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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 consorts 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_2S-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 *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 consorts. Recently, hydrogen sulphide has also been observed in the pycnocline of the oxygenated upper ocean by both indirect (Cutter & Krahforst 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 C_1 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 C_1 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 pellicle 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 C_1 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 methane 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 *Methylophaga* 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 C_1 bacteria are MMA-oxidizers, methane-producers, and methane-oxidizers. The C_1 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 C_1 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 O_2-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 H_2S-tolerances. The third sub-population used sulphide 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 (σ_t), salinity (‰), temperature (°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 µm 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

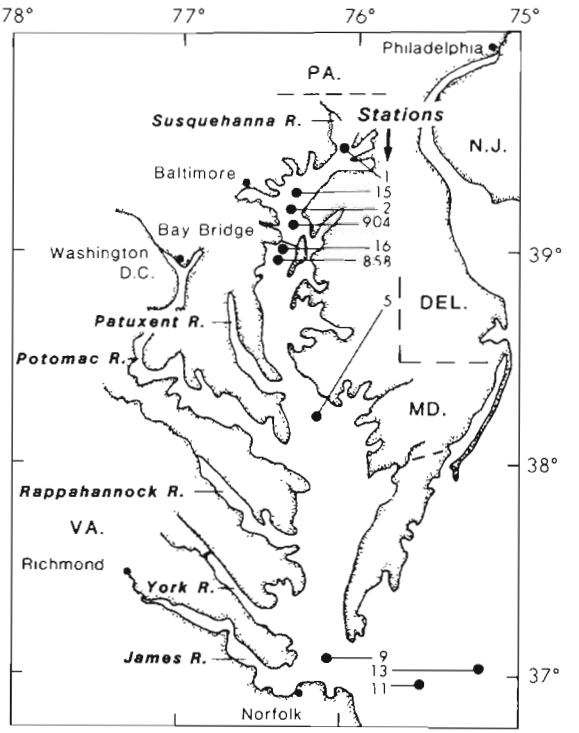

Fig. 1. Sampling locations in Chesapeake Bay and on the adjacent continental shelf. Stns 858 and 904 are from PROPHOT XVIII, May 1986, and Stns 1 to 16 are from CORSAIR XII, May 1987

100 ml while the volumes used in 1987 were 5, 50 and 500 ml. The larger seawater samples in appropriate-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

Stn	Latitude, N	Longitude, W
1	39° 26.44'	76° 01.74'
15	39° 13.05'	76° 15.15'
2	39° 07.84'	76° 19.47'
904	39° 04.00'	Mid-channel
16	38° 58.60'	76° 22.15'
858	38° 58.00'	Mid-channel
5	38° 09.98'	76° 13.90'
9	37° 04.42'	76° 05.11'
11	36° 53.01'	75° 29.72'
13	37° 03.00'	75° 16.70'

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 seawater 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 production 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 comparison 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 production of methane, a rubber septum was installed in the flask tops and methane was determined in the headspace 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 colourless leuco form, methane concentrations in the headspace 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 seawater 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 H_2S, but did not indicate any C_2 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 concentrations 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 enrichments 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 concentrations of methane. The GC methane analyses, unlike 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 O_2-tolerant, these methanogens were also H_2S-sensitive. Although these O_2-tolerant/H_2S-sensitive methanogens are an interesting group, the long incubations required and their failure to transfer to chemically reduced media did not permit us

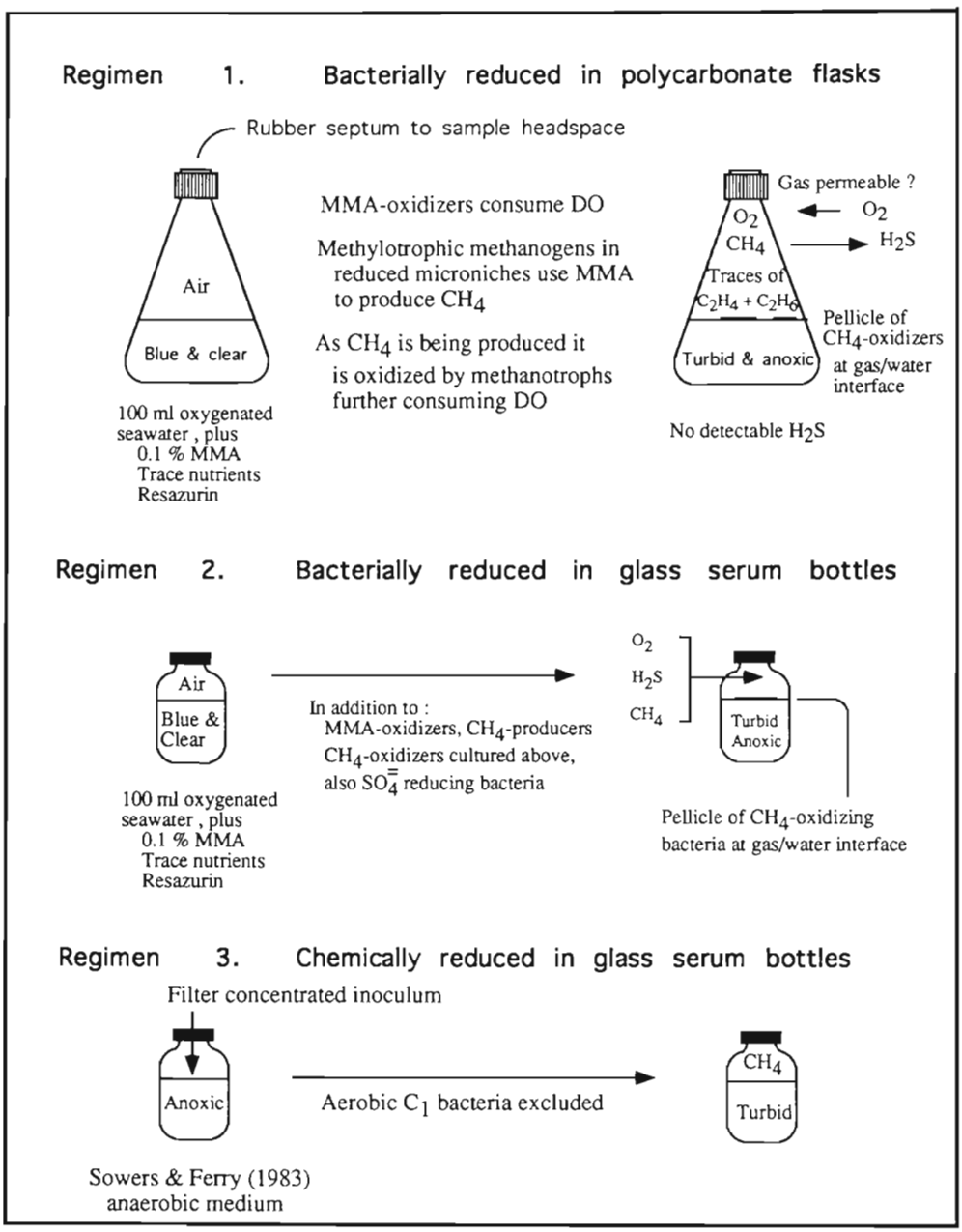

Fig. 2. A pictorial description of the 3 cultural regimens used to enrich different sub-populations of methylotrophic methanogens from Chesapeake Bay. Regimen 1 was designed to enrich *Methylophaga*-like monomethlyamine (MMA)-oxidizing bacteria (Janvier et al. 1985) from oxygenated seawater, and therefore 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 O_2-tolerant/H_2S-sensitive methanogenic enrichments produced traces of ethane and ethylene in addition to methane, but no H_2S. In Regimen 2, the oxygenated seawater sample was prepared identically as in Regimen 1, but was housed in glass serum bottles. Unlike in Regimen 1, H_2S was usually produced, ethane and ethylene were not, and both the anaerobic bacteria and their aerobic bacterial consorts listed were cultured from these O_2- and H_2S-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 O_2- and H_2S-tolerant methanogens enriched in glass bottles were further studied by Sieburth et al. (1993b).

The ability of the O_2- and H_2S-tolerant MBC in glass bottles (Regimen 2) to survive repeated oxygenations at monthly intervals during subculture in fresh, oxy-

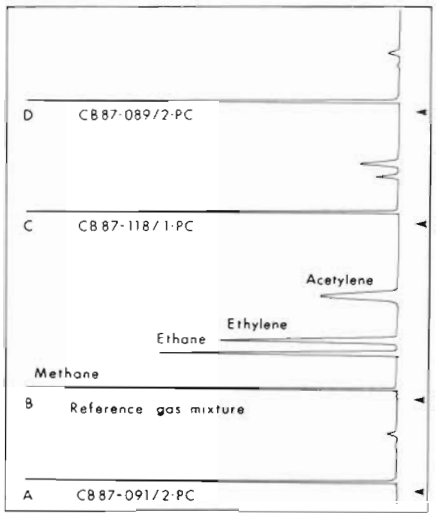

Fig. 3. Gas chromatographic traces of the hydrocarbons in the headspace of methanogenic enrichments made with oxygenated seawater samples supplemented with monomethylamine (MMA) and contained in polycarbonate flasks fitted with rubber septa (Regimen 1 in Fig. 2). (A, C & D) The unexpected smaller trace 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

genated media as well as weeks of anoxia, is shown in Fig. 4. The survival of obligate aerobes under anaerobic conditions for weeks at a time is indicated by 2 observations. The rapid resumption of methanogenesis would presumably require the depletion of dissolved oxygen by both MMA- and methane-oxidizers, while the rapid decrease in methane after a presumed exhaustion of MMA would be caused by methane-oxidizers at the gas/liquid interface presumably utilizing undissolved oxygen from the headspace. Before this project ended, hundreds of MBC were sucessfully transferred at 1 to 2 mo intervals for over 3 yr.

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.

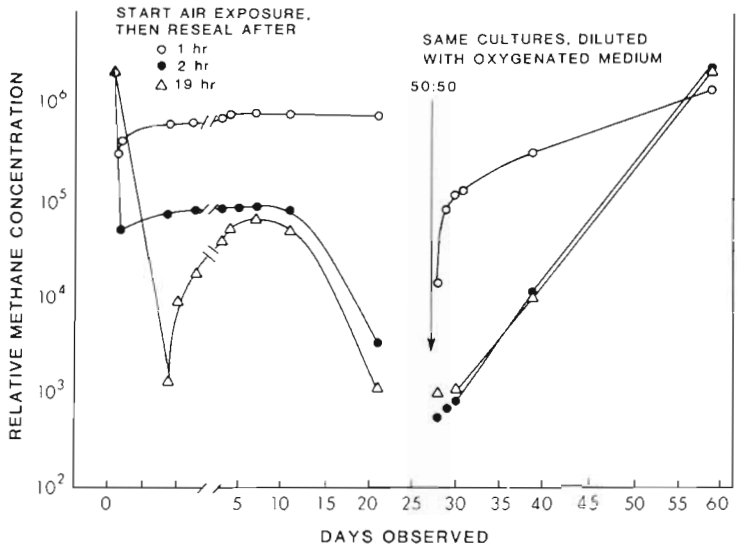

Fig. 4. The ability of the aerobic and anaerobic components of the methanogenic bacterial consortia to survive alternate oxic and anoxic conditions in Regimen-2-type enrichments were observed in 500 ml serum bottle enrichments that had incubated for 36 d and were reduced and methanogenic. Rubber caps were removed and the enrichments were exposed to air during which time the resazurin became oxidized. Without replenishing the partially spent medium, replicate enrichments were resealed after 1, 2 and 19 h exposure to air, and the relative concentration of methane was determined for an additional 20 d. After methane production dropped sharply in 2 of the replicates, half the volume of the spent culture medium was replaced with oxygenated fresh medium, capped and the relative methane concentrations observed for another 31 d. After resealing, the pink media rapidly became reduced, presumably by MMA- and methane-oxidizers. There was marked methane production for 1 wk in the bottle aerated for 19 h, at which time methane concentrations in it and the 2 h exposed bottle rapidly declined, indicating that methane-oxidizers were still active. The rapid reduction of all replicates after replenishment with oxygenated media as well as the rapid increase in methane, indicated that both the aerobes and the anaerobes were still viable after alternating aerobic and anaerobic conditions in the liquid phase

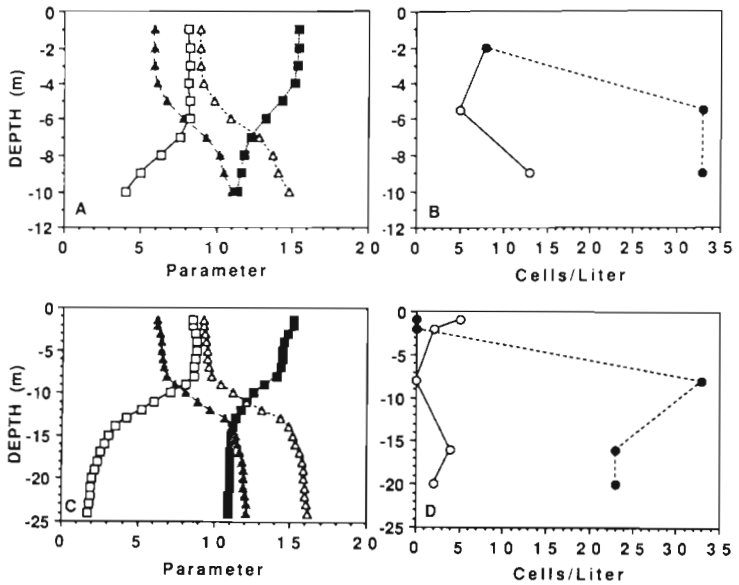

Fig. 5. Comparison of the hydrography and the distribution of methanogens at upper bay Stns 904 (A & B) and 858 (C & D), May 1986. The properties profiled were: temperature, °C (···■···); salinity, ‰ (····Δ····); density, σ_t (---▲---); and dissolved oxygen, ‰ (-□-). Most probable number (MPN) estimates for methanogens were determined for whole unfiltered water (---●---), as well as filtered particulates(-○-)

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 betwen 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_2S-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_2S-tolerant MBC, which are presumably enriched in reduced microniches 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

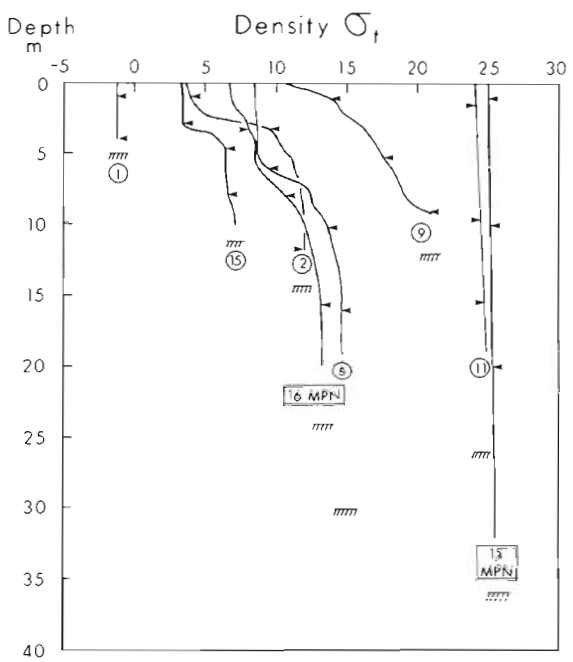

Fig. 6. Density profiles, water column depths, and depths sampled (arrowheads) for each station occupied during CORSAIR XII, May 1987. The end members are the completely mixed freshwater and continental shelf water at Stns 1 and 11 & 13, respectively. These contrast with the waters in between that are 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

Water layer:	Top			Pycnocline			Bottom			Stn %		
Regimen:	3	2	1	3	2	1	3	2	1	3	2	1
1	4/4	3/4	0/4	–	–	–	–	–	–	100	75	0
15	1/4	1/4	2/4	1/4	4/4	0/4	0/4	3/4	1/4	16	67	25
2	4/4	3/4	1/4	4/4	4/4	4/4	4/4	3/4	2/4	100	83	58
16	1/6	8/9	–	2/6	7/9	–	6/6	9/9	–	50	89	–
5	2/4	2/4	2/4	1/4	3/4	2/4	0/4	2/4	3/4	25	58	58
9	0/4	2/4	0/4	0/4	2/4	0/4	0/4	3/4	0/4	0	58	0
11	0/4	0/4	1/4	0/4	1/4	0/4	2/4	0/4	0/4	16	8	8
13	0/6	5/9	–	0/6	1/9	–	0/6	0/9	–	0	22	–
Total	12/36	24/42	6/24	8/36	22/39	6/20	8/36	21/39	6/20			
%	33	57	25	22	56	30	22	54	30	38	58	25

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 (González et al. 1992), would indicate an *in situ* population of 10^2 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 10^6 ml^{-1} (Davis et al. 1985), this would indicate *in situ* populations of up to 10^4 cells ml^{-1}. Methanogen populations of 10^2 to 10^4 cells ml^{-1} would be abundant enough to be part of the microlitersphere (Sieburth & Davis 1982), and could be ecologically significant.

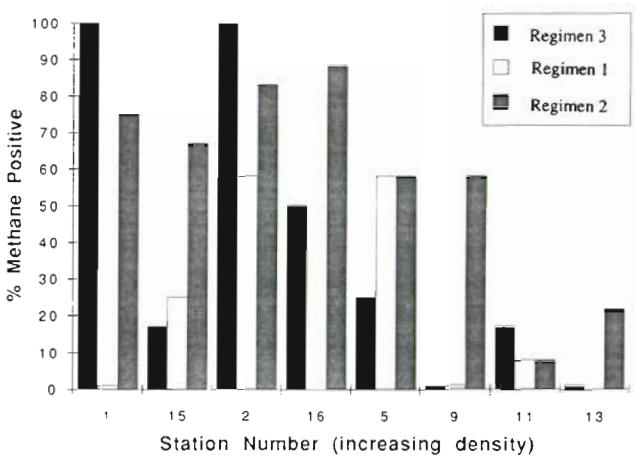

Fig. 7. Distribution of the 3 methanogenic sub-populations obtained with different methods of reduction (see Fig. 2), seaward, down Chesapeake Bay. The chemically-reduced methanogen sub-population of Regimen 3 that must develop in the absence of oxygen and the presence of marked sulphide, as do those from anoxic sediments, were dominant up the Bay. Oxygen-tolerant methanogen sub-populations that developed in seawater reduced through oxygen consumption by the ambient MMA oxidizers, persisted throughout the Bay. The O_2- and H_2S-tolerant methanogen sub-population of Regimen 2 enriched in glass had a wider distribution than the O_2-tolerant/H_2S-sensitive methanogen sub-population enriched in the polycarbonate flasks (Regimen 1)

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 C_1 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 H_2S-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 *Methanococcoides 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 methylotrophs that bacterially reduced the oxygenated seawater in glass bottles and permitted the accumulation of H_2S. In the following paper (this issue, Sieburth et al. 1993b) we show that all of the O_2- and H_2S-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_2S 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.

Anaerobes 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 consorts (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 bacterivores in 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 compostion 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_2S formed could diffuse away or be inactivated by oxygen. The O_2- and H_2S-tolerant methanogens of Regimen 2 must also be part of the phycosphere since they can be enriched from xenic

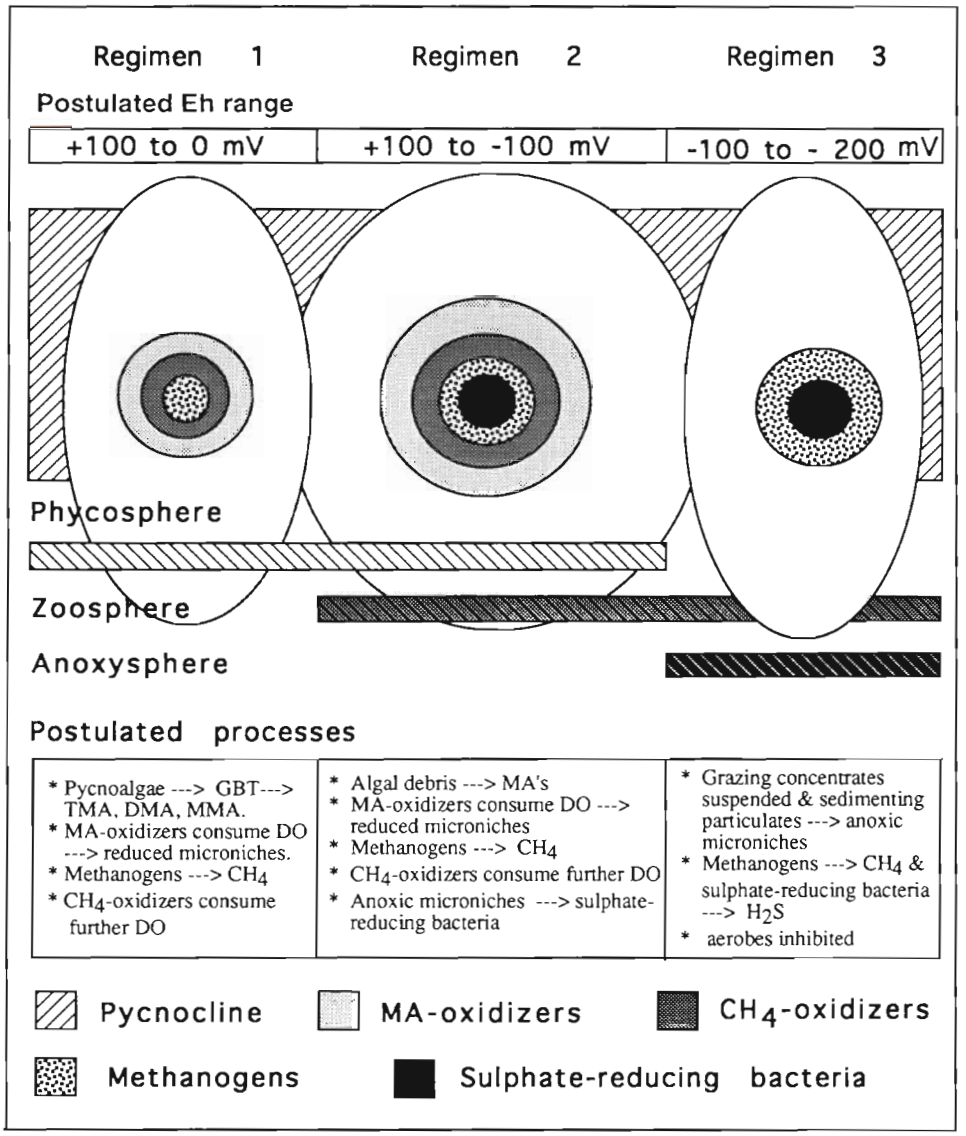

Fig. 8. A 'top down' working hypothesis based on algal symbiotrophy of the MBC *in situ* is used to explain the bacteria observed to be present in the methanogenic enrichments of the 3 regimens of Fig. 2. This speculative diagram indicates the different processes that appear to occur in each of the 3 regimens, and could explain their vertical and horizontal distribution in Chesapeake Bay as shown in Table 2 and Fig. 7. The O_2-tolerant/H_2S-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 O_2- and H_2S-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 H_2S-tolerant methanogens and could occur wherever anoxia and H_2S 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 O_2- and H_2S-tolerant methanogens of Regimen 2, that are versatile enough to also occur in the *phycosphere*, where only the O_2-tolerant/H_2S sensitive methanogens enriched by Regimen 1 can exist. The symbiotrophic nurturing of the MBC by their algal hosts in the phycosphere, that is amplified by their consumption in the zoosphere, may 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

algal cultures (Sieburth 1988), in which they can persist through decades of subculturing. This symbiotrophic (Lewis 1974) maintenance of the mineralizing MBC by their algal hosts must be the key to sustained *in situ* productivity in the open sea during its productive seasonal stratification (Sieburth 1991). The MBC enriched in Regimens 1 and 2 may be thought of as artifical phycospheres, where excess dissolved

MMA in the fluid and undissolved oxygen in the headspace 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_2S of their sulphate-reducing bacterial consorts to accumulate and select these H_2S- tolerant methanogens. Likewise, fermenting faeces, their fragments and similar particulates which can go anaerobic, could allow sufficient H_2S 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_2S-rich sediments on the nearshore sea floor and H_2S-rich waters in anoxic basins (Sieburth 1987, 1991, Donaghay et al. 1992) that can be considered to be part of an *anoxysphere*. If this 'symbiotrophic 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 nearby pycnocline processes on the sediment, where anoxia would excludes the aerobes responsible for the oxidative C_1 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 subpopulations 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. 1988). 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 'anoxysphere' that would exclude aerobiosis. King (1984) in his benchmark paper, showed the *in situ* dominance of C_1 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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