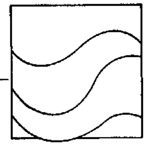

Methanoarchaea associated with sinking particles and zooplankton collected in the Northeastern tropical Atlantic

Methanoarchaea
Particles
Zooplankton
Fecal pellets
Sea water column

Methanoarchaea
Particules
Zooplancton
Pelotes fécales
Colonne d'eau océanique

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ABSTRACT

During the "Eumeli-4" cruise in the northeastern tropical Atlantic Ocean, the occurrence and activity of methanoarchaea in the water column were examined, whether in association with sinking particulate material collected by means of sediment traps and in situ pumps, or with zooplankton organisms harvested with plankton nets and their freshly egested fecal pellets. Besides highlighting the necessity to use adequate particle samplers, the results confirm the presence of viable pelagic methanogens in particulates; likely to be present in zooplankton, in the form of enteric flora, these archaea would be released in the sea water column within egested fecal pellets.

RÉSUMÉ

Methanoarchaea associées au matériel particulaire en suspension et au zooplancton collectés dans l'océan Atlantique tropical nord-est.

Au cours de la campagne "Eumeli-4" effectuée dans les eaux tropicales du nord-est de l'Océan Atlantique, nous avons cherché à mettre en évidence la présence et l'activité de populations productrices de méthane, soit associées au matériel particulaire en suspension récolté avec des pièges à sédiment et des pompes *in situ*, soit associées aux organismes zooplanctoniques, récoltés avec des filets, et à leurs pelotes fécales. Outre la nécessité d'utiliser des collecteurs de particules efficaces, les résultats confirment la présence de méthanogènes pélagiques viables, dans le matériel particulaire. Ces archaea seraient, à l'origine, présentes dans les tractus digestifs des organismes zooplanctoniques, en tant que microflore entérique, et éjectées dans la colonne d'eau océanique à l'intérieur des pelotes fécales.

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INTRODUCTION

Since a slight supersaturation of methane in open ocean surface waters was first reported (Lamontagne *et al.*, 1973), dissolved methane in the upper regions of the ocean water column has been shown to be commonly present at concentrations higher (30 to 70 %) than would be expected from the solubility of methane in sea water and known atmospheric concentrations (Kiene, 1991). This worldwide phenomenon of methane supersaturation, frequently associated with the pycnocline, at a depth between 50 and 200 m, is apparently due to in situ biological processes (Sieburth, 1987).The mechanism for this production of methane in the essentially aerobic environment of the

upper ocean is enigmatic, because methane production is mediated by methanogenic archaebacteria (Woese, 1987), that require strict anaerobic conditions.

Scranton and Brewer (1977) suggested that this paradoxical methanogenic activity stemmed from methanogens living within reducing microenvironments, such as suspended particles, fecal pellets, or the intestinal tracts of pelagic animals. In 1979, Traganza *et al.* reported a correlation between excess methane and zooplankton blooms in near-surface waters of the western Mediterranean Sea and subtropical north Atlantic Ocean. The same year, Oremland demonstrated the presence of methanogens in the intestinal tracts of fish, and suggested an association of methanogens with the digestive tract of zooplankton (Oremland, 1979).

As methane concentration generally peaks at the pycnocline, an alternative methane source could be derived from the association of methanogens with phytoplankton. Some data have shown occasional correlations between methane and chlorophyll a (Brooks *et al.*, 1981), but there has been no consistent identification of phytoplantkonic material as a source of methane production, suggesting that methane and chlorophyll a maxima are probably secondarily associated with nutrient-rich waters (Burke *et al.*, 1983; Owens *et al.*, 1991).

Since then, it has been clearly established that both suspended microparticulates and large aggregates harbour rich communities of bacteria and protozoa, at densities two to five orders of magnitude greater than populations freely dispersed in the surrounding sea water (Taylor *et al.*, 1986; Alldredge and Cohen, 1987). Also, the presence of commensal microflora has been evidenced in the digestive tracts of marine zooplankton (Sochard *et al.*, 1979; King *et al.*, 1991), as has the release of gut flora via fecal pellets (Pomeroy and Deibel, 1980). However, the nature of the methane-generating processes in the sea water column is yet poorly understood.

A methanoarchaea has been isolated from live plankton collected in oxygenated surface waters of the open sea off southern California (Cynar and Yayanos, 1991), and active methanogens have been detected in particulates collected in the northwestern Mediterranean Sea, with *in situ* pumping or sediment traps, as well as in zooplankton biomass and fecal pellets (Bianchi *et al.*, 1992; Marty, 1993).

Here we report on observations of the occurrence and activity of methanogens in the oxygenated mixed layer. These studies were conducted during the 'Eumeli-4' cruise of the R/V *L'Atalante* to the northeastern tropical Atlantic Ocean (May-June 1992), in an attempt to determine, on one hand, whether active methanogens are present in particulate material, and on the other hand, whether these methanogens would be associated with sinking particles, or with zooplanktonic organisms and their freshly egested fecal pellets.

MATERIALS AND METHODS

Sampling sites

Experiments were performed during the 'Eumeli-4' cruise (leg 2 - June 1992) on board R/V *L'Atalante* in the northeastern tropical Atlantic Ocean. Three sites were occupied during this study, one eutrophic (20° 32′ N - 18° 34′ W), one mesotrophic (18° 30′ N - 21° 10′ W) and one oligotrophic (21° 03' N - 31° 08′ W).

At each site, vertical profiles of temperature, salinity, density, and fluorescence were recorded down to 500 m, with a standard CTD-rosette (conductivity-temperature-depth), permitting localization of the fluorescence peak and the pycnocline.

The water depth was 1,900 m, 3,000 m, and 4,500 m, at the eutrophic, mesotrophic and oligotrophic sites, respectively. The salinity in the upper layers fluctuated around 36-38 (eutrophic site), 34-35 (mesotrophic site) and 37-37.4 (oligotrophic site), reaching 35-36 (eutrophic and mesotrophic sites) and 37 (oligotrophic site) in deeper layers (200 m). The temperature ranged between 17-18 °C, 19-21 °C and 23-24 °C in the upper layers, and 15-16 °C, 13-14 °C and 20 °C at 200 m depth, at the eutrophic, mesotrophic and oligotrophic sites, respectively. Fluorescence peaked at depths between 40 and 60 m at the eutrophic site, above 50 m depth at the mesotrophic site, and fluctuated between 100 and 170 m depth at the oligotrophic site.

Sample collection

Sediment traps

Material falling through the water column was collected in free-floating time-series sediment traps (Heussner *et al.*, 1990). During the study, three traps were deployed at 200 m and allowed to drift for 24 h. The 200 m depth was chosen to be below the photic zone and the mixed layer at the three sites during the sampling period. During these deployments, no poison or preservative were used in the traps. The PPS4 (Piège à Particules Séquentiel, model 4), a small-scale model (0.05-m^2 collecting area) using a 6-cup carousel, was moored at the eutrophic site, and the PPS5 (Piège à Particules Séquentiel, model 5), a baffled 1-m^2 conical trap with eight receiving cups, at the mesotrophic and oligotrophic sites.

Upon recovery, the receiving cup carousels were at once unscrewed from the traps, and the samples immediately processed. Subsampling of each cup was carried out, and each subsample was carefully hand-picked to remove zooplankton 'swimmers' which often entered the traps. Aliquots (~10 ml) of the remaining particulate samples were removed and injected into "Hungate type" anaerobic culture tubes (Bellco Glass, Inc.), and processed as indicated below.

Six different samples of particulate material, and two samples of swimmers were obtained from the PPS4 time-series sediment trap moored at the eutrophic site (ISIS21); two sets of particulate material samples were obtained from the PPS5 time-series sediment trap moored at the mesotrophic (PD09) and oligotrophic (PD12) sites.

In situ pumps

Particulate material sedimenting along the water column was also collected with *in situ* particle samplers (Challenger

Oceanic), using 142-mm diameter filters (Nuclepore 10 μm), with or without prefilters. Pumps were deployed at depths corresponding to the fluorescence maximum and/or the pycnocline, and at 200 m, corresponding to sediment-trap depth. The pumps were activated for periods between 30 and 90 min, and the filtered volumes ranged from 60 litres in the eutrophic area to 1,300 litres in the oligotrophic area.

On recovery, the housings were opened and filters removed. In some cases, filters and prefilters were cut off, and portions transferred into Hungate tubes containing 10-ml 0.2 μm filtered sea water collected at the same depth. In other cases, particles were washed off with 0.2 μm filtered seawater, and 10-ml subsamples injected into Hungate tubes. Unfiltered sample waters were used as controls.

At the eutrophic site, two *in situ* pumps were running for 45 min at a depth of 60 m (PIS20: 60 litres filtered) and 60 min at a depth of 200 m (PIS21: 449 litres), respectively. At the mesotrophic site, five *in situ* pumps were running for 30 to 45 min at 60 m (PIS22: 452 litres; PIS24: 816 litres; PIS26: 555 litres) and 60 min at 200 m (PIS23: 990 litres; PIS25: 1,000 litres), respectively. At the oligotrophic site, eight *in situ* pumps were running for 30 to 45 min at 80 m (PIS30: 670 litres), 110 m (PIS28: 784 litres), 140 m (PIS32: 644 litres; PIS33: 829 litres), 160 m (PIS29: 877 litres), 170 m (PIS34: 923 litres), and 200 m (PIS27: 1,365 litres; PIS31: 1,283 litres), respectively.

Plankton samples

Plankton samples were collected from vertical tows with a WP2 net (0-200 m), or horizontal tows of an Omori net (50 m). Zooplankton specimens were sorted, identified, and placed in Hungate tubes (1 to 30 individuals per tube). In other cases, sorted specimens of selected species were placed in large 80-litre collector systems, described by La Rosa (1976), which permitted the recovery of fecal pellets in the bottom of container, while the animals themselves remained in a cylindrical tergal bag. The containers, filled with sea water, were covered and allowed to stand for 12 h, as an average, at *in situ* temperature, while the organisms defecated. At the end of incubation time, fecal pellets were recovered with the aid of a pipette, and transferred into Hungate tubes.

At the eutrophic site, incubations were performed with fecal pellets freshly egested, mixed zooplankton specimens (essentially copepods and gelatinous), and isolated copepod specimens (*Calanus helgolandicus* and *Pleuromamma* sp.).

At the mesotrophic site, incubations were performed with fecal pellets freshly egested, and isolated Euphausiid, pteropod, and salp specimens: *Euphausia* sp., 30 individuals, in triplicate; *Diacra* sp., 20 individuals; *Clio* sp., 20 individuals; *Cavolinia* sp., 7 individuals; *Cyclosalpa* sp., 1 individual, in tetraplicate.

At the oligotrophic site, incubations were performed with fecal pellets freshly egested, and isolated copepod, decapod, salp and Euphausiid specimens: *Calanides*, 30 individuals; *Sergestes* sp., 4 individuals; *Iasis zonaria*, 1 individual; *Euphausia* sp., 30 individuals; *Stylocherion* sp., 20 individuals, in duplicate; small *Euphausia* sp. + *Nematoscelis* sp., 5 + 2 individuals. At this site, filamentous cyanobacteria were frequent in the plankton samples; one incubation was done with 60 packets of this blue-green bacteria.

Methane production measurements

All experiments were carried out on shipboard within 1 to 4 h of sample collection, and performed anaerobically, to minimize oxygen contamination from the air. Samples of homogenized particulates, samples of filters, zooplankton specimens, or fecal pellets were transferred into 15-ml Hungate tubes, and headspaces were flushed immediately with O_2-free various gas phases for 10 min prior to sealing with flange-type butyl rubber stoppers and screw caps. Sets of tubes were prepared with the following treatments: (i) 10-ml sea water under 100% H_2; and for some tubes, (ii) 10-ml sea water amended with 0.5 ml of a sterile stock solution containing a pool of methanogenic substrates (formate, final concentration 5 g l^{-1}, acetate 2.5 g l^{-1}, methanol 5 ml l^{-1}, and trimethylamine 60% 16 ml l^{-1}). The headspaces of tubes were flushed with H_2-CO_2 (Marty *et al.*, 1990), before the tubes were incubated in the dark at 15-18 °C, which approximated the *in situ* temperature throughout the sampling period, and periodically assayed for CH_4 production in the headspace.

During the cruise period, on board, 1-ml gas samples were taken weekly with a gas-tight pressure lock syringe and injected, through the stopper, into 3-ml Venojet tubes. In the laboratory, the gas-phase of the Venojet tubes and the headspace of each Hungate tube were analysed for CH_4: 10-μl subsamples were injected into a Girdel Gas Chromotograph (Series 30) equipped with a Chromosorb G AWDMCS column and a flame ionization detector. Argon was used as the carrier gas, and temperatures of column, injector, and detector were set at 80°C, 190 °C, and 220 °C, respectively (Marty, 1993). Afterwards, the headspace of the various Hungate tubes was assayed for CH_4 production every 7 days for a 2-month period.

From these various natural samples, which did or did not produce CH_4, enrichment cultures were performed by regrouping samples from the same sources, and inoculating 1-ml to 5-ml in a carbonate-buffered mineral medium supplemented with methanogenic substrates, trace vitamins, trace minerals, yeast extracts, and reducing agents, under H_2-CO_2: 80-20, or N_2-CO_2: 80-20 (Hungate, 1969; Ferrara-Guerrero *et al.*, 1993; Marty, 1995). Cultivation was performed in the dark at 30 °C.

RESULTS AND DISCUSSION

We used a variety of sampling devices during the cruise in order to detect methane production by the microflora colonizing the corresponding niches (*i.e.* sinking particles, zooplankton organisms, and egested fecal pellets). Besides the impossibility of comparing the results obtained from the different samplers, bacterial activity studies did not allow the use of poison or preservative in sediment traps; as a

result, the deployment times were as short as possible, and the amount of material collected was insufficient to perform physico-chemical analyses as well as activity experiments. Furthermore, methane production occurred at very low rates; in many samples, it was below detection limits for a period of 10-15 days after the incubations were started.

For all these reasons, the results were expressed as the amount of methane produced in the headspace of the Hungate tubes (nmol CH_4 per tube) after one-month period of incubation (Table).

Samples collected with sediment traps

Among the 20 Hungate-tubes inoculated with particulate samples collected with the 3 sediment traps, the 13 tubes containing material collected at the mesotrophic and oligotrophic sites did not exhibit production of detectable amounts of methane; little methane evolved in the headspaces of six out of the seven tubes containing either particulates (3 to 5 nmol CH_4 per tube) or swimmers (11 nmol CH_4 per tube) collected at the eutrophic site.

All seven enrichment cultures prepared from these 20 natural samples produced detectable amounts of methane in their headspace; methane accumulated up to 88 nmol CH_4 in one of the enrichments from the mesotrophic site, while the other enrichments exhibited similar and lower methane concentrations (7-14 nmol of CH_4 per tube).

Thus, in the three studied sites, particulate material collected with sediment traps contained methanogens. At the eutrophic site, the sediment trap contained numerous

Table

Production of methane by natural particulate samples and zooplankton organisms collected during the "Eumeli-4" cruise and their enrichment cultures.

Samples & Sites	1 = Natural samples 2 = Enrichments	Positive tubes/number of tubes	Methane activity expressed as the number of cultures where methane was detected in headspace (nmol CH_4/tube)				
			< 10	< 50	< 100	< 1000	> 1000
Sediment traps	1- eutrophic	6/7	5	1			
	2- eutrophic	3/3	2	1			
	1- mesotrophic	0/6					
	2- mesotrophic	2/2		1	1		
	1- oligotrophic	0/7					
	2- oligotrophic	2/2	1	1			
In situ pumps	1- eutrophic	0/12					
	2- eutrophic	2/2		1			1
	1- mesotrophic	2/22	2				
	2- mesotrophic	5/5	2	3			
	1- oligotrophic	2/35	2				
	2- oligotrophic	6/8	3	2		1	
Zooplankton	1- eutrophic	6/8	1	3			2
	2- eutrophic	4/6		2		1	1
	1- mesotrophic	8/10	8				
	2- mesotrophic	6/10	6				
	1- oligotrophic	0/7					
	2- oligotrophic	5/7	4				1
Cyanobacteria	1- oligotrophic	1/1	1				
	2- oligotrophic	1/1	1				
Fecal pellets	1- eutrophic	4/10	1		2	1	
	2- eutrophic	5/5	3	2			
	1- mesotrophic	0/1					
	2- mesotrophic	1/1	1				
	1- oligotrophic	5/14	5				
	2- oligotrophic	3/3	1	1			1
TOTAL	1- eutrophic	16/37	7	4	2	1	2
	2- eutrophic	14/16	5	6		1	2
	1- mesotrophic	10/39	10				
	2- mesotrophic	14/18	9	4	1		
	1- oligotrophic	8/64	8				
	2- oligotrophic	17/21	10	4		1	2

particles that allowed methanogens to develop; in contrast, at the mesotrophic and oligotrophic sites, the particulates collected with sediment traps, which were very sparse, did not allow methanogens to develop although the archaea were present, as demonstrated by the methane production observed in all enrichment cultures.

Samples collected with in situ pumps

Of 69 Hungate-tubes inoculated with natural particulate samples collected with the 15 sets of in situ pumpings, four unamended tubes exhibited trace quantities of methane in their headspace (3 to 6 nmol CH_4 per tube), whereas no methane production could be detected in the headspace of all other tubes, either amended or unamended with methanogenic substrates. The four methane-producing tubes contained particulates collected at 60-m depth at the mesotrophic site (PIS26 and PIS22), and 110-m depth at the oligotrophic site (PIS28).

Fifteen enrichment cultures were prepared from these 69 natural samples; no methane was produced in two of the enrichment cultures originating from the oligotrophic site; very little methane production was observed in five others (4-9 nmoles CH_4 per tube); methane production, ranging from 10 to 17 nmol CH_4 per tube, occurred in six other enrichment cultures, with the highest productions (335 nmol per tube, and up to 20 μmol CH_4 per tube) recorded in enrichment cultures obtained from materials collected at 110-m depth at the oligotrophic site (PIS28), and 60-m depth at the eutrophic site (PIS20), respectively.

Despite numerous Hungate-tube tests performed with natural samples collected during the 15 *in situ* pumping sets, very few original samples were found to be methane-producers (PIS22, PIS26, PIS28). When it did occur, methane production was detected in Hungate tubes unamended with methanogenic substrates, suggesting that organic matter present in the samples was sufficient to allow methanogens to develop. It should be noted that PIS28, which was running at 110 m depth at the fluorescence peak of the oligotrophic site, collected material deriving from phytoplankton, as verified by relatively high chlorophyll *a* concentrations (~0.04 μg per litre of filtered sea water; Descolas-Gros, pers. comm.), whereas PIS22 and PIS26 which were set at 60 m depth, in the thermocline, and below the fluorescence peak of the mesotrophic site, collected material with lower chlorophyll *a* concentrations (~0.014-0.018 μg $litre^{-1}$). In the latter case, the presence of methanogens appeared not to be correlated with the presence of phytoplanktonic material.

In contrast to the low number of methane-positive natural samples, methane production was recorded in 87% of enrichment cultures performed from these natural samples, suggesting that methanogens were present, but inactive, in almost all particulate materials collected with *in situ* pumps.

Zooplankton organisms

Of twenty-five Hungate tubes containing zooplanktonic organisms, 14 tubes exhibited detectable amounts of methane in their headspace. Six of the eight tubes from the eutrophic site produced methane, only 5 nmol CH_4 per tube for one tube, between 10 and 30 nmol CH4 per tube for 3 tubes, and up to 4-18 μmol CH_4 per tube for 2 tubes containing copepods and gelatinous organisms. Eight of the ten tubes from the mesotrophic site produced methane in small quantities (3 to 9 nmol CH_4 per tube).

Among the 23 enrichments performed, 15 exhibited methane accumulation in their headspace, from 3 to 35 nmol CH_4 per tube for 12 tubes, 130 nmol CH_4 per tube for one tube containing copepods and gelatinous organisms collected at the eutrophic site, and up to 260 and 580 μmol CH_4 per tube for two tubes containing 30 copepods collected at the oligotrophic site, and mixed copepods collected at the eutrophic site, respectively.

Fifty-six per cent of the natural samples of zooplankton, corresponding to the highest percentage reported in this study, and involving either mixed cultures or selected individuals, were methane-producers, suggesting an association between zooplankton and methanogens. All of the four sets of mixed cultures tested produced methane, three of them from natural samples, and the latter only from enrichment culture. Among the 18 sets containing selected individuals, only three did not produce methane from natural samples nor enrichment cultures, but the three natural samples contained few individuals: 4 *Calanus helgolandicus* (copepods), 5 *Euphausia* sp. + 2 *Nematoscelis* sp., and 4 *Sergestes* sp. (decapods), respectively. Conversely, almost all Hungate tubes containing 30 to 32 *Euphausia* or pteropods produced methane, whereas enrichment cultures derived from them are not all methane-producers. The highest methane productions were observed in Hungate tubes containing copepods, either as mixed species or as selected individuals, with methane accumulation 10^3 to 10^6 times greater than in other tubes, up 4-18 μmol CH_4 per tube for two natural samples containing mixted cultures of copepods, and 260 to 580 μmol CH_4 per tube for enrichment cultures.

Cyanobacteria

It is interesting to note that the Hungate tube containing filamentous cyanobacteria collected at the oligotrophic site produced methane, in trace quantities (3 nmol CH_4 per tube), as well as enrichment performed from this sample (9 nmol CH_4 per tube). These results suggest that packets of filamentous blue-green bacteria could retain and provide microenvironments for particulate methanogens floating in the water column.

Fecal pellets

Twenty-five Hungate-tubes were inoculated with fecal pellets freshly egested by zooplankton organisms. Methane production was undetectable in 16 of these tubes; trace quantities of methane (4-6 nmol CH_4 per tube) were produced in six other tubes, whereas 80 to 125 nmol CH_4 accumulated in the headspace of the remaining 3

tubes, which contained fecal pellets egested by organisms collected at the eutrophic site.

All nine enrichment cultures performed produced methane, between 4 to 14 nmol CH_4 per tube for eight tubes, and up to 210 µmol CH_4 per tube for one enrichment containing fecal pellets egested by organisms collected at the oligotrophic site.

Our observations of methane production activity associated with 36 % of the natural samples of freshly egested fecal pellets, and 100 % of their enrichement cultures, demonstrated the presence of methanogens inside fecal pellets produced by zooplanktonic organisms, and reinforced the hypothesis of a relationship between methanogens and zooplankton.

CONCLUSION

Among the 195 cultures performed with particulates and zooplankton collected during the "Eumeli-4" cruise, and containing either natural samples (140 tubes) or enrichment cultures (55 tubes), 40 % were methane producers, whereas control cultures, containing sea water collected at the same sites and depths, never produced methane. These results confirm the presence of methanogens in the globally oxygenated sea water, where, as a consequence of their incapacity to survive as free-living microorganisms, they are associated with particulate materials. Furthermore, our results demonstrate, once again, the inadequacy of Niskin bottles to collect these inhabited particles, which could be only collected with submersible pumps or sedimentation traps.

When the percentages of methane-positive cultures containing natural samples were calculated for each site studied, the highest was obtained from the eutrophic site (43 %), next from the mesotrophic site (26 %), and finally from the oligotrophic site (12 %); in addition, the highest methane productions (>1000 nmol CH_4 per tube) were registered in samples collected at the eutrophic site. In contrast, the percentages of methane-positive enrichment cultures are rather similar for the 3 sites (78 to 87 %).

Only 24 % of the natural samples, containing particles or zooplankton organisms dispensed in unamended sea water, produced detectable amounts of methane, whereas methane production was observed within 82 % of the enrichment cultures performed from these natural samples. Although methanogens seem to be widely distributed within suspended particulates (including dead cells, fecals pellets or zooplanktonic organisms), most of these archaea are inactive under natural conditions. These inactive methanogens were not metabolically limited by nutrient availability, since on the one hand, methane production occurred in unsupplemented tubes and, on the other hand, the addition of a pool of methanogenic precursors in some Hungate tubes did not initiate or stimulate methane production. It should be noted that methanogens were not affected by the low incubation temperature, as increasing temperature until 30 °C neither initiated nor increased methane production, which suggested an adapatation of these marine methanogens to low *in situ* temperatures.

We can hypothesize that in inactive natural samples, methanogens were not dead, but were able to become active only when transferred into enrichment cultures. In addition to an incubation temperature of 30 °C and the presence of methanogenic substrates, although they did not seem to be limiting factors in the field, enrichment cultures are supplemented with reducers, *i.e.* Na_2S and cystein, and growth factors, both of them have a stimulatory effect on methanogenic activity.

Another possible explanation is that methane was not detected in Hungate tubes containing natural samples, because it was immediately oxidized by methane-oxidizing bacteria surviving in the globally anaerobic sea water. However, in the enrichment cultures, where the presence of reducers led to better anaerobic conditions, methane-oxidizing bacteria were killed or inhibited, allowing methane to be produced and to accumulate in the headspace of culture-tubes.

This enrichment culture medium might be used for successive transfers of mixed marine methanogenic cultures, originating from marine sediments, over periods of months or years (Ferrara-Guerrero *et al.*, 1993; Marty, 1995); however, it seemed to be not very convenient for pelagic particulate methanogens. Although 82 % of the enrichment cultures produced methane, it was impossible to maintain these viable methanogens, as all of them failed in the end to develop in successive transfers, after the first- to the fifth-transfer, in the new medium. This 'lack of survival' suggests that these pelagic methanogens need specific growth factors or syntrophic bacteria (aerobic bacteria as suggested by Sieburth and Donaghay, 1993), occurring naturally in the particulates but absent from the culture medium.

An alternative host for pelagic methanogens could be anaerobic protozoa, since it has been demonstrated that protozoa-populated particulates (Caron *et al.*, 1982), on the one hand, and many anaerobic protozoa living in anoxic environments, on the other hand, harbour symbiotic methanogens (Fenchel and Finlay, 1992). However, anaerobic protozoa have so far only been evidenced in globally anaerobic marine biotopes, such as sapropel, anoxic water or sediment (Coleman, 1991), and no data are available about their occurrence and role in sinking particulates.

Our experiments, designed to test the presence of viable and active methanogens in various particulate materials, did not provide information on the rate of *in situ* methane production. However, the observations were important as they confirmed the hypothesis of Oremland (1979) and Traganza *et al.* (1979), that the paradoxical methane supersaturation in the upper water column could be due to active methanogens associated with particulate material, essentially originating from zooplankton. Release of methane by zooplankton, presumably from gut microflora, has been evidenced with marine (Fuhrman and Capone, 1991), and freshwater animals (Schmidt and Conrad, 1993), suggesting that the intestinal tracts of zooplankton provide an anaerobic an environment that allows methanogens to develop. Our results are, moreover, consistent with previous observations (Gowing and Silver, 1983; Marty,

1993), and confirm the presence of viable methanoarchaea within zooplankton organisms, probably in the form of enteric flora in the digestive tract (De Angelis and Lee, 1994). Subsequent release of these enteric methanogens in freshly egested fecal pellets provides the condition for the presence of these strictly anaerobic organisms in the oxygenated water column.

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