Geomicrobiology Journal
January 2009 ; Volume 26, Issue 1 : Pages 31-43
http://dx.doi.org/10.1080/01490450802599284

The original publication is available at http://www.tandf.co.uk/journals/

Archaeal Methane Cycling Communities Associated with Gassy Subsurface Sediments of Marennes-Oléron Bay (France)

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Abstract:

In Marennes-Oleacuteron Bay, a macro-tidal bay located on the French Atlantic coast, kilometer-scale acoustic turbidity reveals an accumulation of free gas in the sediment. Large concentrations of organic matter and rapid sedimentation rates provide ideal settings for biogenic methane cycling. We integrate seismic, sedimentologic, biogeochemical and molecular genetic approaches to determine whether microbial methane cycling is involved in this process. Here we show that the acoustic turbidity upper boundary matched with X-ray facies displaying fissures with the highest methane concentrations, demonstrating the existence of methane bubbles in the sediment. 16S rRNA and mcrA gene clone libraries were dominated by sequences affiliated to the three known ANME lineages and to putative methanogens. Sequences related to the marine benthic group B (MBG-B) and miscellaneous crenarchaeotal group (MCG) were also detected. However, the highest methane concentration facies was the only section where active Archaea were detected, using reverse-transcribed rRNA, indicating that these communities were involved either directly or indirectly in the methane cycling process. Moreover, three metabolically active novel uncultivated lineages, related to putative methane cycling Archaea, could be specifically associated to these methane bearing sediments. As methane cycling Archaea are commonly retrieved from deep subseafloor and methane seep sediment, the study of coastal gassy sediments, could therefore help to define the biogeochemical habitats of deep biosphere communities.

Keywords: Archaea; 16S rRNA; mcrA; methane; sediment

Introduction

Over 85 % of the annual emissions of methane are biogenically produced by methanogenesis in various environments (Reeburgh, 2007; Valentine et al., 2004), such as marine sediments which contain the largest global reservoir of methane (Kvenvolden, 1988; Hovland et al., 1993). Deltas and estuaries commonly constitute appropriate settings for methane production due to the rapid accumulation of sediments with high organic matter concentrations (Fleischer et al., 2001). Recent studies on Marennes-Oléron Bay, a macro-tidal bay located on the French Atlantic coast, have revealed a kilometer-scale acoustic turbidity (Bertin and Chaumillon, 2005). The acoustic turbidity, interpreted as an accumulation of free gas in the sediment, is correlated with a high sediment gain area in excess of 3 m since 1824 and could be related to anthropic processes (Bertin and Chaumillon, 2005; Bertin et al., 2005). The lack of thermogenic activity in the bay, and the high primary production could indicate the generation of biogenic methane on decade to century time-scales (Bertin and Chaumillon, 2005). The present study aims at determining whether the acoustic turbidity in an estuarine environment associated with high sedimentation rate is a consequence of methane accumulation and whether active methane cycling microbial communities are involved in this process. Methanogenesis is mediated by methanogenic Archaea and only occurs in anoxic environments

(Wolfe, 1971). In all known methanogens, the methyl-coenzyme M reductase (MCR) enters the final step in methane synthesis (Ellermann *et al.*, 1988). Therefore, the *mcrA* gene can be used to target methanogens by using molecular techniques (Springer *et al.*, 1995; Luton *et al.*, 2002). However, over 80 % of the biogenic methane that rises from anoxic marine sediment is consumed by anaerobic methane oxidization (AOM) (Orphan *et al.*, 2001*a*), therefore representing 5 to 20 % of the net modern atmospheric methane flux (Valentine and Reeburgh, 2000). Molecular techniques have recently shown that AOM is mediated by anaerobic methane oxidizers (ANME) (Boetius *et al.*, 2000; Hinrichs *et al.*, 1999). Three archaeal clades (ANME-1, ANME-2, ANME-3) oxidize methane under anaerobic conditions either as single cells or associated to sulfate-reducing *Bacteria* (SRB) (Boetius *et al.*, 2000; Niemann *et al.*, 2006; Orphan *et al.*, 2002). ANME could reverse methanogenesis using a modified MCR (Hallam *et al.*, 2003; Hallam *et al.*, 2004). Although, none of these groups have been obtained in pure culture, a few studies have attempted to investigate the effect of environmental factors on AOM activities of different ANME communities (Nauhaus *et al.*, 2007; Nauhaus *et al.*, 2002*b*; Valentine and Reeburgh, 2000).

It is therefore crucial to target the metabolically active communities in order to correlate their phylogeny with variable environmental factors (Biddle *et al.*, 2006; Sorensen and Teske, 2006; Lloyd *et al.*, 2006; Treude *et al.*, 2005*a*). However, DNA-based molecular approaches do not distinguish between living and dead microorganisms (Damste and Coolen, 2006; Dell'Anno and Corinaldesi, 2004). Alternatively, fluorescent in situ hybridization has commonly been used to identify metabolically active microorganisms, but this approach does not provide an overall picture of the active microbial communities. In order to target the active cells from subsurface sediments (Inagaki *et al.*, 2005), extractable archaeal rRNA can be used, as rRNA has a rapid turnover rate (Danovaro *et al.*, 1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp *et al.*, 1993). Only a few studies have used rRNA-derived clone libraries to investigate the diversity of active methane cycling communities in subsurface sediments (Biddle *et al.*, 2006; Sorensen and Teske, 2006). Moreover, few studies, to date, have found microbial methane cycling communities in non seep or gas hydrate coastal sediments (Parkes *et al.*, 2007; Treude *et al.*, 2005*b*; Thomsen *et al.*, 2001). Hence, in this study we conducted an integrated seismic, sedimentologic, biogeochemical and molecular genetic survey of the subsurface gassy sediments of the Marennes-Oléron Bay.

Materiel and methods

Site description and sampling

The Marennes-Oléron Bay is located on the French Atlantic coast, immediately north of the Gironde Estuary. This bay is a 150 km^2 wide semi-enclosed environment connected to the Atlantic Ocean through the Pertuis d'Antioche to the North and to the Maumusson inlet to the South. Intertidal areas represent 60 % of the bay. Two small rivers, the Charente and the

Seudre, flow into the Marennes-Oléron Bay. Tides affecting the study area are semi-diurnal and range from less than 2 m to more than 6 m. Swells are strongly attenuated by the narrow entrances of the bay, but wind-waves can produce high turbidity in the water column due to wind-driven resuspension.
The Marennes-Oléron Bay, the first oyster farming area in Europe, is an area of high primary production (466 $g.m^{-2}.yr^{-1}$) (Bertin and Chaumillon, 2005). Most of the suspended sediments supplied to the bay are derived from the Gironde Estuary (Froidefond *et al.*, 1998). The sediment budget, computed by subtracting the 1824 profile from the 2004 bathymetric digital elevation model, shows a volume difference of $+120.10^6$ m^3 for the last 181 years (Bertin and Chaumillon, 2006). This positive sediment budget shows high sedimentation rates, in agreement with 210 Pb measurement results made on the eastern mudflat of the bay (Gouleau *et al.*, 2000). Very high resolution seismic profiles were recorded in the central Marennes-Oléron Bay during the MOBIDYC5 cruise (2006, CNRS-INSU on board the RV Côte de la Manche). Two cores, M5VC42 and M5VC43 (45°57'N, 01°10'W) were collected on the seismic profile m4b31 within the acoustic turbidity area (Figure 2.3a). M5VC42 was used for sedimentary analysis and M5VC43 for biogeochemical and molecular genetic approaches.

Sedimentary analysis

The 4 m long M5VC42 core was cut into 1.5 m slabs that were opened transversely, directly photographed, and described according to the Munsell colour chart. Slabs were X-radiographed using the Scopix system (Migeon *et al.*, 1999). Subsamples for grain-size analyses were performed only where X-ray images showed noticeable changes. Grain-size analyses were carried out using a Malvern Mastersizer diffractometer. Correlation between sedimentologic and seismic data was done on the basis of a depth-to-time conversion of the core datasets, according to the relationships between P-wave velocities and sediment grain size (Hamilton, 1972).

Geochemical analysis

Methane analyses were performed on core M5VC43 at 30, 60, 90, 120, 150, 180, 210, 240 and 300 centimeters below the seafloor (cmbsf) using the headspace technique (Kolb, 1999). The cores were immediately sub-sampled on board using 5 mL syringes (luer end removed) and added to headspace vials (20 mL) filled with a $NaCl/HgCl_2$ work solution. Methane concentrations were determined using a HP 7694 automatic headspace sampler connected to a HP 5890 gas chromatograph equipped with FID and TCD detectors. The 2-sigma uncertainty was better than 4 %. However, methane concentration measurements have to be considered as minimum values as gas escapes occur during core handling. Results were expressed as microlitre per litre wet sediment (μL/L wet sediment).
Carbon isotope ratio analyses were carried out on sediment samples collected at different levels: 30, 60, 90, 120, 150, 180, 210, 240 and 300 cmbsf in M5VC42. Inorganic carbon was removed

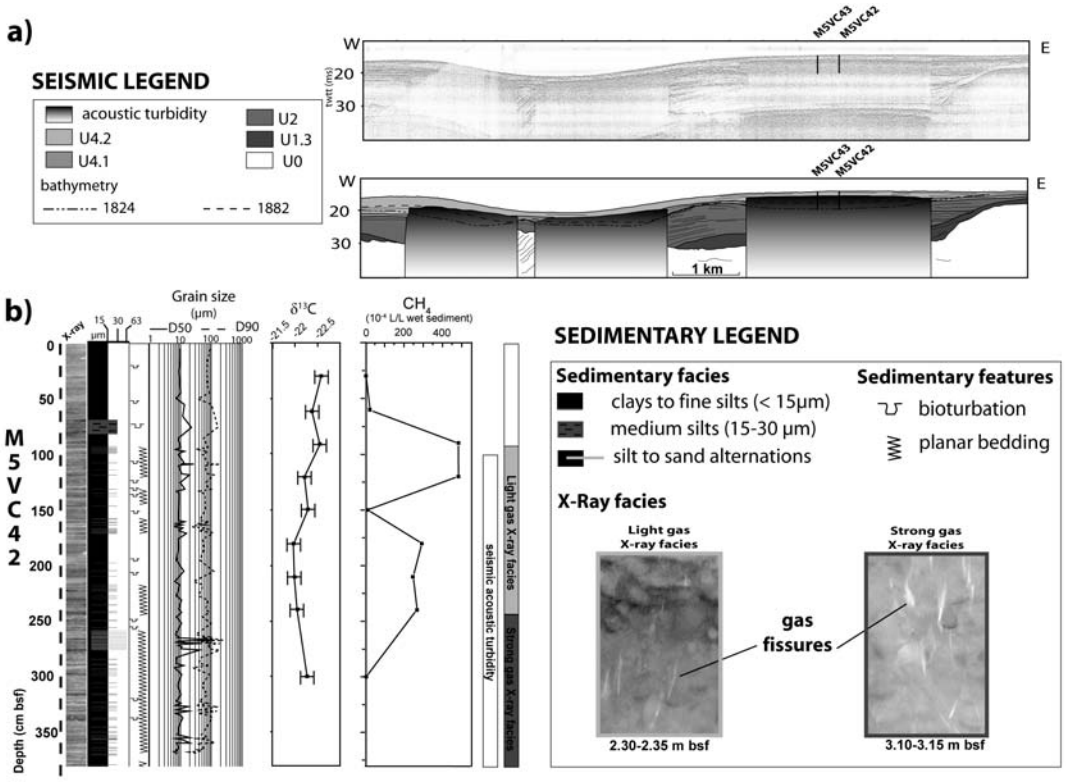

FIG. 2.3: (a) VHR seismic profile m4b31 and its interpretation showing the geometry of the sediment infilling of the northern Marennes-Oléron Bay. Locations of the cores VC42 and VC43 are indicated. Seismic unit U0 corresponds to the bedrock, and seismic units U1.3, U2, U4.1 and U4.2 correspond to the soft sediment incised valley-fill. Locations of the acoustic turbidity areas are indicated. (b) Detailed description of the core VC42. From left to right: elevation in meters (NGF), X-ray image, sedimentary facies, sedimentary features, median grain-size in micrometers, variations in δ^{13}C and methane (CH_4) concentration. Correlation with acoustic turbidity and X-ray facies are also indicated.

by acidification (HCl 2M ; 1 mL/100 mg DW). To evaporate excess acid, samples were dried overnight at 60°C under a hood. They were then mixed to 1 mL MilliQ water, freeze-dried and finely grounded using a ball mill. Analyses were performed by CF-IRMS using an Isoprime mass spectrometer (Micromass, UK). Results were reported in the standard δ^{13}C notation relative to the Vienna PeeDee Belemnite standard (VPDB) where $\delta^{13}C = [(R_{sample}/R_{PDB})\text{-}1] \times 10^3$, with $R = {}^{13}C/{}^{12}C$. Sample analytical precision was ±0.1 ‰.

DNA extractions and PCR amplification

DNA was extracted from 5×1 g of uncontaminated frozen sample, core M5VC43 at 0, 60, 90, 125, 185 and 305 cmbsf, following a modified FastDNA® Spin Kit for Soil (Bio101 Systems, MP Biomedicals™) protocol (Webster *et al.*, 2003). Replicate extractions were pooled, purified

and concentrated in an YM-100 Microcon (Amicon/Millipore™) centrifugal device to give a final volume of 100 μL.
For the amplification, all manipulations were carried out in a PCR cabinet, using Rnase/Dnase Free Water (MP Biomedicals™) and performed using a GeneAmp PCR system 9700® (Applied Biosystems™). All PCR mixtures (50 μL) contained 5 μL of DNA template, 1X Taq DNA polymerase buffer (MP Biomedicals™), 1 μL of dNTP (10 mM of each dATP, dCTP, dGTP and dTTP), 10 μM of each primer and 0.5 μL of Taq DNA polymerase (MP Biomedicals™). Negative controls were also carried out with DNA extractions performed with no sample. For all negative controls, no PCR products were observed. Inhibition of PCR amplification by soluble contaminants in the DNA extracts was also tested as described elsewhere (Juniper *et al.*, 2001). For all DNA extracts, no PCR inhibition was observed.
Archaeal 16S rRNA gene amplification was conducted by nested PCR with combination of primers A8f (5'-CGG TTG ATC CTG CCG GA-3') and A1492r (5'-GGC TAC CTT GTT ACG ACT T-3') in the first round (Casamayor *et al.*, 2000), with A344f (5'-AYG GGG YGC ASC AGG SG-3') and A915r (5'-GTG CTC CCC CGC CAA TTC CT-3') in the second round (DeLong, 1992; Eden *et al.*, 1991). PCR cycles for the first round (A8f/A1492r) were as follows: 1 cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at 51°C and 2 min at 72°C and 1 cycle of 6 min at 72°C. PCR cycles for the second round (A344f/A915r) were: 1 cycle of 3 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at 57°C and 2 min at 72°C, and 1 cycle of 5 min at 72°C. Five independent PCR products from the first round were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and used as template for the second round. This nested PCR was necessary to obtain visible PCR products on a 0.8 % (w/v) agarose gel stained with ethidium bromide. A portion of the *mcrA* gene was amplified using the ME primers (Hales *et al.*, 1996). To obtain visible amplification products, two rounds with the following reaction conditions were performed: 1 cycle of 5 min at 94°C, 30 cycles of 45 s at 94°C, 1.5 min at 50°C and 2 min at 72°C, and 1 cycle of 10 min at 72°C. An aliquot (5 μL) of 3 pooled PCR products of the primary amplification was used as templates for the second amplification round.

RNA extractions and RT-PCR amplification

Total RNA was extracted from each uncontaminated frozen sample (5×1 g) of the M5VC43 core (0, 60, 90, 125, 185 and 305 cmbsf) using the FastRNA® Pro soil direct Kit (Bio101 Systems, MP Biomedicals™) following the manufacturer's instructions. To digest trace amounts of DNA, the extraction products were pooled and 150 μL were incubated 1 hour at 37°C with 1X of TURBO DNase® buffer and 18U of TURBO DNase® (Ambion™). The digestion was stopped by adding EDTA to a final concentration of 15 mM and heating 10 min at 65°C. The product was finally concentrated and purified with the RNeasy minikit (Qiagen™) according to the manufacturer's instructions to give a final volume of 100 μL.
The purified RNA product was immediately serially diluted (1 to 50 times) and reverse transcribed using the OneStep RT-PCR kit (Qiagen™), according to the manufacturer's instructions,

with combination of 16S rRNA primers for *Archaea* with A8f (5'-CGG TTG ATC CTG CCG GA-3') and A1492r (5'-GGC TAC CTT GTT ACG ACT T-3') and the following touchdown PCR protocol: 30 min at 50°C, 15 min at 95°C followed by 20 cycles of 1 min at 94°C, 1 min at 58°C (decreasing 0.5°C every cycle), 4 min at 72°C, then followed by 20 cycles of 1 min at 94°C, 1 min at 51°C, 4 min at 72°C and a final amplification step of 10 min at 72°C. To obtain visible products, a nested PCR was performed as described for the 16S rRNA gene amplification. Nested PCR assays, using the 16S rRNA primers for *Archaea*, without the reverse transcription step, showed no DNA contamination.

PCR-DGGE analysis

In order to characterize the general archaeal community depth structure, a 16S rRNA gene PCR-DGGE analysis was undertaken. Nested PCR was performed as described for the archaeal 16S rRNA gene amplification using primers Saf and PARCH 519R as previously described (Nicol *et al.*, 2003). PCR products were analyzed by DGGE using a DCode Universal Mutation Detection System® (BioRad™) on a 1 mm thick (16×16 cm) 8 % (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 40 %, 37, 5:1, BioRad™). The gel had a denaturant gradient between 30 and 60 % and was prepared with 1X TAE buffer (pH 8, 40 mM Tris Base, 20 mM acetic acid, 1 mM EDTA, MP Biomedicals™) and poured "Gradient maker" (Hoefer SG30®). Electrophoresis was carried in 1X TAE buffer at 60°C for 330 min at 200 V (initially at 80 V for 10 min). The gel was stained with SYBRGold® nucleic acid gel stain for 45 min, washed for 10 min with 1X TAE buffer and scanned using a Phospho fluorimager Typhoon 9400® (Amersham Biosciences™).

Cloning and sequencing

According to archaeal DGGE profiles, clone libraries were constructed: a) three DNA-derived 16SrRNA gene (DNA65, DNA125 and DNA185), b) two RNA-derived 16S rRNA gene (RNA95 and RNA125), and c) six DNA-derived *mcrA* gene (mcr0, mcr60, mcr90, mcr125, mcr185 and mcr305). The number indicates the depth of each sample (cmbsf). To minimize PCR bias (Polz and Cavanaugh, 1998), five independent PCR products were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and cloned into *Escherichia coli* (XL10-Gold®; Stratagene™) using the pGEM-T Easy vector system I (Promega™) following the manufacturer's instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Plasmid extraction, purification and sequencing of the insert, were carried out by the sequencing OUEST-Genopole platform® of Roscoff Marine laboratory (France).

Phylogenetic analysis

All the 16S rRNA base pair sequences (∼553 bases) were checked for chimeras, using the CHIMERA-CHECK version 2.7 algorithm from the RDP-II (Cole *et al.*, 2003). Out of a

total of 376 sequences (including those from the 16S rRNA gene and *mcrA* gene), 2 were found to be chimeras and were excluded from the phylogenetic analyses. The phylogenetic placement was carried out using NCBI BLAST search program within GenBank (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.*, 1990). The 16S rRNA sequences were then edited in the BioEdit 7.0.5.3 program (Hall, 1999) and aligned using CLUSTALW (Thompson *et al.*, 1994). The phylogenetic trees were constructed by the PHYLO-WIN program (http://pbil.univ-lyon1.fr/) (Galtier *et al.*, 1996) with neighbour-joining method (Saitou and Nei, 1987) and Jukes and Cantor correction. The nonchimeric *mcrA* sequences (∼770 bases) were translated into amino acids using BioEdit and then aligned using CLUSTALW. The PHYLO-WIN program with neighbor-joining algorithm and PAM distance (Dayhoff *et al.*, 1978) was then used for phylogenetic tree construction. For the 16S rRNA and *mcrA* gene phylogenetic reconstruction, the robustness of inferred topology was tested by bootstrap resampling (500), values over 50 % are shown on the trees. The richness from the clone libraries was estimated, with the rarefaction curves at 99 %, 97 % and 95 % sequence identity levels, using the DOTUR program (Schloss and Handelsman, 2005). Operational taxonomic units (OTUs), using a 97 % sequence similarity, were generated with the SON program (Schloss and Handelsman, 2006) and the percentage of coverage (Cx) of the clone libraries was calculated by Good's method (Good, 1953) as described by Singleton and colleagues (Singleton *et al.*, 2001). Statistical estimators, the significance of population differentiation among clone libraries (F_{ST}) (Martin, 2002), and the exact tests of population genetic differentiation (Raymond and Rousset, 1995), were calculated using Arlequin 3.11 (Excoffier *et al.*, 2005).

Nucleotide sequence accession numbers

The sequences are available from GenBank database under the following accession numbers: 16S rRNA gene and rRNA (AM942119 to AM942178) and *mcrA* gene (AM942079 to AM942118).

Results and discussion

High methane concentration in the gassy sediments

X-ray images revealed that physical structures were dominated by millimeter to centimeter-thick planar bedding corresponding to strong and low X-ray attenuations. X-ray also showed that sediment was slightly bioturbated (Figure 2.3b). Three major X-ray facies were identified. From the top of the core to 95 cmbsf, F1 X-ray facies consisted of poorly laminated sediments. From 95 to 245 cmbsf, F2 X-ray facies consisted of laminated sediments showing sparse sub-vertical and elongated fissures (about 1 cm long and less than 1 mm wide). The transition from F1 to F2 X-ray facies was close to the acoustic turbidity upper boundary observed on m4b31 seismic profile at about 100 cmbsf (Figure 2.3). From 245 to 380 cmbsf, F3 X-ray facies consisted of laminated sediments showing several sub-vertical and elongated fissures (about 1

cm long and more than 1 mm wide). No such fissures were observed in cores collected outside acoustic turbidity areas (Billeaud *et al.*, 2005). Grain size analyses showed that sediments were mainly composed of black clay to fine silts (< 15 μm) alternating with dark greenish (5Y 4/1) coarse silt (30 to 63 μm) and sparse very fine sand (63 to 125 μm) layers. These grain-size alternation layers were often correlated with X-ray attenuation. However the transitions between F1-F2 and F2-F3 X-ray facies did not match with any grain size change.
The acoustic turbidity layer (Figure 2.3a), evidenced by seismic profiles, was correlated with the highest sedimentation rates of the bay (Bertin and Chaumillon, 2005). Moreover, the acoustic turbidity upper boundary matched with the top of F2 X-ray facies displaying fissures and with the highest methane concentrations (Figure 2.3 and 2.4). In contrast, other studies have found the acoustic turbidity upper boundary below the methane peak concentration (Abegg and Anderson, 1997; Thiessen *et al.*, 2006). Hence, both acoustic turbidity and elongated fissures demonstrated the existence of methane bubbles in the sediment (Abegg and Anderson, 1997). The occurrence of gas bubbles suggests that the sediment pore-fluids were saturated with methane. In oversaturated cores, it is well-known that most (up to 95 %) of the methane escapes during core recovery due to the significant decrease of methane solubility related to pressure and degassing (Hensen *et al.*, 2007). This gas loss during ex situ sampling could explain the variation in methane concentration below 120 cmbsf because the bubble repartition and the degassing were probably not homogeneous in the sediment core.

However, the general methane concentration profile in sediment pore-fluids (Figure 2.4b) was similar to the ones found in coastal marine sediments or tidal flats (Parkes *et al.*, 2007; Wilms *et al.*, 2007; Thomsen *et al.*, 2001). At 30 cmbsf, the CH_4 concentration was relatively low ($\sim$4 μL/L wet sediment), probably reflecting the regional background. At 60 cmbsf the CH_4 concentration increased with depth, reaching a maximum peak value of 483 μL/L wet sediment between 90 and 120 cmbsf, which matched the acoustic turbidity upper boundary, and which generally corresponds to the sulfate methane transition zone (SMTZ) (Iversen and Jorgensen, 1985; Thomsen *et al.*, 2001). However, this hypothesis would need to be confirmed by sulfate analyses.
Values of δ^{13}C (-22.0 and -22.6 ‰) were intermediate between values in shallower depths (15-30 cmbsf) of the intertidal mudflats lining the bay (Figure 2.3b): -20.2 ‰ on the western side (Oléron Island), -23.0 ‰on the continental side. Inputs from the continental side thus seem to have more influence on the organic matter origin in the studied cores, assuming negligible changes occurred during diagenesis (Sackett, 1964). On the continental side of the bay, organic matter was mainly of microalgal origin in the surficial layers and consisted of a mixture of sedimented estuarine phytoplankton (-22.7 ‰), of a larger fraction of microphytobenthos (-16 ‰), and of ^{13}C-depleted terrestrial material (-28 ‰) (Riera and Richard, 1996), while in deeper layers this later became more abundant. A slight and irregular decrease in the organic carbon content was observed with increasing depth. In average 0.83 % C was measured

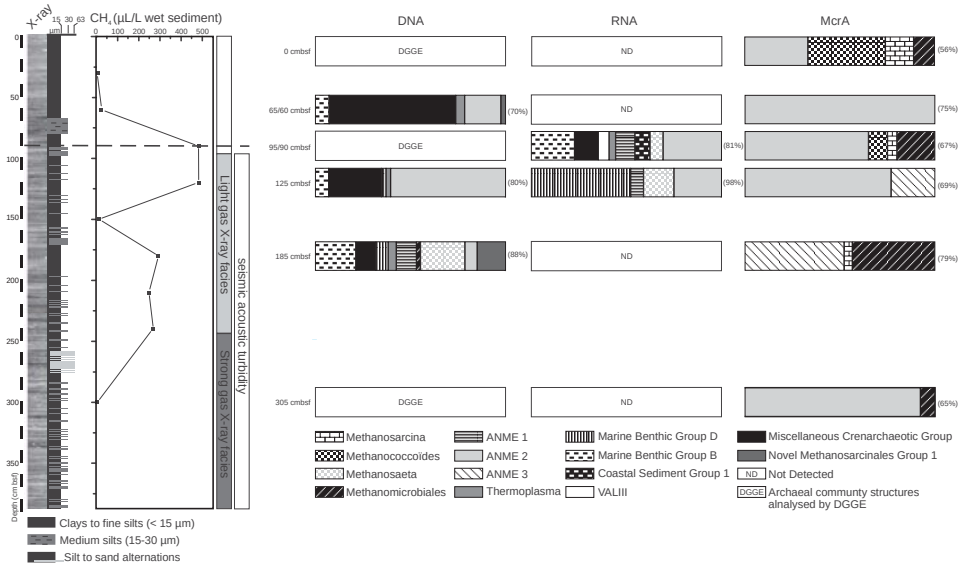

FIG. 2.4: Depth distribution of the archaeal phylogenetic community structures based on 16S rRNA, crRNA and *mcrA* gene compared to X-ray image, sedimentary facies and methane (CH_4) concentration. The percentage of coverage of each clone library examined is indicated in brackets. The phylogenetic affiliation of each clone sequence was determined by similarity analysis. The relative abundance of each phylotypes in the clone library was calculated and represented in a column diagram. DNA designs the DNA-derived libraries; RNA, the RNA-libraries; McrA, the *mcrA* gene libraries.

(dry sediment collected in M5VC42 core) which was about twice the 0.46 % C detected in a core collected outside the acoustic turbidity. These relatively large amounts of organic matter combined with the highest sedimentation rates of the bay (Bertin and Chaumillon, 2005) could create ideal settings for active methanogenic microbial communities producing high amounts of biogenic methane (Valentine, 2002).

Archaeal diversity depth distribution

Analysis of the clone libraries. Eleven different clone libraries were constructed, representing a total of 376 sequences. All the DNA-derived 16S rRNA PCR products from all depths were screened by DGGE prior cloning, in order to select the most representative phylogenetic diversities of the core (Webster *et al.*, 2003). Hence, three depths were chosen to construct DNA-derived clone libraries. The whole 16S rRNA sequences, derived from RNA and DNA, were assigned to 48 OTUs based on a 95 % genus level of phylotype differentiation (Schloss and Handelsman, 2004). After technical optimisation, archaeal amplifiable DNA was only retrieved by nested PCR from all depths and 17, 11 and 14 OTUs were respectively assigned to DNA65, DNA125 and DNA185 clone libraries. Amplifiable RNA was detected only from 95 cmbsf and 125 cmbsf depths, which are correlated with the highest methane concentrations. The

two RNA-derived clone libraries from these depths were respectively assigned with 27 OTUs and 5 OTUs. The coverage values for the 16S rRNA clone libraries ranged from 70 to 98 % (Figure 2.4). Rarefaction curves were strongly curvilinear for all the libraries attesting for a sufficient sampling effort (data not shown).
The molecular techniques (PCR and cloning), used to build clone libraries, are known to be inherently biased (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). However, the ratios between the 16S rRNA gene and rRNA per cell have been reported to be proportional to the metabolic activity of the cells, the rRNA content per cell increasing with metabolic activity (Danovaro *et al.*, 1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp *et al.*, 1993). As ANME-1 and *Methanosaeta* lineages were detected in RNA125 library, but absent in DNA125 library, we hypothesized that the sediments at 125 cmbsf could harbor low cell concentrations of very active ANME-1 and *Methanosaeta* lineages. Moreover, insignificant F_{ST} and P tests ($P < 0.01$) suggested that the sequences from the DNA185 library, compared to the RNA95 and RNA125 libraries, were from similar lineage distributions and were indistinguishable from the combined communities (Martin, 2002). DNA derived libraries in marine sediments, built from short 16S rRNA fragments, could result partially from the DNA amplification of dead, quiescent cells (Dell'Anno and Danovaro, 2005; Corinaldesi *et al.*, 2005). For this reason, we suggest that the DNA derived from the different lineages detected at depth was also the result of the accumulation, during the sedimentation process, of the undegraded DNA from dead, quiescent and active communities from the shallower depths. Hence, the ANME-1 and *Methanosaeata* lineages detected in the DNA185 library could be the consequence of an accumulation of DNA from dead or quiescent cells from the shallowest depths (95-125 cmbsf) where they were found active.
The 16S rRNA clone libraries were, on the whole, dominated by sequences related to *Euryarchaeota* (65 %), except for DNA65 where *Crenarchaeota* were a majority (74 %; Figure 2.4). The archaeal phylogenetic diversity was quite high, forming a total of 11 different lineages: ANME-1, ANME-2, *Methanosaeta, Methanomicrobiales, Thermoplasmales*, Marine Benthic Group D (MBG-D), Miscellaneous Crenarchaeotal Group (MCG), Marine Benthic Group B (MBG-B), and 3 novel lineages (Figure 2.5 and 2.6). Unexpectedly, no sequences related to the Marine Group 1 (MG-1) *Archaea*, an ubiquitous *Archaea* in marine sea beds were detected. Furthermore, the general phylogenetic diversity of the shallow gassy sediments of Marennes-Oléron Bay was similar to the one retrieved from methane bearing environments such as cold seeps and gas hydrates (Kendall *et al.*, 2007; Knittel *et al.*, 2005), mainly characterized by a large proportion of methanogens and methane oxidizers. A majority of sequences were related to uncultured environmental clones from these environments (highest similarity to pure culture, 98 %).

Archaeal methane oxidizing communities. ANME *Archaea* mediate AOM (Boetius *et al.*, 2000; Hinrichs *et al.*, 1999), and dominated the 16S rRNA (34 %) and the *mcrA* libraries (74 %)

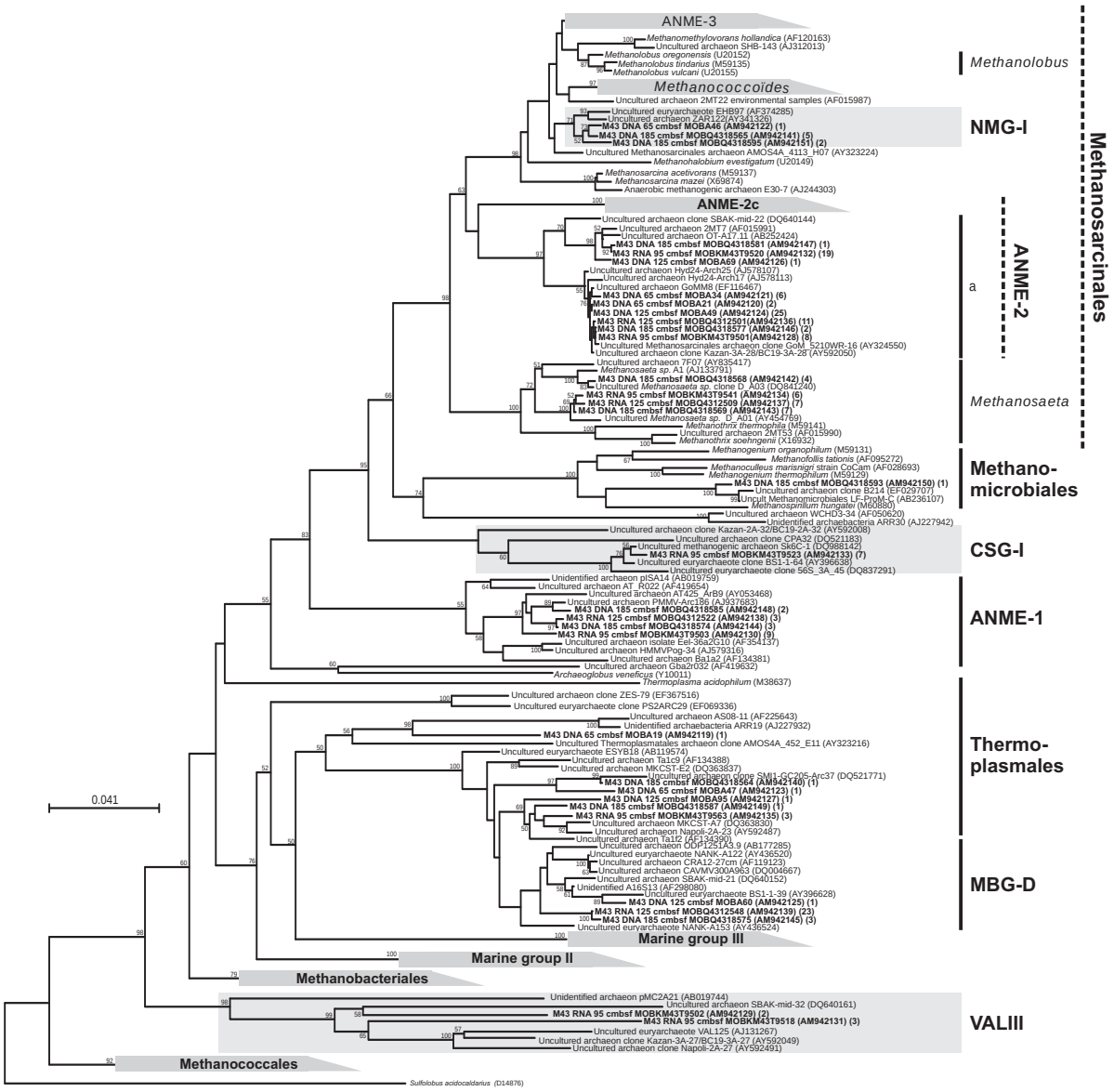

FIG. 2.5: Phylogenetic tree representing the *Euryarchaeota* 16S rRNA gene sequences DNA- and RNA derived, designed by DNA and RNA respectively. Each phylotype is represented by one sequence with > 97 % similarity grouping. The tree was constructed using the neighbour-joining method with Jukes and Cantor correction. Bootstrap values < 50 % are not shown. CSG-1: Coastal Sediment Group 1, MBG-D: Marine Benthic Group D, NMG-1: Novel Methanosarcinales group 1, VALIII: VAL III cluster (Jurgens *et al.*, 2000).

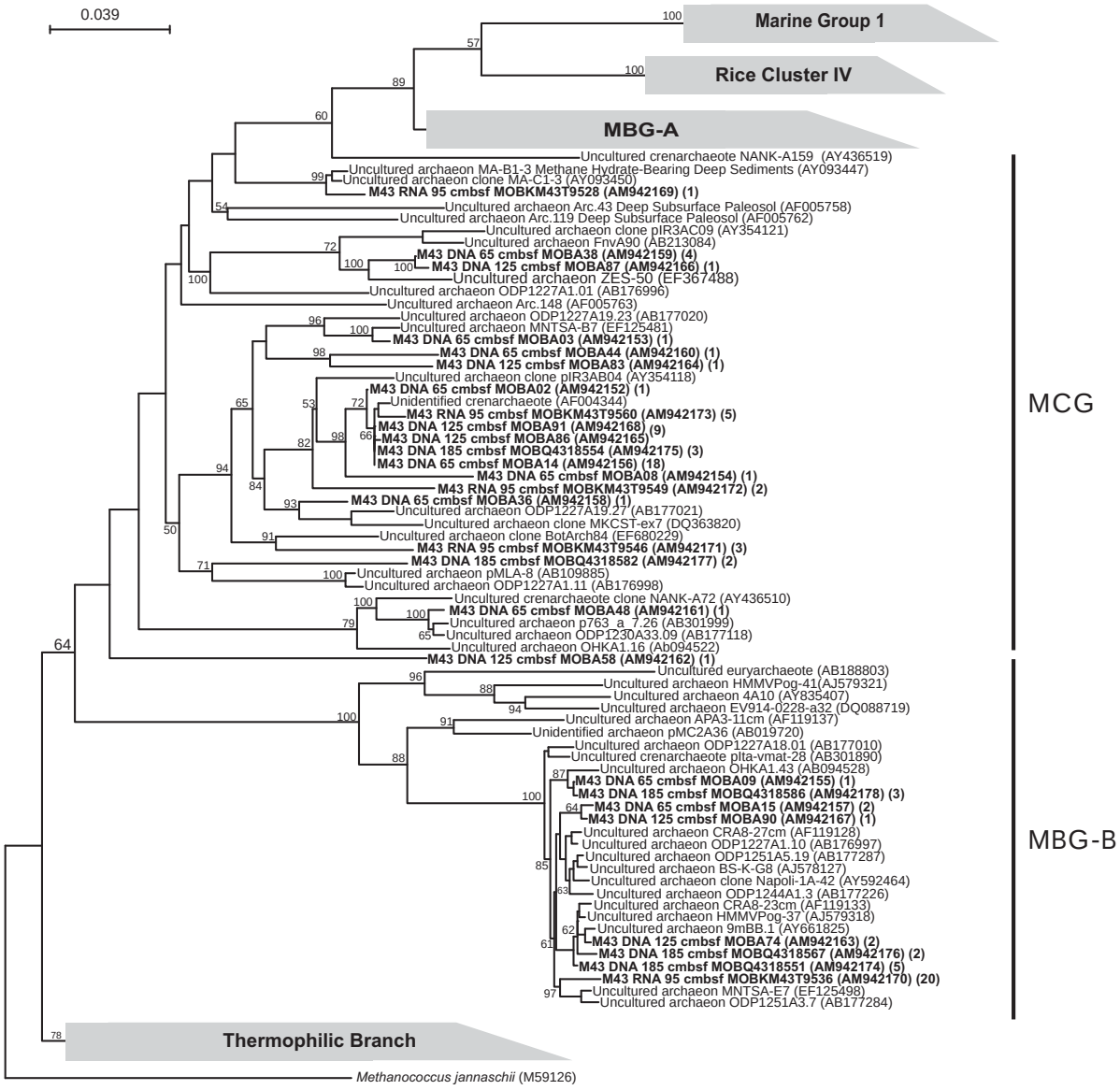

FIG. 2.6: Phylogenetic tree representing the *Crenarchaeota* 16S rRNA gene sequences DNA- and RNA derived, designed by DNA and RNA respectively. Each phylotype is represented by one sequence with > 97 % similarity grouping. The tree was constructed using the neighbour-joining method with Jukes and Cantor correction. Bootstrap values < 50 % are not shown. MCG: Miscellaneous Crenarchaeotal Group, MBG-B: marine benthic group B, MBG-A: Marine Benthic Group A.

(Figure 2.4). Sequences related to ANME-2a, representing 28 % of the clones in the libraries, were most abundant in the DNA125 (61 %). These sequences were shown to form 2 clusters (Figure 2.5); the first cluster is affiliated to sequences from cold seep environments (Heijs *et al.*, 2007). The second cluster is related to sequences from salt marshes (unpublished) and CO_2-hydrate-bearing sediments (Inagaki *et al.*, 2006*a*). Moreover, the 6 *mcrA* libraries (n = 112, coverage = 69 %) were also dominated by sequences related to ANME-2a (61 %), except at 185 cmbsf where they were replaced by ANME-3 (52 %) (Figure 2.4). The sequences related to ANME-2 formed also two clusters within the McrA group e (Figure 2.7). However, ANME-2a sequences from DNA-derived libraries were also present at all depths, whereas ANME-1 were detected only in the deeper parts of the core, (below 95 cmbsf) as previously described in other environments (Knittel *et al.*, 2005; Nunoura *et al.*, 2006). The depth-dependant distribution of ANME-1 tends to support the hypothesis that they are more sensitive to oxygen and to lower temperatures than AMNE 2 (Knittel *et al.*, 2005; Nauhaus *et al.*, 2005). Active ANME-1 were also detected at the highest methane concentrations suggesting that ANME-1 are present at high methane flow rates (Girguis *et al.*, 2005)(Figure 2.4). Moreover, Orcutt and colleagues have recently proposed that ANME-1 could also be involved in methanogenesis depending on the environmental conditions (Orcutt *et al.*, 2005). Although the operon coding for the MCR-I, which includes McrA subunit, is found in all known methanogens (Reeve *et al.*, 1997), ANME-1 and ANME-3 were detected only by one type of primer couple (Figure 2.4), whereas ANME-2a were found among the *mcrA* and 16S rRNA libraries. The absence of *mcrA* sequences related to ANME-1, detected at the same depth 16S rRNA clone libraries, could be the consequence of the lower percentages of coverage of the mcrA libraries or due to a higher number of copies per genome of 16S rRNA gene than *mcrA* (Nunoura *et al.*, 2006).
Moreover, RNA-derived libraries (RNA95 and RNA125) were also dominated by sequences related to these ANME lineages (36 %; Figure 2.4), suggesting that these *Archaea* were also metabolically active. These *Archaea* were detected between 95 and 125 cmbsf, where the methane concentration was the highest. The archaeal communities associated with the other depths were either below the detection limit or less metabolically active. Therefore, AOM could represent one of the major archaeal microbial activities in these sediments.

Archaeal methanogenic communities. Sequences affiliated to archaeal methanogenic lineages represented an important component of the total libraries (26 %) and were composed of *Methanococcoïdes, Methanosarcina, Methanosaeta* and *Methanomicrobiales* (Figure 2.4). However, putative methanogens represented less than 10 % of the 16S rRNA libraries and were found only below the highest methane concentration zone (95 cmbsf). A large proportion of these sequences (9 %) were related to the *Methanosaeta* lineage. Only one sequence is related to the *Methanomicrobiales* lineage. In *mcrA* libraries, the depth distribution of the methylotrophic *Methanococcoïdes* and *Methanosarcina* decreased with depth and seems to be therefore restricted to the shallowest part of the core. The methanogens utilizing C1 compounds usually

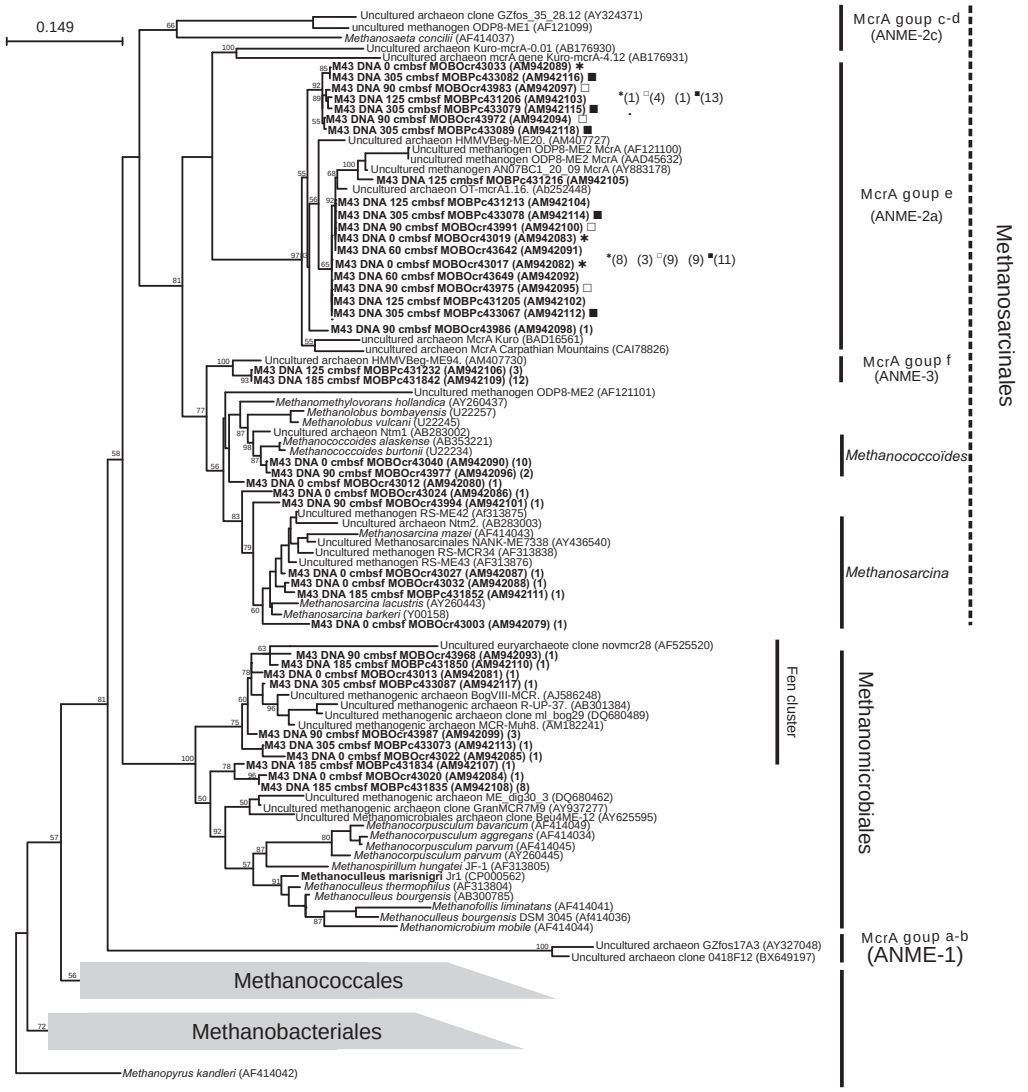

FIG. 2.7: Phylogenetic tree based on translated, partial amino acid sequences of *mcrA* gene ($<$ 260 amino acids). The tree was constructed using the neighbour-joining method using PAM distance (Dayhoff *et al.*, 1978). The robustness of inferred topology was tested by the bootstrap. Bootstrap values $<$ 50 % are not shown.

dominate the marine methanogens within the zone of sulfate reduction since sulfate reducing *Bacteria* (SRB) do not compete for the same substrates (Purdy *et al.*, 2003). Conversely sequences related to *Methanosaeta*, an acetoclastic methanogens (Ma *et al.*, 2006), were detected only below 95 cmsf (Figure 2.4), given that SRBs out-compete acetoclastic methanogens in the zone of sulfate reduction. Nine sequences related to the H_2/CO_2-utilizing *Methanomicrobiales* (Zellner *et al.*, 1999) were detected at all depths even though they were found in higher proportions in the libraries at 185 cmbsf and branched in the Fen cluster (Juottonen *et al.*, 2006), a group of environmental clones from peatlands (Figure 2.4 and 2.7).

Subsurface sediment Archaeal communities. The *Crenarchaeota* sequences were related to the Marine Benthic Group B (MBG-B) and Miscellaneous Crenarchaeotic Group (MCG) lineages (Figure 2.6), representing 35 % of the 16S rRNA clone libraries, and were also detected among the core (Figure 2.4). The proportion of MBG-B sequences in the libraries increases with depth (Figure 2.4) and forms two distinct clusters with environmental clones (Figure 2.6) . The MBG-B *Archaea*, a deep-branching lineage within *Crenarchaeota*, were found to be most active where the methane concentration was the highest (95 cmbsf). MBG-B, also synonymous of Deep Sea Archaeal Group (DSAG), have been previously detected in a wide range of anoxic marine environments (Takai and Horikoshi, 1999; Lloyd *et al.*, 2006; Biddle *et al.*, 2006; Sorensen and Teske, 2006). Recent studies have also revealed that MBG-B were metabolically active within the SMTZ, suggesting that MBG-B were directly or indirectly linked to AOM (Biddle *et al.*, 2006; Inagaki *et al.*, 2006*b*; Sorensen and Teske, 2006; Teske and Sorensen, 2008). MCG sequences dominated the shallowest library (DNA65) (67 %) and were also found, in smaller proportions, in all libraries (Figure 2.4) . The MCG, an ubiquitous lineage in marine sediments, were also active at 95 cmbsf, and are thought to be heterotrophic anaerobes utilizing complex organic substrates (Teske and Sorensen, 2008).
Sequences related to uncultured *Thermoplasmales* and MBG-D lineages were also found in small proportions in the DNA-retrieved libraries (7 %), at all depths. However, sequences related to MBG-D dominated (52 %) the RNA-derived library below 95 cmsf. This lineage within the *Thermoplasmatales* is also frequently found in methane seep sediments (Knittel *et al.*, 2005; Orphan *et al.*, 2001*a*) and deep marine sediments (Parkes *et al.*, 2005; Sorensen and Teske, 2006).

Novel archaeal lineages. In the present study, three potential new lineages with no closely related culture isolates were detected in high methane concentration zones. The first novel lineage, formed an independent cluster within the methane cycling *Methanosarcinaceae* family. This group of 8 sequences were closely related to environmental clones from low salinity methane-harboring environment such as a sulfide rich spring (Elshahed *et al.*, 2004) and estuary sediments (Purdy *et al.*, 2002). This new potential cluster, distantly related to the methylotrophic *Methanolobus oregonensis* (96 % similarity), was named Novel *Methanosarci-*

nales group 1 (NMG-1).

The detection by RNA-based approaches of active novel lineages in methane-bearing environments led to the discovery of the ANME lineages (Niemann *et al.*, 2006; Hinrichs *et al.*, 1999; Orphan *et al.*, 2001*b*). Interestingly, the RNA-derived sequences usually fell with the same phylotypes as the DNA-derived sequences, except for 2 groups of sequences that formed novel lineages (Figure 2.5). These two novel lineages were found metabolically active with a majority of the methane cycling communities (AMNE, MBG-B and *Methanosaeta*), where the sediments harbored the highest methane concentrations. Moreover the second novel lineage, designed as the Coastal Sediment group-1 (CSG-1), deeply branching within *Euryarchaeota* phylum, was distantly related to the *Mehanosarcinales* family (Figure 2.5). The CSG-1 only grouped with a few environmental clones, and was distantly affiliated to the new methylotrophic methanogen *Methermicoccus shengliensis* (highest similarity to pure culture, 86 %). The KM43T9523 sequence, which groups with the CSG-1 novel lineage, was closely related (98 % similarity) to the environmental clones from tidal flats (Kim *et al.*, 2005) and coastal sediments (Parkes *et al.*, 2007) where methane cycling *Archaea* also occurred (Figure 2.5). The third cluster groups with the VAL III sequences, a novel lineage distantly related to *Methanobacteriales* and *Methanococcales* (Jurgens *et al.*, 2000). The KM43T9502 and KM43T9518 sequences (highest similarity to cultured relative, 76 %) were related to environmental clones (85 % similarity) from mud volcanoes and deep marine sediments where methane also occurred (Heijs *et al.*, 2007; Sorensen and Teske, 2006). As all three lineages were related to methanogens or environmental clones found in methane-bearing environments, and as two were only found active at the highest methane concentrations, we suggest these lineages could be methane cycling *Archaea*.

Summary

In Marennes-Oléron Bay, the areas with the highest sedimentation rates and the highest organic matter concentrations harbored kilometer-scale acoustic turbidity zones related to the presence of free methane. In these gassy sediments, the archaeal diversity was dominated by metabolically active methane cycling communities (ANME, methanosarcinales, methanomicrobiales and MBG-B) congruently with the highest methane concentrations. Moreover, metabolically active novel uncultivated lineages, associated to putative methane cycling *Archaea*, could be specifically associated to methane-bearing sediment habitats. As these lineages are also typical deep subseafloor and methane seep sediment communities, the study of coastal gassy sediments submitted to high environmental and anthropic variables, which modify the microbial niches, would help to define the biogeochemical habitats of the deep biosphere communities.

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