Archaeal Populations in Hypersaline Sediments Underlying Orange Microbial Mats in the Napoli Mud Volcano

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Abstract:

Microbial mats in marine cold seeps are known to be associated with ascending sulfide- and methane-rich fluids. Hence, they could be visible indicators of anaerobic oxidation of methane (AOM) and methane cycling processes in underlying sediments. The Napoli mud volcano is situated in the Olimpi Area that lies on saline deposits; from there, brine fluids migrate upward to the seafloor. Sediments associated with a brine pool and microbial orange mats of the Napoli mud volcano were recovered during the Medeco cruise. Based on analysis of RNA-derived sequences, the "active" archaeal community was composed of many uncultured lineages, such as rice cluster V or marine benthic group D. Function methyl coenzyme M reductase (mcrA) genes were affiliated with the anaerobic methanotrophic Archaea (ANME) of the ANME-1, ANME-2a, and ANME-2c groups, suggesting that AOM occurred in these sediment layers. Enrichment cultures showed the presence of viable marine methylotrophic Methanococccoides in shallow sediment layers. Thus, the archaeal community diversity seems to show that active methane cycling took place in the hypersaline microbial mat-associated sediments of the Napoli mud volcano.

1. Introduction

Over 200 mud volcanoes have been found along the northern flank of the Mediterranean Ridge in the Eastern Mediterranean Sea (13). The formation of the Mediterranean Ridge is linked to the collisional tectonics between the African and Eurasian plates, resulting in intensive faulting (19). Within the Mediterranean Ridge, the Olimpi area, situated south of Crete, is a dynamic environment containing active seepage of mud, fluid, and brines. During the Messinian salinity crisis, evaporites
were deposited in the Mediterranean Basins (70), resulting in continuous evaporite
dissolution and brines migrating upwards in the Olimpi area (14). Mud volcanism is
often associated with brine seeps in this area (70). The Napoli mud volcano is a
submarine circular dome situated in the Olimpi area (Fig. 1). Subsurface brines
reaching the seafloor of the mud volcano create brine pools and lakes with diameters
ranging from centimeters to meters (13). The highest fluid flows are located near the
physical center of the mud volcano where mud mixed with brine enriched in biogenic
methane are mostly expelled (13).

Most of the methane rising up does not reach the seafloor because it is mainly
consumed by an efficient microbially mediated process known as anaerobic
oxidation of methane (AOM) (37). AOM has been documented in various anoxic
marine sediments, such as sediments of mud volcanoes (45), hydrothermal vents
(62), and hypersaline environments (36). AOM is driven by ANaerobic
MEthanotrophs (ANME) of the Archaea, and is mainly coupled to sulfate reduction
driven by Sulfate Reducing Bacteria (SRB). ANME are divided into three
phylogenetic groups, ANME-1, ANME-2 and ANME-3. The ANME-1 Archaea are
distantly affiliated with the methanogenic orders Methanosarcinales and
Methanomicrobiales, the ANME-2 with the methanogenic order Methanosarcinales,
and the ANME-3 with the methanogenic genera Methanococcoscoides/Methanolobus.

Alternative electron acceptors such as NO₂⁻ (55), Fe³⁺, and Mn⁴⁺ (5) have been
recently reported to be coupled to AOM with higher energy yields, based on
thermodynamic estimations. So far, no pure culture or defined consortium of ANME
has been isolated, and the biochemical pathways of AOM remain unknown. In the
current reverse methanogenesis hypothesis, i.e. CO₂ reduction to CH₄, methane
oxidation is catalyzed by a modified methyl coenzyme M reductase (MCR) (22, 23,
which in methanogens catalyzes the final step of methanogenesis (63). The
mcrA gene, encoding the MCR, is unique and found in all methanogens and
anaerobic methanotrophic Archaea (63). Phylogenetic mcrA based trees mirror the
phylogeny of the 16S rRNA genes for all known methanogens (20, 38). And, the
mcrA genes are conserved making them specific and useful functional gene markers,
targeting methanogens and methanotrophs in the environment.

Dense filamentous microbial mats on the seafloor of cold seep sediments are visible
to the naked eye. These mats are mainly composed of multicellular filaments
(diameter of 12 to 160 µm (41)), pigmented (e.g. orange or white) and unpigmented.
Microbial communities in sediments underlying microbial mats have been shown to
support high rates of sulfate-reduction (8, 27), sulfur-oxidation (50), nitrate-reduction
(8, 27) and anaerobic methane oxidation (8, 27). Members of these communities
have been previously identified as filamentous sulfur-oxidizing bacteria of the
Beggiatoa, Thioplaca, Leucothrix, Thirotrix and Desmanthos genera (24), as well as
diverse Proteobacteria (24, 43) and Archaea (42, 43). Interestingly, the archaeal
communities in sediments underlying seep-associated microbial mats are dominated
by methanogens and methane oxidizers (35). Thus, sediments underlying mats
provide alternative niches for diverse active archaeal communities adapted to
dynamic changes of fluid flow regimes.

This study analyzes archaeal community structure and diversity with depth, in
hypersaline sediments associated with orange-pigmented mats of the Napoli mud
volcano. The main objectives were to characterize the archaeal communities in
hypersaline sediments underlying dense microbial mats. Vertical distribution patterns
of archaeal communities were assessed using PCR-DGGE. Total RNA was extracted
from 0 to 4 cm below seafloor (cmbsf) and 6 to 10 cmbsf sediment layers and
subjected to reverse-transcription PCR with primers specific of the archaeal 16S rRNA genes. Then, archaeal methanotroph and methanogen diversity was determined based on mcrA genes from two different sediment depths (2 to 4 and 8 to 10 cmbsf). Finally, as methane was previously shown to mainly have a biogenic origin in the Napoli mud volcano, enrichment cultures for methanogens were carried out at all depths.

MATERIALS AND METHODS

Sediment sampling and porewater analysis.

Sediment samples were collected in the Napoli Mud Volcano, in the Eastern Mediterranean Sea during the Ifremer Medeco cruise with the research vessel Pourquoi Pas? in October/November 2007. Sediment pushcore CT-21 (Fig. 1) was recovered during dive PL 331-10 by the remotely operated vehicle (ROV) VICTOR 6000 (Ifremer) from 1938 metres of water depth (N 33°43.4397, E 24°41.0385). In the sampled area, sediments were recovered with dense orange microbial mats. Brine pools and rivers were observed in close proximity to the microbial orange mats. The sediment push-core sample contained bacterial orange filaments that penetrated the first 2-3 cm layers. Immediately after retrieval, the sediment core (10 cm long) was sectioned aseptically in 2 cm thick layers in the cooling room (4°C), and frozen at −80°C for nucleic acid extractions.

Depth profiles of dissolved porewater sulfate and chloride were quantified from diluted pore waters. Porewater was obtained by centrifuging approximately 10g of crude sediment, 15 minutes, 3000 x g at 4°C. The porewater was then stored at -20°C. Depth profiles of dissolved porewater sulfate and chloride were quantified from diluted pore waters. Sulfate and chloride concentrations were measured using ion
exchange chromatography, with a isocratic DX120 ion chromatography system (DIONEX Corporation, Sunnyvale, CA) fitted with Ionpas AS9-SC columns and a suppressor (ASRS-ultra II) unit in combination with a DS4-1 heated conductivity cell. Components were separated using a sodium carbonate gradient, with a flow of 1.5 mL/min.

**Culture media for enrichment of methanogens.**

One volume of sediment subsample (10 cm$^3$) was transferred into an anaerobic cabinet and then into 50 mL vials containing one volume (10 mL) of sterile and reduced Artificial Sea Water (ASW) medium. ASW corresponded to medium 141 of DSMZ devoid of organic carbon substrates. Enrichments were performed anaerobically in 50 mL vials according to Balch and Wolfe (3). Medium 141 from the DSMZ was used with slight modifications: organic substrates were omitted except yeast extract with a concentration adjusted to 0.2 g/L. The medium was prepared and sterilized under 80% N$_2$ and 20% CO$_2$ gas atmosphere. In order to enrich CO$_2^{-}$ reducing, acetoclastic and methylotrophic methanogens, three enrichment media supplemented with H$_2$ (200 kPa), acetate (10 mM), and trimethylamine (TMA, 20 mM) were used. One g of sediment from the different sections of CT21 was inoculated into 9 mL of medium (pH 7), the suspension was mixed and serially diluted until 10$^{-3}$. The cultures were incubated at 15°C to mimic in situ conditions. Cultures were periodically checked for methane production for one year. Methane was detected directly in the headspace of vial cultures by a micro MTI M200 Gas Chromatograph equipped with MS-5A capillary column and Poraplot U capillary column. Positive enrichment dilutions of methanogens were monitored by PCR-
DGGE. For dilutions showing one DGGE band on the fingerprint, 16S rRNA genes were amplified cloned and sequenced using the A8F and A1492R primers.

**Nucleic acids extraction and purification.**

Total genomic DNA was directly extracted and purified from 5g of wet sediment for all sections in duplicates, using the Zhou *et al.* (69) method with modifications. Sediment samples were mixed with DNA extraction buffer as described (69), followed by 3 cycles of freezing in liquid N$_2$ and thawing at a 65°C. The pellet of crude nucleic acids obtained after centrifugation, was washed with cold 80% ethanol, and resuspended in sterile deionized water, to give a final volume of 100 µL. Crude DNA extracts were then purified using the Wizard DNA clean-up kit (Promega, Madison, WI). DNA extracts were aliquoted and stored at –20°C until required for PCR amplification. Total RNA was directly extracted and purified from 2g of wet sediment from pooled sediment sections 0 to 4 and 6 to 10 cmbsf, using the RNA PowerSoil Total RNA Isolation Kit (MO BIO Labs. Inc., Carlsbad, CA) according to manufacturer recommendations. Aliquots of RNA extracts were treated by Turbo DNAse (Applied Biosystems, Foster City, CA) and purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The quality of RNA samples was examined by agarose-gel electrophoresis and concentrations were determined using spectrophotometry (Nanodrop ND-100, NanoDrop Technologies Wilmington, DE, USA).

**Archaeal 16S rRNA PCR-DGGE amplification.**

Archaeal 16S rRNA genes were amplified by PCR from purified DNA extracts using the primers pair 8F (5’-CGGTTGATCCTGCTTGGGA-3’) and 1492R (5’-
GGCTACCTTGGTACGACTT-3’) (10). All PCR reactions (total volume reaction 25 μL) contained 1 μL purified DNA template, 1X PCR buffer (Promega, Madison, WI), 2 mM MgCl₂, 0.2 mM of each dNTP, 0.4 mM of each primer (Eurogentec) and 0.6 U GoTaq DNA polymerase (Promega, Madison, WI). Amplification was carried out using the GeneAmp PCR 9700 System (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: denaturation at 94 °C for 1 min, annealing at 49 °C for 1 min 30 s, and extension at 72 °C for 2 min for 30 cycles. All the archaeal 16S rRNA gene PCR products were then re-amplified in a nested PCR with primers 340F (5’-CCCTACGGGGYGCASCAG-3’) (65) containing a GC clamp (5’-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3’) at the 5’ end, and primer 519R (5’-TTACCGCGGCKGCTG-3’) (51). The PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 72 °C to 62 °C (touchdown -0.5 °C per cycle) for 30 s, and extension at 72 °C for 1 min, for 20 cycles, then denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min, for 10 cycles, and a final extension at 72 °C for 30 min (25).

To restrict contamination to a minimum, PCR experiments was carried out under aseptic conditions (Captair® bio, Erlab, Fisher Bioblock Scientific) using autoclaved and UV-treated plasticware and pipettes, and only sterile nuclease-free molecular grade water (MP Biomedicals, Solon, OH, USA). Positive (DNA extracted from pure cultures) and negative (molecular grade water) controls were used in all PCR amplifications.

Denaturing Gradient Gel Electrophoresis fingerprinting analysis.

DGGE was carried out as described by Toffin et al. (64) with some modifications. PCR products were separated by DGGE using the D-Gene™ System (Bio-Rad
Laboratories, Hercules, CA) on 8% (w/v) polyacrylamide gels (40% acrylamide/bis solution 37.5:1 Bio-Rad) with a gradient of denaturant between 20% and 60%. A denaturing gradient gel consists of [100% denaturant equals 7M urea and 40% (v/v) formamide]. Gels were poured with the aid of a 30 mL volume Gradient Mixer (Hoefer SG30, GE Healthcare, Buckinghamshire, UK) and prepared with 1 X TAE buffer (MP Biomedicals, Solon, OH, USA). Electrophoresis was carried out at 60°C, 200 V for 5 hours (with an initial electrophoresis for 10 min at 80 V) in 1 X TAE buffer. Polyacrylamide gels were stained with SYBRGold nucleic acid gel stain (Invitrogen, San Diego, CA) for 30 min, and viewed using the Typhoon 9400 Variable Mode Imager (GE Healthcare, Buckinghamshire, UK).

Construction of RNA-derived 16S rRNA gene libraries.

RNA-derived cDNA was synthesised by reverse transcription using the 16S rRNA archaeal primer 915R (5’-GTGCTCCCCCGCCAATTCCT-3’) (9) and the Moloney Murine Leukaemia Virus reverse transcriptase (M-MuLV, MP Biomedicals, Irvine, CA) according to the manufacturer’s protocol. Purified RNA (100-150 ng) was initially denatured at 65°C for 10 min, and 7.7 µM primer 915R was added to the denatured RNA. The mixture was incubated at 70°C for 10 min. The reverse transcription reaction mixture (total volume of 22 µL) consisted of denatured RNA, 1X M-MuLV buffer, 200 µM of deoxynucleoside triphosphate mix, and 10 mM DTT (dithiothreitol). The reverse transcription reaction mix was incubated at 42°C for 2 min. A 200-unit aliquot of M-MuLV reverse transcriptase was added prior to a 80 min incubation at 42°C for the reverse transcription of the RNA into complementary DNA (cDNA). The reaction was then stopped by heating at 70°C for 15 minutes. The cDNA end product was used as a template for archaeal 16S rRNA gene based PCR using the primer
set 340F/915R. The PCR amplification involved 20 cycles of 94 °C for 1 min, 71 °C to 61 °C (touchdown -1 °C per cycle) for 1 min, and 72 °C for 2 min. PCR products were purified with the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany) and analyzed on 1% (w/v) agarose gels in 1 X TAE buffer and stained with ethidium bromide and UV-illuminated. Purified PCR products were cloned into TOPO® XL PCR Cloning Kit, and transformed into Escherichia coli TOP10 One Shot® cells (Invitrogen, San Diego, CA) according to the manufacturer’s recommendations. Control PCR using the purified RNA and the same primers were performed to monitor possible DNA contamination of the RNA templates. No contaminating DNA was detected in any of these reactions.

Construction of mcrA environmental gene libraries.

The mcrA genes were amplified using the ME1 (5’-GCMATGCARATHGGWATGTC-3’) and ME2 (5’-TCATKGCRTAGTTDGGRTAGT-3’) primers (20). The PCR conditions were as follows: denaturation at 94 °C for 40 s, annealing at 50 °C for 1’30 min, and extension at 72 °C for 3 min, for 30 cycles. PCR products were purified on a 1% agarose gel using the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany) and cloned using the TOPO XL PCR Cloning Kit (Invitrogen, San Diego, CA) according to the manufacturer’s protocols.

Phylogenetic analysis of DNA.

The gene sequencing was performed by Taq cycle sequencing and determined on a ABI PRISM 3100-Genetic Analyzer (Applied Biosystems, Foster City, CA) using the M13R (5’-CAGGAAACAGCTATGAC-3’) universal primer. RNA-derived cDNA, DNA-derived mcrA and enrichment culture-derived 16S rRNA gene sequences were...
analyzed using the NCBI BLASTN search program within GeneBank (http://blast.ncbi.nlm.nih.gov/Blast) (2). Potential chimeric sequences in the clone libraries were identified with the CHIMERA CHECK program of the Ribosomal Database Project II (Center for Microbial Ecology, Michigan State University, http://wdcm.nig.ac.jp/RDP/html/analyses.html). Potential chimeras were eliminated before phylogenetic trees were constructed. The RNA-derived 16S rRNA sequences and the enrichment culture-derived 16S rRNA gene sequences were then edited in the BioEdit v7.0.5 programm (21) and aligned using the SINA webaligner (http://www.arb-silva.de/, (53)). The \textit{mcrA} sequences were translated into amino acid sequences using BioEdit and aligned using ClustalX (33). Sequence data were analysed with the MEGA4.0.2 program (61). The phylogenetic trees were calculated using the neighbour-joining method. The robustness of the inferred topology was tested by bootstrap resampling (1000x). Rarefaction curves were calculated for the RNA-derived 16S rRNA, and \textit{mcrA} gene libraries using the RarFac program (http://www.icbm.de/pmbio/), and a 97% similarity cutoff value for sequence-based OTUs. Gene library coverage was calculated using the following formula: \( C = \left[1-\left(\frac{n_1}{N}\right)^2\right] \times 100 \), where \( n_1 \) is the number of unique OTUs, and \( N \) is number of clones in the library (58).

**Statistical analyses of DGGE banding patterns.**

The DGGE profile was analyzed as described by Fry \textit{et al.} (15), using a presence/absence scoring of the DGGE bands. After making a grid to determine whether bands were present (score=1) or absent (score=0) for each lane on a same line of the DGGE profile, a presence/absence matrix was obtained. This matrix was then used to build a similarity matrix based on the Jaccard coefficient, using the
vegan package within the R software (54). Finally, a dendrogram was obtained using the ward agglomeration method within the hierarchical clustering package of the R software.

**Nucleotide sequence accession numbers.**

The sequence data reported here will appear in GenBank nucleotide sequence databases under the accession No. HM004785 to HM004825, and HQ443429 to HQ443514 for RNA-derived 16S rRNA gene sequences, HM004828 to HM004903 and HQ454430 to HQ454493 for *mcrA* gene sequences, and HM004826 to HM004827 for enrichment culture-derived 16S rRNA gene sequences.

**RESULTS and DISCUSSION**

**Geochemical and biological characteristics.**

Observation of large orange-pigmented mats on the surface of the sampled sediment core and direct microscopic examination of filamentous morphology indicated that the bacteria were possibly members of genera *Beggiatoa* or *Thioploca*, as reported elsewhere (46, 56).

Profiles of sulfate and chloride concentrations in porewater sediments underlying the orange microbial mats were anticorrelated. The chloride porewater profile showed an increase in concentration with depth (Fig. 2), reaching 1200 mM at 10 cmbsf, which is more than 2 times higher than normal seawater concentrations (600 mM, (13)). The sodium concentrations showed a similar increase with depth (Supplementary material. SM1), reaching 1224 mM at 10 cmbsf, which is also more than 2 times higher than normal seawater concentrations (492 mM, (13)). Furthermore, Charlou et al. characterized the brines in Napoli as being enriched in Cl and Na. Hence,
increases in chloride and sodium were presumably linked with the upflowing brines from deep sources in the Napoli mud volcano. The surface sediment layers colonized by the orange-pigmented mat bacteria showed chloride concentrations of 700 mM, and could be influenced by the brine pools contiguous to the filamentous bacteria on the seafloor. Moreover, bacterial mats are a common feature found in habitats influenced by hypersaline brine fluid intrusions (43, 48).

The sulfate porewater concentrations showed a slight decrease with depth (Fig. 2), which could suggest sulfate reduction. Sediments associated with orange- and white-pigmented *Beggiatoa* mats have been shown to host high rates of sulfate reduction, probably a combination of increased substrate availability in the seep fluids, and of rapid sulfate recycling within the sulfur-oxidizing bacterial mat (43, 48). However, the Napoli sediments did not seem to have a clear sulfate reducing zone. Profiles of the Mg$^{2+}$ and Ca$^{2+}$ porewater concentrations (Supplementary material. SM1) showed decrease with depth. Concentrations reached 34 and 3 mM for Mg$^{2+}$ and Ca$^{2+}$ respectively at 10 cmbsf (whereas seawater concentrations are typically 56 and 11 mM (13), suggesting authigenic carbonate precipitation in these sediment layers. Indeed, anaerobic oxidation of methane increases alkalinity in porewater fluids by producing HCO$_3^-$, that in turn reacts with and precipitates Mg$^{2+}$ and Ca$^{2+}$ cations (11, 30).

**Vertical distribution of the archaeal communities.**

The DGGE fingerprints (Fig. 3A) generated from DNA samples extracted from sediment layers associated with orange-pigmented microbial mats of the Napoli mud volcano displayed a complex and diverse distribution of the archaeal communities. The resulting dendrogram (Fig. 3B) of the DGGE pattern highlighted clear changes in
populations with depth in two separate clusters. The first cluster grouped depths 0 to
4 cmbsf, and the second the 4 to 10 cmbsf sediment layers, suggesting a change in
archaeal populations at 4 cmbsf, with increasing depth and salinity. This shift could
be linked to the presence of the bacterial filaments in these sediments. Indeed these
filaments belonging to what is commonly called "Big Bacteria" could have locally
modified the geochemical conditions in the surrounding sediments (56), therefore
affecting the microbial community diversity in the upper 4 cm. Salt concentrations
might also have affected the depth distribution of the microbial communities, as
reported elsewhere (28).

According to the statistical analysis of the DGGE pattern indicating a shift in the
archaeal community at 4 cmbsf, we constructed two RNA-derived 16S rRNA gene
libraries for depths 0-4, and 6-10 cmbsf. A total of 55 archaeal RNA-derived 16S
rRNA gene sequences were analysed for the 0-4 cmbsf section, and 72 for the 6-10
cmbsf section. Rarefaction curves generated for the RNA-derived 16S rRNA genes
indicated saturation (Supplementary material, SM2), while percent coverage was
determined to be 40% and 72.2% for the 0-4 and 6-10 cmbsf sections respectively.
Hence coverage analysis suggests that the full diversity of archaeal 16S rRNA
sequences was not exhausted and that a greater diversity remains to be detected
within these sediments. Simpson's diversity indices (57) were calculated for each
section, and indicated a decrease in archaeal diversity with depth (D=0.9554 for the
0-4 cmbsf section, and 0.9109 for the 6-10 cmbsf section).

The phylogenetic trees of the RNA-derived 16S rRNA gene libraries showed high
archaeal diversity and a majority of sequences most closely related to environmental
clones from mud volcano sediments of the Mediterranean Sea (i.e. Milano, Kazan,
Chefren), marine sediments and subseafloor sediments (i.e. Peru margin).
eight OTUs belonging to archaeal uncultured groups were detected in the 0-4 cmbsf sediment layer 16S gene library, belonging to two major groups (Fig. 4 and 5): Marine Benthic Group D (MBG-D, 40.5%) and Rice Cluster V (RC-V, 40.5%). The other minor groups that were detected also belong to uncultured archaeal lineages (Fig. 4 and 5), i.e. Deep-sea Hydrothermal Vent Euryarchaeotic group 4 (DHVE-4), Group VI, Terrestrial Miscellaneous Euryarcheotal Group (TMEG), Marine Group II (MG-II), Miscellaneous Crenarchaeotic Group (MCG), Marine Benthic Group B (MBG-B). One clone (NapMat-0_4-rtC09) was not related to any known group. The only sequence closely related to cultured prokaryotes (NapMat-0_4-rtB11b) was related to the methanogenic order of the *Methanosarcinales* (Supplementary material. SM3). Thirty-one OTUs also belonging to archaeal uncultured groups were detected in the 6-10 cmbsf sediment layer 16S gene library, belonging to two major groups (Fig. 4 and 5): MBG-D (55%), and MCG (17.5%). Other minor groups related to uncultured archaeal groups were detected as well (Fig. 4 and 5), i.e. TMEG, Marine Benthic Group E (MBG-E), and MBG-B. Two clones were affiliated with the methanogenic order *Methanosarcinales*, one with the anaerobic methanotrophic group ANME-3, one with the Rice Cluster II, which are surmised to be involved in methane production (18), and finally one clone (NapMat-6_10-rtH01) was affiliated with an extreme halophilic *Archaea* of the *Halobacteria* (47). Most of the sequences only detected in the 6-10 cmbsf section (i.e. MBG-B, MCG, and TMEG) were closely related with sequences retrieved from subsurface sediments of the Peru margin (7, 60).

Intriguingly, RC-V members were presumably active in the 0-4 cmbsf sediments underlying orange-pigmented mats. The RC-V were first discovered in an anoxic flooded rice paddy soil (18), detected in many freshwater sediments (12, 17, 67, 68),
and were recently shown to be active in tubeworm populated sediments of the Storegga Slide (34). The intralineage levels of rDNA similarity of the RC-V sequences were low highlighting that this group seems to be phylogenetically very diverse. This could suggest diverse metabolic activities and physiologies. RC-V were also previously observed in cold coastal waters of the Mackenzie River in northwestern Canada, rich in suspended particles (16). The authors suggested that RC-V were linked to detrital decomposition. The Napoli sediments in which the RC-V were detected had a high organic matter content (data not shown), which could support the hypothesis of an organotrophic metabolism. This study is the first to report occurrence of probable active members of the RC-V in hypersaline sediments. Also, sequences affiliated with the RC-V were not detected in the 6-10 cmbsf sediment layers where salinity reached 1300 mM, suggesting that members of the RC-V probably do not tolerate high salt concentrations.

Sequences affiliated with the archaeal uncultured MBG-D were detected in many saline or hypersaline environments (6, 26, 36, 48, 59). Jiang et al. proposed that salinity could play a role in controlling the distribution of marine benthic groups, such as the MBG-D (26). Furthermore, the MBG-D was the main archaeal group presumably "active" in both sections (0-4 and 6-10 cmbsf) where Cl\(^-\) and Na\(^+\) porewater concentrations were high (834 and 792 mM respectively).

**Diversity and distribution of the ANME.**

In order to show if ANME and/or methanogen affiliated sequences in hypersaline sediments are phylogenetically distinct from sequences in non hypersaline conditions, \(mcrA\) gene libraries were constructed for representative sediment sections characterized by increasing Cl\(^-\) and Na\(^+\) porewater concentrations, \textit{i.e.} 2-4
and 8-10 cmbsf. A total of 75 mcrA sequences were analysed for the 2-4 cmbsf sediment section, and 65 for the 8-10 cmbsf layers (Fig. 6). Rarefaction curves generated for the mcrA clones of the two libraries indicated saturation (Supplementary material. SM2); while percent coverages were determined to be 81.3% and 87.7% for the 2-4 and 8-10 cmbsf gene libraries. Simpson's diversity indices (57) were calculated for each section, and indicated a decrease in the methanotrophic/methanogenic diversity with depth (D=0.7952 for the 2-4 cmbsf section, and 0.7096 for the 8-10 cmbsf section).

Three dominant mcrA phylotypes were present (Supplementary material. SM4), i.e. mcrA group a/b (ANME-1 as defined by Hallam et al., (22)), mcrA group c/d (ANME-2c) and mcrA group e (ANME-2a). The majority of the mcrA clones were related to the ANME-2a at 2-4 cmbsf (58.3%), followed by the ANME-1. The ANME-2c sequences represented only a small portion of the ANME in the gene library. At the 8-10 cmbsf section, the ANME-1 became the dominant group (65.6%), followed by the ANME-2c, and the ANME-2a represented only a small proportion of the ANME in the gene library.

Sediments underlying bacterial mats seem to constitute hot spots for AOM (43), as a consequence of high methane flux in the upwardly moving subsurface fluids, combined with sulfate availability in the surficial sediments aided by the mats (35). ANME-2a Archaea have been found as the unique methanotrophic representative in sediments of the active center of the Napoli mud volcano (Lazar et al., unpublished data), dominated marine sediments of Skagerrak (52), and in sediments covered with white-pigmented mats of the Gulf of Mexico (43). It has been suggested that ANME-2 may be more active at low temperatures compared to ANME-1 (44). Temperature gradient measurements of the active center of the Napoli mud volcano showed little
increase of temperature with depth down to 160 cmbsf, with an average temperature of 14°C (Lazar et al., unpublished data). It has also been suggested that ANME-2 dominate sediment layers with high sulfate concentrations whereas ANME-1 seem to be found in sediment layers with low sulfate concentrations (31). Therefore, the distribution of the ANME-2 in the Napoli mud volcano sediments could support these assessments (Fig. 7).

ANME-1 Archaea have been detected in various environments. In hypersaline sediments of the Gulf of Mexico, the community of ANME was found to be limited to the ANME-1b, which were probably active (36). The authors suggested that the ANME-1b preponderance could be explained by the high salinity of the site (chloride concentration was 2200 mM), and that ANME-1b could be a high salinity adapted subpopulation. The increase in ANME-1 sequences with depth and with chloride concentrations (at the 8-10 cmbsf section, chloride concentration was 1256 mM) in the Napoli sediments could support this assumption.

**Culturable methanogenic diversity.**

Methane production was detected in media designed to enrich methylotrophic methanogens on trimethylamines (TMA) in the shallow sulfate-rich 0-2, and 2-4 cmbsf sediment sections. Microscopic observations of positive enrichments from the medium designed to enrich hydrogenotrophs suggested that methanogens were cocci-shaped cells. Under UV light, autofluorescent cells were detected as free cells. Total genomic DNA was extracted from the TMA enriched medium inoculated with the 0-2 and 2-4 cmbsf sediment sections. Phylogenetic affiliation of clone NapMat-0_2-enr30 showed showed 99% sequence similarity with clone Tommo05_1274_3_Arch90 of the Euryarchaeota (FM179838) recovered from the
Tommeliten methane seep, in the North Sea (66), and 98% of sequence similarity with the closest cultured methanogen *Methanococcoides methylutens* (M59127). Phylogenetic affiliation of clone NapMat-2_4-enr31 showed 98 % sequence similarity with the cultured methanogen *Methanococcoides burtonii* (CP000300).

These results are in agreement with previous studies detecting methylotrophic *Methanococcoides*-type methanogens in saline or hypersaline habitats such as a brackish lake (4), marine sediments in Skan Bay (29), anaerobic sediments of mangroves (39, 40), brine seeps of the Gulf of Mexico (28), and recently sediments of the center of the Napoli mud volano (Lazar et al., unpublished data). Representative species of methylotrophic methanogens in culture collections take up methylated compounds as substrates that are not used by other competitive microorganisms such as sulfate-reducing bacteria. Methylated compounds could derive from organic detritus from the microbial mat (Fig. 7). And, known cultured methylotrophic methanogens, belonging to the *Methanohalophilus* and *Methanohalobium* genera, have been shown to efficiently tolerate high salinity environments (up to 25 % NaCl) (49).

Surprisingly, in this study, only one 16S rRNA gene sequence of a known halophilic Archaea of the *Halobacteria* was detected, despite the high measured chloride concentrations. The same observation was also reported for brines from the Gulf of Mexico (28). However, as most of the sequences detected (*i.e.* DHVE4, MBG-D, MCG, RC-V, ANME) belong to as yet uncultured archaenal lineages, we can assume that some of the Napoli sequences represent unknown halophilic or halotolerant microorganisms. Moreover, Simpson’s diversity indices indicated a decrease in archaenal and methanotrophic/methanogenic diversity with depth hence with
increasing salinity. This could suggest that salt adapted \textit{Archaea} dominated the deeper layers of the Napoli mud volcano.

\textbf{Conclusion.}

In this study, culture-dependent and –independent techniques were employed in order to assess the distribution of the "active" RNA-derived 16S rRNA archaeal sequences in sediments associated with orange-pigmented mats of the brine impacted Napoli mud volcano. In the shallow sulfate-rich sediment layers of the Napoli mud volcano, the active fraction of the archaeal community was mainly represented by sequences belonging to as yet uncultured lineages similar to those present in cold seeps and deep subseafloor sediments, but unexpectedly also rice paddies. \textit{mcrA} gene libraries suggested that AOM might have occurred in the Napoli mud volcano sediments. Enrichment cultures indicated that viable methanogens were present in the shallow sulfate-rich sediment layers. Therefore, a complex archaeal community was observed in this hypersaline habitat, possibly intertwining sulfur and methane cycles.

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microbial consortium couples anaerobic methane oxidation to denitrification.


Figure 1. A) Location map showing the Olimpi area in the Eastern Mediterranean Sea. From Aloisi et al. (1). B) Closer view on the Napoli mud volcano and the position of the sampled push core (Bénédicte Ritt, pers. comm.).
Figure 2. Sulfate (filled squares) and chloride (open circles) porewater concentrations (mean ± standard deviation) in mM with depth for core CT-21 of the Napoli mud volcano sediments in cmbsf. The scale represents sediment depth below the seafloor. Sediment sections dedicated to *mcrA* and RNA derived 16S rRNA are indicated on the right.
Figure 3. A) DGGE analysis of archaeal 16S rRNA genes obtained by nested PCR in the Napoli mud volcano. B) Dendrogram obtained from clustering analysis of DGGE banding profiles and scoring bands as present or absent, using the R software. Bar indicates dissimilarity values.
Figure 4. Phylogenetic analysis of the Crenarchaeal RNA-derived 16S rRNA genes of the Napoli mud volcano sediments based on the neighbour-joining method with 575 homologous positions. Bootstrap values (in percent) are based on 1000 replicates and are indicated at nodes for branches values ≥50% bootstrap support. Gene sequences recovered in this study from Napoli mud volcano sediments are in
boldface type. Clones with designation beginning NapMat-0_4 and NapMat-6_10 are from the sediment section 0 to 4 and 6 to 10 cmbsf respectively. Numbers in parentheses indicate the number of analyzed clones that have more than 97% sequence identity. The scale bar indicates five substitutions per 100 nucleotides. MCG, Miscellaneous Crenarchaeotic Group, MBG-B, Marine Benthic Group B, MBG-A, Marine Benthic Group A, MG-I, Marine Group I.
Figure 5. Phylogenetic analysis of the Euryarchaeal RNA-derived 16S rRNA genes of the Napoli mud volcano sediments based on the neighbour-joining method with 575 homologous positions. Bootstrap values (in percent) are based on 1000 replicates and are indicated at nodes for branches values $\geq$50% bootstrap support. Gene sequences recovered in this study from Napoli mud volcano sediments are in boldface type. Clones with designation beginning NapMat-0_4 and NapMat-6_10 are from the sediment section 0 to 4 and 6 to 10 cmbsf respectively. Numbers in parenthese indicate the number of analyzed clones that have more than 97 % sequence identity. The scale bar indicates two substitutions per 100 nucleotides. RC-V, Rice Cluster V, MBG-D, Marine Benthic Group D, TMEG, Terrestrial Miscellaneous Euryarchaeotal Group, MBG-E, Marine Benthic Group E, MG-II, Marine Group II, RC-II, Rice Cluster II, DHVE-4, Deep Sea Hydrothermal Vent Euryarchaeotal Group 4.
Figure 6. Phylogenetic analysis of MCR amino acid sequences from the Napoli mud volcano sediments based on the neighbour-joining method with approximately 258 amino acid positions. Bootstrap values (in percent) are based on 1000 replicates and are indicated at nodes for branches values ≥50% bootstrap support. Gene sequences from the Napoli mud volcano sediments obtained in this study are in boldface type. Clones with designation beginning NapMat-2_4 are from the sediment section 2 to 4 cmbsf, and NapMat-8_10 from sediment section 8 to 10 cmbsf. Numbers in parentheses indicate the number of analyzed clones that have more than 97% nucleotide sequence identity, and more than 99% amino acid sequence identity. The scale bar indicates 10% estimated difference. ANME, Anaerobic Methanotroph.
Figure 7. Schematic illustrating the potential interactions between anaerobic methanotrophic Archaea (ANME) probably mediating anaerobic oxidation of methane, methanogens, and uncultured Archaea in different hypersaline sediment sections of the Napoli mud volcano. Sediment depth below the seafloor is indicated at the right of the illustration.