (1.0, 5.0 and 10 ml) and the filters containing the particles

Table 1. Summary of the latitude and longitude for each station shown in Fig. 1 for the Chesapeake Bay and adjacent shelf

<table>
<thead>
<tr>
<th>Str</th>
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<th>Longitude, W</th>
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<td>15</td>
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</tr>
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</tr>
<tr>
<td>11</td>
<td>36°53.01'</td>
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</tr>
<tr>
<td>13</td>
<td>37°03.00'</td>
<td>75°16.70'</td>
</tr>
</tbody>
</table>

Fig. 1. Sampling locations in Chesapeake Bay and on the adjacent continental shelf. Stns 856 and 904 are from PROPHOT XVIII, May 1986, and Stns 1 to 16 are from CORSAIR XII, May 1987.
were used to inoculate 30 ml of sterile 10% seawater in
60 ml serum bottles that contained the supplements
used above. Sterile 3.0 μm porosity filters (Whatman
GF/D) were used in sterile polysulphone filter holders
(Nalgene #300-4100) under low vacuum (<5 cm Hg)
in an attempt to differentiate between the particle-
associated and the non-particle-associated bacteria, as
described above, in which either the filter or the sea-
water filtrate, respectively, was used as the inoculum.
The reduced enrichments were analyzed weekly for
methane until no further cultures became reduced,
usually within 4 wk. The number of MBC per liter was
estimated by scoring the number of replicates in each
series that were positive and negative for the produc-
tion of methane, and then calculating the MPN from
standard tables (Anon. 1976).

1987 enrichment regimens for the comparison
of methanogen sub-populations. A diagram that
describes the 3 regimens used to obtain enrichments of
different sub-populations of methanogens is presented
in Fig. 2. In their basic configuration they use 100 ml
portions of raw oxygenated seawater samples that
serve as both the medium and inoculum. By necessity
the anaerobic medium of Regimen 3 can only be
inoculated by filter concentrates. For the 1987 com-
parison of the 3 regimens, half the replicates of
Regimens 1 and 2 that used raw water as the inoculum
and half that used sterilized natural seawater (15% S)
inoculated with filter concentrates, were combined to
give a larger number of replicates. Filter concentration
undoubtedly added a bias, but this should have been
consistent between regimens and should allow a
comparison between regimens down the axis of the
estuary. The following description of the 3 regimens is
for their usual mode of inoculation.

The first MBC were fortuitously enriched from
100 ml samples of seawater supplemented with MMA
(0.1 % wt/vol final concentration) as substrate and a
dilute mineral/vitamin mix (Sieburth et al. 1987) in
Corning ‘Orange Top’ polycarbonate flasks. This is
shown as Regimen 1. The unlikely possibility that
methanogenesis was occurring in these gas-permeable
flasks was indicated by a bacterial pellicle at the
liquid/gas interface (Sieburth 1987, 1988) that was
suggestive of those produced by methane-oxidizing
bacteria (Sieburth et al. 1987). The presence of
methane-oxidizers was verified by the isolation of pure
cultures on agarose plates in an atmosphere of 50% methane (Sieburth et al. 1987). To detect the produc-
tion of methane, a rubber septum was installed in the
flask tops and methane was determined in the head-
space by gas chromatography (GC) (King et al. 1983).
Subsequently, 0.0001 % (wt/vol) resazurin was added
to indicate when the enrichments had been bacterially
reduced. After the resazurin was reduced to its colour-
less leuco form, methane concentrations in the head-
space were determined. Subcultures made into both
oxygenated seawater as well as into the reduced
medium of Sowers & Ferry (1983) in glass serum bottles
failed to become methanogenic.

Regimen 2 also used 100 ml of raw oxygenated sea-
water as the medium and inoculum, and received the
same supplements. The difference was that they were
made in glass serum bottles that were stoppered
and crimped. Unlike Regimen 1 that took months to
develop, these became reduced and methanogenic
within weeks, usually produced H2S, but did not
indicate any C2 hydrocarbons. Regimen 3 was the
anaerobic medium of Sowers & Ferry (1983) that
received an equivalent inoculum as a filter concentrate.

Persistence of aerobic and anaerobic processes in
MBC during alternate exposure to air and anoxia
in Regimen 2. The effect of aeration on the MBC was
observed by following changes in the concentration of
headspace methane in triplicate 36-d-old enrichment
cultures, after they were exposed to air for 1, 2, 19 h,
respectively. After following changes in methane con-
centrations in the headspace of the essentially spent
medium for 28 d, 50% of the culture volume was
replaced with fresh oxygenated medium, and methane
concentrations were again followed.

RESULTS

The polycarbonate enrichment flasks fitted with
rubber septa (Regimen 1) formed a pellicle at the
gas/liquid interface long before the resazurin became
reduced to the leuco form, and methane accumulated
to significant concentrations (up to 3 mo). The enrich-
ments in glass bottles (Regimen 2) also underwent
the same sequence of events, but in only 1 to 4 wk.
Methane-oxidizing bacteria that form a pellicle at the
gas/liquid interface appear to be a very sensitive early
indicator of methanogenesis, before the reduction of
the bulk water and the production of unequivocal con-
centrations of methane. The GC methane analyses, un-
like those for the glass bottle enrichments, also showed
a trace of ethane and a slightly larger peak of ethylene
as shown in Fig. 3. Hydrogen sulphide was never
detected in these methanogenic enrichments and they
were unable to be successfully transferred into the
chemically reduced medium of Sowers & Ferry (1983),
or even into the glass bottles of Regimen 2 where
hydrogen sulphide does accumulate. This indicates
that in addition to being O2-tolerant, these methanogens
were also H2S-sensitive. Although these O2-tolerant/
H2S-sensitive methanogens are an interesting group,
the long incubations required and their failure to
transfer to chemically reduced media did not permit us
Regimen 1. Bacterially reduced in polycarbonate flasks

- MMA-oxidizers consume DO
- Methylophaga-like monomethyamine (MMA)-oxidizing bacteria (Janvier et al. 1985) from oxygenated seawater, and therefore used polycarbonate flasks not intended for anaerobic culture. A pellicle of bacteria that were cultured as methane oxidizers (Sieburth et al. 1987), developed at the gas/liquid interface and was the first indication that methane would eventually accumulate to appreciable concentrations in the headspace. The O₂-tolerant/ H₂S-sensitive methanogenic enrichments produced traces of ethane and ethylene in addition to methane, but no H₂S. In Regimen 1, H₂S was usually produced, ethane and ethylene were not, and both the anaerobic bacteria and their aerobic bacterial consorts were cultured from these O₂- and H₂S-tolerant methanogenic enrichments. Regimen 3 was similar to the traditional anaerobic medium used to enrich methanogens from anoxic sediments (Sowers & Ferry 1983), and precluded the coenrichment of the aerobic consorts of the methanogens to study them further. Only the O₂- and H₂S-tolerant methanogens enriched in glass bottles were further studied by Sieburth et al. (1993b).

Regimen 2. Bacterially reduced in glass serum bottles

- MMA-oxidizes, CH₄-producers
- CH₄-oxidizers cultured above, also SO₄²⁻ reducing bacteria

Fig. 2. A pictorial description of the 3 cultural regimens used to enrich different sub-populations of methylotrophic methanogens from Chesapeake Bay. Regimen 1 was designed to enrich Methylophaga-like monomethylamine (MMA)-oxidizing bacteria (Janvier et al. 1985) from oxygenated seawater, and therefore used polycarbonate flasks not intended for anaerobic culture. A pellicle of bacteria that were cultured as methane oxidizers (Sieburth et al. 1987), developed at the gas/liquid interface and was the first indication that methane would eventually accumulate to appreciable concentrations in the headspace. The O₂-tolerant/ H₂S-sensitive methanogenic enrichments produced traces of ethane and ethylene in addition to methane, but no H₂S. In Regimen 1, H₂S was usually produced, ethane and ethylene were not, and both the anaerobic bacteria and their aerobic bacterial consorts were cultured from these O₂- and H₂S-tolerant methanogenic enrichments. Regimen 3 was similar to the traditional anaerobic medium used to enrich methanogens from anoxic sediments (Sowers & Ferry 1983), and precluded the coenrichment of the aerobic consorts of the methanogens to study them further. Only the O₂- and H₂S-tolerant methanogens enriched in glass bottles were further studied by Sieburth et al. (1993b).

The ability of the O₂- and H₂S-tolerant MBC in glass bottles (Regimen 2) to survive repeated oxygenations at monthly intervals during subculture in fresh, oxy-
Gas chromatographic traces of the hydrocarbons in the headspace of methanogenic enrichments made with oxygenated seawater samples supplemented with monomethylamine (MMA) and contained in polycarbonate flasks fitted with rubber septa (Regimen 1 in Fig. 2). (A, C & D) The unexpected smaller trace of ethane and larger trace of ethylene in the methanogenic enrichments is shown for 3 enrichment cultures. (B) The presence of methane, indicated by a pellicle of methane-oxidizers, was confirmed by GC with reference gases. The C$_2$ hydrocarbons were not detected in the headspace of methanogenic enrichments made in glass containers. Each sample injection of 250 µl is shown by arrowheads.

In an attempt to determine if the MBC were associated with particulates, MPN estimates were made in 1986 using different volumes of untreated seawater or the particulates concentrated on 3 µm filters from different volumes as the inocula for enrichment cultures. As can be seen in Fig. 5, both estimates were equally low in the surface mixing-layer, but the unfiltered inocula in the pycnocline and bottom water samples had much higher MPN estimates of the MBC than the filter-concentrated preparations. Filtrates were essentially negative. The MBC that accumulate in the pycnocline and sediment through the bottom water layer where they persist in similar populations, are definitely sensitive to filter concentration.
Fig. 5. Comparison of the hydrography and the distribution of methanogens at upper bay Stns 904 (A & B) and 858 (C & D), May 1986. The properties profiled were: temperature, °C (--; salinity, % (-----Il-----); density, $\sigma _{1}$ (---); and dissolved oxygen, % (O--). Most probable number (MPN) estimates for methanogens were determined for whole unfiltered water (----$\circ$----), as well as filtered particulates(O--)

To extend our observations in 1987, samples were obtained down the estuarine density gradient from mixed freshwater to mixed seawater as shown in Fig. 6. The inoculum was equivalent to the particles in 100 ml of sample. The criteria for positive cultures was the same as for the MPNs, and the number of positive enrichments as well as the total was recorded. All 3 enrichment procedures diagramed in Fig. 2 were compared for the 3 water layers at all the stations down the density gradient, and the results are summarized in Table 2. Enrichments made for MPN estimates at Stns 13 and 16 that are not reported here, were incorporated into Table 2, and explain the larger number of cultures at these stations. When the 3 enrichment procedures were compared on the basis of the water layer, the results were similar for all 3 water layers. The bacterially reduced enrichments in glass (Regimen 2) yielded twice the percentage of positive methanogenic enrichments as did either the chemically reduced medium (Regimen 3) or the bacterially reduced medium in polycarbonate flasks (Regimen 1). If there was a bias between regimens, then it was consistent for each water mass.

When the percentage of methane-positive cultures were calculated for each treatment at each station down the estuary and plotted (Fig. 7), a striking trend was observed. The highest yield of methanogenic enrichments was in the chemically reduced enrichment of Regimen 3 at the head of the estuary above the Bay Bridge, which tapered off down the density gradient of the Bay. This indicates that the methanogens tolerant of chemical reduction by sulphide had a tendency to do well in the mixed waters up the estuary and may be of benthic origin. The most consistent methanogen recovery along the density gradient was in the bacterially reduced enrichments in glass. The bacterially reduced methanogenic enrichments in polycarbonate flasks that appear to select for O$_{2}$-tolerant/H$_{2}$S-sensitive methanogens did as well as those in glass at the mid-bay Stn 5, but had a much narrower distribution up and down the estuary.

DISCUSSION

The O$_{2}$- and H$_{2}$S-tolerant MBC, which are presumably enriched in reduced micro-niches through the bacterial consumption of dissolved oxygen initiated by MMA oxidizers in the glass bottles of Regimen 2, have given us a reliable procedure for the

Fig. 6. Density profiles, water column depths, and depths sampled (arrowheads) for each station occupied during CORSAIR XII, May 1987. The end members are the completely mixed freshwater and continental shelf water at Stns 1 and 11 & 13, respectively. These contrast with the waters in between that are undergoing varying states of stratification.
**Table 2.** The effect of chemical and bacterial reduction in the 3 regimens on the enrichment of methanogenic cultures along an increasing density gradient of the Chesapeake Bay shown in Fig. 6. Stn %: percent of methane-positive cultures for each regimen at each station. Regimen 1: bacterially reduced in polycarbonate flasks; 2: bacterially reduced in glass serum bottles; 3: chemically reduced in glass serum bottles.

<table>
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<th>Bottom</th>
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<td>3</td>
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<tr>
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MPN estimation of methanogenic particulates (Fig. 5). This procedure has allowed us to observe an accumulation of culturable methanogens in the pycnocline that persist into the bottom layer of water. The cultured methanogen populations of some 30 cells l-1, if they are the usual 4 orders of magnitude underestimate of in situ populations (Gonzalez et al. 1992), would indicate an in situ population of 102 cells ml-1. Undescribed Archaea that would include bacteria in the order Methanomicrobiales were found to account for up to 2% of the total ribosomal RNA extracted from the bacterioplankton of oxygenated coastal waters (DeLong 1992). Assuming that total 4',6'-diamidino-2-phenyl-indole (DAPI) counts of these populations are 106 ml-1 (Davis et al. 1985), this would indicate in situ populations of up to 104 cells ml-1. Methanogen populations of 102 to 104 cells ml-1 would be abundant enough to be a part of the micro-literosphere (Sieburth & Davis 1982), and could be ecologically significant.

The 1986 MPNs of the filter-concentrated MBCs were much lower than those made from unfiltered seawater. The filtrates did not account for the lowered MPN of the filter concentrates (Table 1 in Sieburth 1987). Filtration could have either destroyed the anaerobic microniches and exposed the MBC to increased concentrations of atmospheric oxygen, or filtration may have concentrated bacterivorous protozoa (Sieburth 1984, Cyanar et al. 1985) able to consume C1 microbes (Sieburth et al. 1987). The follow-up studies in 1987 allowed a comparison of methanogen enrichment with 3 procedures that appear to select for methanogenic sub-populations with differing H2S-tolerances (Table 2, Fig. 7).

One sub-population consisted of those methylotrophic methanogens which overcame the inhibitory effect of direct enrichment into a sulphide-reduced medium such as that of Sowers & Ferry (1983) used in Regimen 3. Although this population is maximal in the fresher waters at the head of the bay, and decreased seaward, it also appears to be a component of the methane cycle in the upper ocean. The methylotrophic methanogen enriched from the oxygenated upper layer of the ocean (Cynar & Yayanos 1991) was serologically indistinguishable from Methanococoides methylutens which was originally obtained in the same medium from anoxic inshore mud (Sowers & Ferry 1983).
A second sub-population, obtained with Regimen 2, consisted of those methylotrophic methanogens which were coenriched with the aerobic MMA-oxidizing methyloptrophs that bacterially reduced the oxygenated seawater in glass bottles and permitted the accumulation of H$_2$S. In the following paper (this issue, Sieburth et al. 1993b) we show that all of the O$_2$- and H$_2$S-tolerant methanogens analyzed were different immunologically from extant species, with many of those developing in the pycnocline and sedimenting to bottom waters apparently belonging to new taxa.

The third sub-population, obtained with Regimen 1, consisted of those methylotrophic methanogens which developed in gas-permeable polycarbonate flasks with bacterial reduction, and were unable to be successfully transferred to a chemically reduced medium or to glass bottles after enrichment where H$_2$S can accumulate. They appear less abundant than the bacterially reduced methanogens above, and require a prolonged period of incubation for enrichment. They can be differentiated from methanogens developing in glass bottles by the presence of trace amounts of ethane and ethylene in addition to methane. The literature on the microbiological production of trace amounts of ethane and ethylene in sewage, feces and sediments is not clear on how rigidly oxygen was excluded. Ethane and ethylene among other volatiles have been found in cores of surface sediments (Bernard et al. 1978, Whelan et al. 1980), while incubated slurries of sediments have been used to study the formation of ethane (Oremland 1981) as well as the other C$_2$ and C$_3$ hydrocarbons (Vogel et al. 1982). The absence of ethane and ethylene in the methanogenic enrichments made in glass, as well as their presence in semipermeable polycarbonate flasks that might allow oxygen to flux in and hydrogen sulphide to flux out, indicates that these 2 C$_2$ hydrocarbons may be indicators of methanogenesis in the presence of oxygen or the absence of hydrogen sulphide.

An aerobes appear widespread in the oxygenated sea (see ‘Introduction’). The ability of oxygen hypersensitive anaerobes to occur in reduced microniches of seemingly aerobic habitats has been attributed to the presence of oxygen-consuming aerobic bacterial consort (Schlegel & Jannasch 1981). In the MBC, MMA oxidizers must initiate the process. But the early appearance of a pellicle of methane-oxidizers before the bulk medium becomes completely reduced or methane concentrations become appreciable, indicates that methane-oxidizers probably assist in the creation of reduced microniches. It also indicates that methanogenesis and methanotrophy can occur simultaneously and at the same site. This is in contrast to the accepted model that shows methane only being produced in the sediment and methane only being oxidized in the thermocline (pycnocline) of stratified waters (Hanson 1980, Large 1983, Brock 1985).

In my experience, methane oxidizers were more successfully cultured in the MBC (Sieburth et al. 1993a) than with the classical procedure of enriching with an atmosphere of 50:50 air and methane (Whittenbury & Dalton 1981, Sieburth et al. 1987). This may be due to the exclusion of aerobic bacteria from the reduced enrichments. This exclusion of the aerobic consumers may also explain why methane production is recognized in anoxic basins and sediments where oxidative consumption is depressed, and is not generally recognized in the oxygenated water column (Oremland 1988, Cicerone & Oremland 1988, Kiene 1991) where production appears to be closely balanced by consumption as it is in the early stages of the enrichment cultures of Regimes 1 and 2, before dissolved oxygen is consumed and the enrichments behave like anoxic basins.

The methanogens obtained with the bacterially-reduced enrichments of Regimens 1 and 2 have a very different distribution than those obtained in the anoxic medium, Regimen 3. This may indicate that the redox potential (Eh) requirements of methane-producing and hydrogen sulphide-producing bacteria in the water column could be very different from the Eh requirements of pure cultures reported in the literature of −200 mV or less for methane-producing bacteria (Mah & Smith 1981) and 0 to −100 mV for sulphate-reducing bacteria (Pfennig et al. 1981). Accordingly, I have illustrated my working hypothesis in Fig. 8, in an attempt to explain the possible sources of these 3 distinct populations of cultured methanogens and where the Eh ranges might be. Assuming that the −100 mV Eh for sulphide reducers (Pfennig et al. 1981) also applies in situ, while the water column methanogens with their essential aerobic consorts must have a very different and higher Eh range of growth than pure cultures of methanogens (Mah & Smith 1981), an Eh range for the methanogens in each regimen has been postulated. What this figure attempts to convey is that not all suspended and sedimenting microparticulates are the same, and that this is due to their different sources and size which may have a significant effect on their bacterial composition and Eh potential.

If this is essentially correct, then the sub-population of sulphide-sensitive methanogens in Regimen 1 would be expected to be selected and thrive in the oxygen-rich phycosphere of Bell & Mitchell (1972) where the methylated amine degradation products from the exuded algal osmolyte GBT could enrich them, but any H$_2$S formed could diffuse away or be inactivated by oxygen. The O$_2$- and H$_2$S-tolerant methanogens of Regimen 2 must also be part of the phycosphere since they can be enriched from xenic
Fig. 8. A 'top down' working hypothesis based on algal symbiotrophy of the MBC in situ is used to explain the bacteria observed to be present in the methanogenic enrichments of the 3 regimens of Fig. 2. This speculative diagram indicates the different processes that appear to occur in each of the 3 regimens, and could explain their vertical and horizontal distribution in Chesapeake Bay as shown in Table 2 and Fig. 7. The O₂-tolerant/H₂S-sensitive methanogenic consortium enriched by Regimen 1 is illustrated as a reduced microniche created by an outer ring of MMA oxidizers, a central core of methane producers and an intermediate layer of methane oxidizers. The O₂- and H₂S-tolerant methanogenic consortium enriched by Regimen 2 differs by having a central core of sulphate-reducing bacteria. The anaerobic Regimen 3 only supports an inner core of sulphate-reducers and an outer ring of H₂S-tolerant methanogens and could occur wherever anoxia and H₂S prevent aerobes from developing. In this 'top-down' hypothesis, they would be the source of methanogens for the anoxysphere of nearshore anoxic sediments and anoxic basins. But Regimen 3 methanogens probably originate in the zoosphere where they coexist with O₂- and H₂S-tolerant methanogens of Regimen 2, that are versatile enough to also occur in the phycosphere, where only the O₂-tolerant/H₂S sensitive methanogens enriched by Regimen 1 can exist. The symbiotrophic nurturing of the MBC by their algal hosts must be the key to in situ mineralization and sustained productivity in the stratified ocean. DMA: dimethylamine; DO: dissolved oxygen; GBT: glycine betaine; MA: methylamine; MMA: monomethylamine; TMA: trimethylamine.
MMA in the fluid and undissolved oxygen in the head-space become the surrogate algal hosts.

The MBC of Regimen 2 would also be expected to endure the digestive process of copepods in the zoosphere that encase faecal pellets in a chitinous peritrophic membrane (Johnson et al. 1982), that could permit the H$_2$S of their sulphate-reducing bacterial consorts to accumulate and select these H$_2$S-tolerant methanogens. Likewise, fermenting faeces, their fragments and similar particulates which can go anaerobic, could allow sufficient H$_2$S to accumulate and enrich methanogens culturable in Regimen 3 (Oremland 1979, Cynar & Yayanos 1991) anywhere in the water column and be part of the zoosphere. The anaerobic Regimen 3, however, prevents the development of aerobes and the sub-population of methanogens enrichable in it could be the basis for creating anoxic H$_2$S-rich sediments on the nearshore sea floor and H$_2$S-rich waters in anoxic basins (Sieburth 1987, 1991, Donaghay et al. 1992) that can be considered to be part of an anoxosphere. If this symbiotic top-down hypothesis for algal fueled methane-production and sulphate-reduction is correct, it means that the accepted ‘bottom-up’ model (Hanson 1980, Large 1983, Brock 1985) and the procedures used to measure their extent and flux rates, must be rethought (Sieburth 1991).

My first paper discussing the upper-ocean methane cycle (Sieburth 1983) presented the concept that the pycnocline was in essence a ‘false benthos’, since the bacterial processes involved in the pycnocline included those that occur in nearshore sediments. The importance of in situ decay in the pycnocline is even better recognized now, with over 90% of decay occurring in situ before sedimentation takes place (cf. Sieburth 1991). Now, I would like to retract my hypothesis of the ‘false benthos’ and propose that the processes in nearshore reduced sediments where the water is shallow, as in Chesapeake Bay, are due to the superimposition of the nearly pycnocline processes on the sediment, where anoxia would exclude the aerobes responsible for the oxidative C$_4$ bacterial processes discussed in this paper. Oxidative processes which essentially balance the production of reduced substances, and make the upper ocean methane cycle elusive to document. In other words, the benthos may be thought of as a ‘false pycnocline’, limited to anaerobic bacterial processes where reduced products can accumulate in the relative absence of photosynthesizing algae and aerobic oxidizers.

The production of hydrogen sulphide by 2 of the sub-populations of methanogens in the water column has broad implications. A widespread occurrence of sulphate-reducing bacteria would explain the paradoxical and ubiquitous presence of sulphide-utilizing strains of Thiobacillus (Tilton et al. 1967a, b) and Thiobacillus-like bacteria (Tuttle & Jannasch 1972) in an otherwise oxygenated sea, that at the time would not be expected to be producing the reduced product hydrogen sulphide that is susceptible to oxidation by dissolved oxygen. The presence of bacteria in the MBC that could dark fix CO$_2$ in reduced microparticulates, could also explain part of the dark fixation of CO$_2$ attributed to nitrogen fixation in sedimenting particles (Karl et al. 1990). In the poorly ventilated nutrient-rich waters of Chesapeake Bay (Malone 1991), the presence of sulphate-reducing bacteria that accompany methanogens may be very important.

The versatile MBC enriched in Regime 2, augmented by the bacteria in Regimen 3, could be capable of inducing and sustaining the suboxia and anoxia reported for Chesapeake Bay (Newcombe & Horne 1938, Officer et al. 1984, Seliger et al. 1985), as postulated in the paradigm recently described by Sieburth (1991). The extremely large riverine input of nutrients into the system supports a very large photosynthetic biomass in the pycnocline in the spring period of our study (Tyler & Seliger 1989, Malone 1991). Grazing on this biomass would provide the in situ detritus for the exhaustion of dissolved oxygen by methylotrophic bacteria (Janvier et al. 1985, Sieburth et al. 1993a) along with the simultaneous production and oxidation of methane, and the production of hydrogen sulphide by their consortial sulphate-reducing bacteria, could turn the bottom layer of water into an ‘anoxosphere’ that would exclude aerobiosis. King (1984) in his benchmark paper, showed the in situ dominance of C$_4$ methanogens in seaweed-enriched sediments. Here we show that in the pycnocline of the water column, there is another algal enriched methanogenic habitat, one that in stratified coastal and open oceans has a potential volume and area much more extensive than either the hydrothermal vents or coastal sediments. This oceanic methanogenic habitat is so different from coastal habitats that the methane-oxidizers it supports may be very different physiologically and taxonomically from those in inshore habitats such as that in Chesapeake Bay (Sieburth et al. 1993a).

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**LITERATURE CITED**


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