Hydrocarbon-related microbial processes in the deep sediments of the Eastern Mediterranean Levantine Basin

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Abstract
During the 2011 exploration season of the EV Nautilus in the Mediterranean Sea, we conducted a multidisciplinary study, aimed at exploring the microbial populations below the sediment–water interface (SWI) in the hydrocarbon-rich environments of the Levantine basin. Two c. 1000-m-deep locations were sampled: sediments fueled by methane seepage at the toe of the Palmachim disturbance and a patch of euxinic sediment with high sulfide and methane content offshore Acre, enriched by hydrocarbon from an unknown source. We describe the composition of the microbial population in the top 5 cm of the sediment with 1 cm resolution, accompanied by measurements of methane and sulfate concentrations, and the isotopic composition of these methane and sulfate (δ¹³CCH4, δ¹⁸OSO₄, and δ³⁴SSO₄). Our geochemical and microbiological results indicate the presence of the anaerobic methane oxidation (AOM) coupled to bacterial sulfate reduction (BSR). We show that complex methane and sulfur metabolizing microbial populations are present in both locations, although their community structure and metabolic preferences differ due to potential variation in the hydrocarbon source.

Introduction
Marine sediments can contain high amounts of organic matter and/or cold hydrocarbon seepage, either of which can create subsurface ‘hotspots’ of microbial activity (Jørgensen & Boetius, 2007; Knittel & Boetius, 2009; Goffredi & Orphan, 2010; and references therein). In turn, the products of the oxidation of this organic matter or other hydrocarbons, such as CO₂, acetate, and methylated compounds, can fuel the production of methane through methanogenesis (Garcia et al., 2000; Reeburgh, 2007). As a result, diverse and active methanogen communities have been found in the sediments of cold seeps and mud volcanoes (e.g. Dhillon et al., 2005; Kendall & Boone, 2006; Lazar et al., 2011a, b). Moreover, sulfate, abundant in the ocean waters, is a dominant electron acceptor for the anaerobic oxidation of short-chain alkanes and complex mixture of hydrocarbons (Kleindienst et al., 2012). The hydrocarbon enrichment of seep sediments results in rates of bacterial sulfate reduction (BSR) several orders of magnitude higher compared with non-seep sediments (Aharon & Fu, 2000; Treude et al., 2003; Joye et al., 2004; Bowles et al., 2011).

Hydrocarbon metabolism and BSR are linked within the sulfate–methane transition zone (SMTZ), where the anaerobic oxidation of methane (AOM) and BSR simultaneously take place (Knittel & Boetius, 2009). The evidence for AOM was initially geochemical, and particularly isotopic: Reeburgh (1982) attributed the enrichment in ¹³C of the dissolved inorganic carbon (DIC) in marine sediments to AOM at the SMTZ, because methane is particularly enriched in ¹³C. This geochemical evidence was followed by biological and lipid evidence, demonstrating that a consortia of anaerobic
methane-oxidizing Archaea (ANME) and sulfate-reducing bacteria (SRB) are the key mediators of AOM (Hoehler et al., 1994; Hinrichs et al., 1999; Boetius et al., 2000). Marine AOM consumes >90% of methane produced in ocean sediments, controlling methane emissions to the atmosphere (Knittel & Boetius, 2009), while the ANME and SRB involved in AOM are intensively studied (Hinrichs & Boetius, 2002; Knittel & Boetius, 2009).

Three euryarchaeal groups of ANME (ANME-1, ANME-2, and ANME-3) related to cultivated Methanosarcinales and Methanomicrobiales orders have been described based on the 16S rRNA gene phylogeny (Lösekann et al., 2007). The ANME-1 and ANME-2 Archaea are usually found in consortia with Desulfoarcina/Desulfococcus group SRB (Knittel et al., 2005; Schreiber et al., 2010), while the Desulfoarculus group SRB is associated with ANME-3 (Niemann et al., 2006a, b; Lösekann et al., 2007). Some ANME species are able to oxidize methane without a bacterial partner (Orphan et al., 2002; Knittel et al., 2005; Pernthaler et al., 2008; Jagersma et al., 2012; Milucka et al., 2012). Recently, nondeltaproteobacterial populations, including Betaproteobacteria, were shown to be associated with ANME consortia (Pernthaler et al., 2008). The microbial ecophysiology at the SMTZ is complex, partially due to the ability of ANME to produce methane (Alperin & Hoehler, 2009a, b; House et al., 2009; Lloyd et al., 2011) and possibly ferment (Alperin & Hoehler, 2009a, b; Bowles et al., 2011) in addition to AOM. High ANME abundance has also been found in the top sediment layers of hydrocarbon-rich sediments above the SMTZ (Pachiadaki et al., 2011).

The complexity of the microbial system comprising the production and consumption of methane often results in overlap of methanogenesis with AOM, which is reflected in the isotope geochemistry measured in pore fluid profiles in sediments (Seifert et al., 2006; Holler et al., 2009). Biological methanogenesis results in a large carbon isotope fractionation of methane, where the produced methane is highly depleted in $^{13}$C (−100‰ to −50‰) and the residual DIC is enriched by more than 50–70‰ (Valentine et al., 2004; Conrad, 2005; Reeburgh, 2007; Penger et al., 2012). This is opposed to the small carbon isotope fractionation [0–10‰] (Alperin et al., 1988; Martens et al., 1999]) by AOM. Thus, the $\delta^{13}$C_DIC tends to decrease in the zone of AOM and increase in the zone of methanogenesis, highlighting the spatial relationship between the two processes.

Beside the coupling of SRB to AOM, nonmethane hydrocarbons are a major electron source for BSR (Bowles et al., 2011; Quistad & Valentine, 2011). Again, isotope geochemistry is a unique tool to explore BSR coupled to either AOM or other hydrocarbon oxidation. SRB preferentially utilize lighter sulfur and oxygen isotopes ($^{32}$S and $^{16}$O respectively), enriching the pore water sulfate with residual $^{34}$S and $^{18}$O. The isotopic partitioning of both sulfur and oxygen isotopes in sulfate is temperature dependent and varies among the SRB species (Brüchert et al., 2001; Canfield et al., 2006). This partitioning can be as high as 72‰ for sulfur isotopes (Wortmann et al., 2001; Brunner & Bernasconi, 2005; Canfield et al., 2010; Sim et al., 2011). The magnitude of this sulfur isotope fractionation is largely a function of the recycling of intracellular sulfur intermediates (such as sulfite; Brunner & Bernasconi, 2005; Canfield et al., 2006). The isotopic fractionation of oxygen during dissimilatory BSR is less understood and can significantly deviate from the previously hypothesized 1 : 4 oxygen–sulfur fractionation ratio, due to a combination of kinetic and equilibrium isotope effects (e.g. Brunner et al., 2012; Antler et al., 2013).

The marked changes in the availability of electron acceptors and carbon substrates within marine sediments impacts the microbial diversity (Lloyd et al., 2010). The study of pore water isotope geochemistry combined with the phylogenetic study of the respective microbial populations can shed light on the complex diagenetic processes within hydrocarbon-rich sediments.

The deep Eastern Mediterranean Sea is a hotspot of hydrocarbon seepage (Coleman & Ballard, 2001; Loncke, 2004; Masle et al., 2006; Heijts et al., 2007; Omoregie et al., 2008, 2009) and thus provides an opportunity for studying these processes in detail. During the 2011 exploration season of the Nautilus E/V, gas/fluid-charged sediments emitting methane and other hydrocarbons, possibly associated with deeper reservoirs of natural gas, were discovered at the depth of c. 1000 m at the area of small faults and scarp in the Palmachim disturbance feature in the Levantine basin (Coleman et al., 2012). These sediments were associated with visible gas bubbling at the sampling location and the presence of biogenic carbonates, resulting from the AOM-induced alkalinity shift (Knittel & Boetius, 2009). Acre sediments were sampled on a slope of a pockmark inside a large pockmark field. No visible gas bubbling or calcium carbonate formation was detected in this location. The geological processes forming both features are still poorly understood. In this study, we use a multidisciplinary geochemical–microbiological approach to describe the diagenetic processes near the sediment–water interface at these locations.

Materials and methods

ROV imaging and sample collection

The samples were collected during the 2011 Nautilus E/V field season. Nautilus E/V is equipped with Hercules and Argus remotely operated vehicle (ROV) systems, which
are able to collect high-resolution video, oceanographic data, and precision sampling. All samples were taken with 7 cm diameter, 30-cm-long pushcores. The 'black patch' was observed and sampled at the Acre location, 32°56.1464′N 34°46.9735′E, at water depth of 1099 m (Acre core). As the patch was present on a slope of a pockmark (70°–90°), the cores taken at this location perpendicularly to the sediment surface by hovering ROV were virtually horizontal. Separate cores were taken for the quantitative and isotopic analysis of methane and sulfate and for the determination of microbial populations. We note that the core for the quantitative and isotopic analysis of methane and sulfate at Acre location entered the sediment completely; therefore, the top 1 cm layer could be compressed or lost. The control core was taken 50 cm from the black patch edge to probe microbiota which could be compressed or lost. The control core was taken from sediments less affected by hydrocarbon enrichment; the sediment completely; therefore, the top 1 cm layer was observed and sampled at the Acre location, 32°08.9668′N 34°07.6177′E, at water depth of 1134 m (Palmachim; Fig. 1). The cores used for the microbial population analysis were immediately sliced to 1 cm fractions, 0.5–1 mL sediment aliquots were collected in triplicates from each layer and flash-frozen in liquid nitrogen for further processing.

**Total sulfide and pH measurements**

H₂S concentrations within the sediment were measured with H₂S-100 sensors (Unisense, Denmark). The signal was amplified with a Microsensor Multimeter (Unisense) and processed in the SENSOTRACE BASIC 3.0 software (Unisense). The total sulfide was calculated accounting for pH, salinity, and temperature according to equations given previously (Millero et al., 1988; Jeroschewski et al., 1996). The pH measurements were performed in pore water extracted by centrifugation, preserved at 4 °C following the addition of Hg to stop biological activity; the total sulfide was measured in the flash-frozen sediment samples.

**Determining sulfate and methane concentration**

Sulfate concentrations were measured by high-performance liquid chromatography (HPLC, Dionex DX500) with an error of 3% between duplicates. DIC concentrations were measured according to the peak height and calibration curve on the gas source isotopic ratio mass spectrometer (GS-IRMS, Thermo, at Ben Gurion University) with an error of 0.2 mM. For methane and δ¹³C CH₄ measurements, a special corer with side holes (1 cm in diameter) has been designed for quick and more precise subsampling to prevent methane loss due to pressure release. Two milliliters of the sediment was taken using cut edge syringe into a flushed argon bottle containing 5 mL NaOH (1.5 N), and the bottle was sealed with crimper. One milliliter headspace sample was taken from the crimped vial with a gas-tight pressure lock after the bottle was shaken vigorously. CH₄ in the headspace was measured on a Focus Gas Chromatograph (Thermo) with ShinCarbon column with precision of 2 μM L⁻¹ (Adler et al., 2011).

**Determination of carbon isotopic composition**

δ¹³C DIC and δ¹³C CH₄ were measured by the GS-IRMS at Ben Gurion University through a GasBench II (GBII) interface. The external errors were 0.1% for δ¹³C DIC and 1% for δ¹³C CH₄ between duplicates. The values were reported vs. Vienna Pee Dee Belemnite (VPDB) standard.

**Determination of sulfur and oxygen isotopic composition in sulfate**

The sulfur and oxygen isotope composition of the pore fluid sulfate was analyzed in the Godwin Laboratory at the University of Cambridge. Supersaturated barium chloride was added to samples to fix the sulfate as barite (BaSO₄). This barite precipitate was pyrolyzed at 1450 °C in a Temperature Conversion Element Analyzer (TC/EA), and the resulting carbon monoxide (CO) was measured by continuous flow GS-IRMS (Delta V Plus) for its δ¹⁸O SO₄. For the δ⁳⁴S SO₄ analysis, the barite was combusted at 1030 °C in a Flash Element Analyzer (EA), and resulting sulfur dioxide (SO₂) was measured by continuous flow GS-IRMS (Thermo, Delta V Plus). Samples for δ¹⁸O SO₄ were run in replicate, and the standard deviation of these replicate analyses was used (1σ < 0.4%ois). The error for δ⁳⁴S SO₄ was determined using the standard deviation of the standard NBS 127 at the beginning and the end of each run (1σ < 0.3%ois). Samples for both δ¹⁸O SO₄ and δ⁳⁴S SO₄ were corrected to NBS 127. (δ¹⁸O SO₄ = 8.6%ois and δ³⁴S SO₄ = 20.3%ois), IAEA SO-6 (δ¹⁸O SO₄ = –32.1%ois), and an internal standard (EMB – δ¹⁸O SO₄ = 15%ois).

**TEFAP 454 pyrosequencing and data analysis**

DNA was isolated from flash-frozen sediments using PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) following manufacturer’s instructions. 16S-based tag-encoded FLX amplicon pyrosequencing (TEFAP) was performed at MR DNA (Shallowater, TX, USA) to determine microbial biodiversity (Dowd et al., 2008a, b, c). The bacterial 16S
rRNA was amplified using the 16S universal eubacterial primer set 104F: 5′ GGCG VCA CGG GTG AGT AA-3′ and 530R: 5′ CCG CNG CNG CTG GCA C-3′ (Wang & Qian, 2009). The archaeal 16S rRNA was amplified using the 16S archaeal primer set 349F 5′-GYG CAS CAG KCG MGA AW-3′ and 806R 5′-GGA CTA CVS GGG TAT CTA AT-3′ (Takai & Horikoshi, 2000). A single-step 30-cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: denaturation at 94 °C for 30 s; annealing at 53 °C for 40 s and elongation at 72 °C for 1 min for 28 cycles. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA). The samples were sequenced utilizing 454 GS FLX titanium (Roche, Penzberg, Germany) following manufacturer’s guidelines.

The data derived from the sequencing were processed using a proprietary analysis pipeline (Dowd et al., 2008a, b, c; Capone et al., 2011; Eren et al., 2011; Swanson et al., 2011) at MR DNA (Shallowater, TX, USA). The sequences were depleted of barcodes and primers, short sequences...
< 200 bp were removed, sequences with ambiguous base calls were removed, and sequences with homopolymer runs exceeding 6 bp were removed. Sequences were denoised and chimeras were removed. Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity). OTUs were then taxonomically classified using BLASTN against a curated Greengenes database (DeSantis et al., 2006) and compiled into each taxonomic level. Most abundant pyrotags were classified using BLASTN against NCBI database.

**Phylogenetic analysis**

Evolutionary history was deduced using the maximum-likelihood method based on Kimura 2-parameter model (Kimura, 1980). A discrete gamma distribution was used to model evolutionary rate differences among sites. The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Initial trees for the heuristic search were obtained automatically as follows: When the number of common sites was < 100 or less than one-fourth of the total number of sites, the maximum parsimony method was employed; otherwise BIONJ method with MCL distance matrix was used. Codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The archaeal sequences were submitted to GenBank with accession numbers KF529982–KF530026; bacterial accession numbers are KF199299–KF199321, KF530027, KF543057–KF543064.

**Results**

**Sampling sites geochemistry**

Large carbonate crust features were found within the pockmarks at the toe of the Palmachim disturbance; no carbonate crust was observed at the Acre location. The Palmachim carbonate has a light carbon isotope composition ($\delta^{13}C = -29.3\%$), which could be indicative of methane-related carbon source (e.g. Heijts et al., 2006). Water temperature at the sampling sites was no higher than 0.6 °C above the ambient 13.5 °C. Active bubbling of gas from the sediment was visually determined at the Palmachim site. Total sulfide concentration ranged from 8.9 mmol L$^{-1}$ close to the sediment surface up to 29.28 mmol L$^{-1}$ at 8 cm below the SWI (Acre); 0.017 mmol L$^{-1}$ close to the sediment surface up to 0.13 mmol L$^{-1}$ at 10 cm (Acre Control); and 0.62 mmol L$^{-1}$ close to the sediment surface up to 4.18 mmol L$^{-1}$ at 5 cm (Palmachim; Fig. 2a). The sulfate concentrations decreased from 26.5 mmol L$^{-1}$ at the SWI to 5.2 mmol L$^{-1}$ at 7 cm at the Acre location, and from 31.9 to 16.5 mmol L$^{-1}$ at the Palmachim location (Fig. 2b). The methane concentration at the Acre site was 269.7 μmol L$^{-1}$ at the SWI and had two peaks: 768 μmol L$^{-1}$ at 1.5 cm and 1429 μmol L$^{-1}$ at 4.5 cm. At the Palmachim site, methane concentrations were 31.7 μmol L$^{-1}$ at SWI and had a peak of 776 μmol L$^{-1}$ at 14 cm (Fig. 2b). The pH was generally more alkaline at the Acre location, with maximum of 8.32 at 3 cm; the Palmachim location had a pH minimum of 7.82 at 3 cm (Fig. 2c).

Total organic carbon (TOC) content was generally higher at the Acre locations (Fig. 2d). TOC decreased gradually from c. 0.8% at the surface to c. 0.6% at 5 cm in the control core; it fluctuated around 0.8% at the Acre core and increased from 0.3% to over 0.5% at 5 cm of Palmachim core.

**Isotopic composition of sulfate and methane**

At the Acre location, the maximum sulfur isotope composition of pore fluid sulfide was observed between 6 and 9 cm below the SWI, with $\delta^{34}S_{SO_4}$ reaching 53.6 $\%_o$ and $\delta^{18}O_{SO_4}$ reaching 20.4 $\%_o$ (vs. seawater values of 20.3 $\%_o$ and 8.6 $\%_o$, respectively; Fig. 2e). The $\delta^{13}C_{CH_4}$ had a minimum of ~80.6 $\%_o$ PDB at 5.5 cm. The sulfate-sulfur and oxygen and methane isotope profiles are mirror images of one another (Fig. 2e). At the Palmachim location, the $\delta^{34}S_{SO_4}$ and $\delta^{18}O_{SO_4}$ increased gradually to 32.3 $\%_o$ and 14.1 $\%_o$, respectively, reaching their maximum at 10.5 cm below the SWI (we were not able to measure these isotopes lower in the sediments due to low concentrations). The increases in the $\delta^{34}S_{SO_4}$ and $\delta^{18}O_{SO_4}$ are linearly correlated with one another at both locations, resulting in slopes of 0.44 ± 0.06 (95% confidence interval) and 0.34 ± 0.06 for the Palmachim and Acre cores, respectively (Fig. 2g). In Palmachim sediments, the $\delta^{13}C_{CH_4}$ reached ~70.4 $\%_o$ PDB at 14 cm and remained stable (Fig. 2f); the $\delta^{13}C_{DIC}$ also decreased sharply, from −19.1 $\%_o$ PDB at 0.5 cm to −52.2 $\%_o$ PDB at 10.5 cm (Fig. 2h).

**Archaeal community composition**

The Acre control sediment was dominated by *Thaumarchaeota* (90–96% from total archaeal sequences; Fig 3a–c). Four major groups of *Thaumarchaeota* were identified in the sediments based on clustering of most common OTUs (Supporting Information, Fig. S1). The relative abundance of pyrotags in Group 1, closely related to *Nitrospumilus* and *Giganthauna* genera, increased next to the SWI, but also in the 3 cm layer of Palmachim core (Fig. 3d–f). Group 2 relative abundance increased from 21% at the sediment–water interface to 31% at 5 cm depth in the control core, decreased from 19% at
SWI to 12% at 5 cm depth in the Acre core and constituted c. 12% in the 1 and 3 cm sections of Palmachim core (Fig. 3d–f). Group 3 had 22% and 14% maximum relative abundance in control and Acre cores, respectively; group 4 had 9% and 5.5% maximum relative abundance in control and Acre cores, respectively (Fig. 3d–f). Groups 3 and 4 were uncommon in all Palmachim sections (below 1.5% relative abundance; Fig. 3d–f). The relative abundance of groups 3 and 4 increased linearly, correlating with TOC ($R^2 = 0.79$ and $R^2 = 0.90$, respectively, Fig. S2).

The hydrocarbon metabolizing Euryarchaeota was abundant in eucinic sediments at Acre and Palmachim and < 10% in control samples, where it was mainly represented by *Methanococcus* and *Methanobacteria*-related pyrotags (Fig. 3g–i). Acre core was dominated by *Methanococcus* genus-related pyrotags (31–49% of total archaeal sequences). Only one more genus, *Methanothermobacter* (11% at 5 cm section) was represented with abundance above 2%, although the pyrotags grouped with this genus were only up to 80% similar to any known sequence. In Palmachim core, euryarchaeal population was more diverse: *Methanococcus* (15–60% of total archaeal sequences), *Methanosarcina* (15% of total archaeal sequences at 4 cm section), *Methanobrevibacter* (5–14% of total archaeal sequences), *Thermococcus* (4–9% of total archaeal sequences), *Methanobacterium* (4–7% of total archaeal sequences), and *Methanococoides* (3–6% of total archaeal sequences) were most common.

We have analyzed in-depth pyrotags representative of the most abundant euryarchaeal OTUs (Fig. S3). ANME-related pyrotags were deduced from clustering with previously identified ANME sequences (Knittel et al., 2005; Lösekann et al., 2007). ANME-2c, ANME-3, and two potential ANME-related groups were identified (Fig. 3j–l). In Acre core, ANME-3 (5–15% of total archaeal sequences), potential ANME group A (11–30% of total archaeal sequences), and potential ANME group B (5% of total archaeal sequences at 4 and 5 cm sections) were abundant. These sequences were virtually absent (< 0.5%) in Palmachim core. On the other hand, ANME-2c-related *Euryarchaeota* was present in Palmachim core (5% of total archaeal sequences at 4 cm section). Interestingly, the most abundant pyrotags in Palmachim core (up to 48% of total archaeal sequences at 5 cm section) grouped in unidentified group A were 22% diverged from closest known cultured *Archaea Methanococcus maripaludis*, and 7% diverged from closest uncultured sequence isolated from hydrothermal sediments of the Bransfield Strait in Antarctica (unpublished). Unidentified group B (possibly MBG-D) was most abundant at the 3 cm section of Palmachim core (4%). Two other MBG-D-related groups were present only in Palmachim sediment. The only OTU making unidentified group C was present only in deeper Acre sediments and was distantly related (76% similarity) to *Methanobrevibacter arborophilus*.

**Bacterial community composition**

bTEFAP analysis of the relative abundance of microbial population was recently described as accurate, with maximum standard deviation of c. 5% (Pilloni et al., 2012), despite several possible biases (Shakya et al., 2013). This method is widely used to describe bacterial population diversity (Frank et al., 2013; Zened et al., 2013). A high complexity of microbial population was detected in all cores (Fig. 4a–c, Figs S4 and S5). The Acre control core was dominated by gamma proteobacterial OTUs (up to 38% from total bacterial sequences at 2 cm layer), mainly represented by *Ectothiorhodospiraceae* and *Halomonadaceae*, that can potentially use reduced sulfur species as electron donors for catabolism (Gorlenko et al., 2004; Sorokin et al., 2007). These OTUs were also common at the top 3 cm of Palmachim core (38–40%). The percentage of δ-proteobacteria, mainly represented by the sulfur-reducing order *Desulfobacterales*, increased with depth from 8.5% to 18.5% of total bacterial sequences. δ-proteobacteria were more abundant in hydrocarbon-rich samples, reaching 73.5% of total bacterial sequences at the 5th cm of the Acre core. *Desulfo bacter*–like species were more common throughout Acre core, while *Desulfococcus*–like species were more common throughout Palmachim seep core. Epsilon proteobacterial OTUs were below 1% at the control core, while they were present in hydrocarbon-rich samples, displaying vertically decreasing profiles at Acre (32% at 1 cm, 0.2% at 5 cm) and fluctuating between 9% and 18% at Palmachim core. Chemolithoautotrophic *Sulfovorum* and *Sulfurimonas* genera (Inagaki, 2003; Inagaki et al., 2004) were the main epsilon proteobacterial OTUs in hydrocarbon-rich sediments, usually abundant at the surface, and showing a decrease in relative abundance toward the deeper sections of the sediment. Moreover, 13% of surface sediment sequences in the Acre core possibly belonged to the *Arcobacter* genus.
Arcobacter nitrofigilis, often involved in sulfide oxidation and nitrogen fixation (Sievert et al., 2007; Lavik et al., 2009; Grünke et al., 2011). Pyrotags representing c. 12% from bacterial sequences at 5 cm sediment depth in Acre core clustered with anaerobic denitrifying *Caldithrix* genus, (Miroshnichenko, 2003). Alphaproteobacteria were highly abundant in control core (5–10.5% clonal frequency), represented by *Rhodospirillaceae* spp. with 93–94% similarity to *Rhodovibrio* genus and 99% similarity to *Rhodospirillaceae* spp. described at polymetallic nodule fields of the Clarion-Clipperton Fracture Zone (Wang et al., 2010). Actinobacterial fermenters *Bifidobacterium* sp., 99% similar to honeybee gut *Bifidobacterium* sp. (Martinson et al., 2011), *Lactobacillus* sp. 99% similar to honeybee gut *Lactobacillus* sp. (Martinson et al., 2011) and gamma-proteobacterial fermenters 99% similar to *Gil-
and 5 cm distance from the sediment surface. SEEP-SRB-1a clonal frequency increased in correlation with distance from sediment surface and reached 12% at 5 cm section. In the Palmachim sediment, SEEP-SRB group of pyrotags, 98% similar to *Calyptogena* clam patch bacteria described as putative ANME synthrophs (Orphan et al. 2001), were most common at 4 and 5 cm sections (8% and 7.5% clonal frequency, respectively). Desulfobulbus-related pyrotags were more abundant in the 1 cm section (3%).

To find potential ANME-SRB syntrophy in the sampled sediments, we have analyzed the correlation between relative abundances and SRB (Table 1). Relative abundances of ANME-2c and *Calyptogena* clam patch SEEP-SRB had significant positive correlation; relative abundances of ANME-3 and potential ANME groups A and B had significant positive correlation with *Desulfobacter*-related OTUs and SEEP-SRB-1a clonal frequencies.

**Discussion**

The pore fluid geochemistry provides evidence for the different microbial processes that occur within the sediment. Methane concentrations increase and sulfate concentrations decrease with distance below the SWI at both the Palmachim and Acre locations (Fig. 2a and b), indicative of methanogenesis, methanotrophy, and BSR. In the Acre sediments, sulfate is consumed c. 2.8 times faster and far higher sulfide concentrations are present next to the SWI compared with Palmachim sediments. Moreover, the increase in methane concentration with depth is c. 2.7 larger in Acre compared with Palmachim. These highly similar values hint at a higher flux of hydrocarbons toward the SWI in Acre sediments, although the top 3 cm section of Palmachim sediments was slightly oxygenated, based on the similarity of microbial composition between 1 and 3 cm sections and on lighter color of the sediment, which may have hindered methanogenesis, AOM, and BSR. This may be a result of oxygenated pore water transport or bioturbation/bioirrigation in the sampled area. Due to the nature of hydrocarbon seeps, however, the determination of sulfate and methane production and consumption based solely on the concentrations profiles can be inaccurate and should be treated with caution. Methane at these sites is often in excess and escapes by ebullition, hence standard diffusion-dominated pore fluid profiles are not
readily established. To explore this further, we use DIC concentration and $\delta^{13}$C$_{DIC}$ to determine the source of DIC (oxidation of methane vs. other hydrocarbons). The DIC concentration at the Palmachim site increases to $c.$ 10.7 mmol L$^{-1}$ (compared with $c.$ 2 mmol L$^{-1}$ at the SWI) with $\delta^{13}$C$_{DIC}$ of $-52.2 \%_{oo}$ (compared with water column DIC at 0%oo, Fig. 2h). A $\delta^{13}$C value between $-73\%_{oo}$ and $-67 \%_{oo}$ similar to the measured $\delta^{13}$C$_{CH4}$ (c. $-70 \%_{oo}$ in the deeper part of the Palmachim core, Fig. 2f), is required to yield the sharp decrease in the $\delta^{13}$C$_{DIC}$ within the Palmachim pore fluid. This suggests that in the Palmachim sediments, the entire DIC pool within the pore fluids is produced from the oxidation of methane. Unfortunately, the DIC and $\delta^{13}$C$_{DIC}$ measurements are absent at the Acre site, so we are unable to calculate the isotope constraints on methane oxidation vs. other organic molecules.

The negative correlation between $\delta^{13}$C$_{CH4}$, $\delta^{18}$O$_{SO4}$, and $\delta^{34}$S$_{SO4}$ with methane and sulfate concentrations lends further support for methanogenesis, AOM, and BSR occurring in these sediments. The rate of BSR can be estimated from the shape of the cross-plot between sulfur and oxygen isotopes in dissolved sulfate ($\delta^{18}$O$_{SO4}$ vs. $\delta^{34}$S$_{SO4}$, Aharon & Fu, 2000; Antler et al., 2013). At the Palmachim and Acre sites, we find moderate slopes in the $\delta^{18}$O$_{SO4}$ vs. $\delta^{34}$S$_{SO4}$ cross-plot, 0.44 ± 0.06 and 0.34 ± 0.06, respectively (Fig. 2g). Given that this slope can vary between 0.27 and 10, we consider a mechanistic similarity in the sulfate turnover at both sites. Lower slopes in a $\delta^{18}$O$_{SO4}$ vs. $\delta^{34}$S$_{SO4}$ cross-plot typically correlate with higher rates of BSR rates (c. $10^{-5}$ mol cm$^{-2}$ year$^{-1}$; Aharon & Fu, 2000). These high rates of BSR suggested from our isotope geochemistry are similar in magnitude to what has been found in previously studied hydrocarbon seeps and hydrocarbon-enriched estuary sediments (Aharon & Fu, 2000).

A marked property of the Acre sediment is an increase in sulfate concentrations in the deeper part of the core (Fig. 2b). Below c. 8 cm section, there is also a reciprocal trend in $\delta^{18}$O$_{SO4}$ and $\delta^{34}$S$_{SO4}$ (Fig. 2e). The $\delta^{13}$C$_{CH4}$ was measured only down to 8.5 cm, and its negative peak was observed in the 4.5–5.5 cm section, yet the methane concentrations increased in the deeper 8.5 cm section. This indicates that a maximum of methane production/transport occurs in c. 5 cm section, followed by a peak in BSR. These profiles are a result of the unique setting of the Acre core that was sampled perpendicular to the surface of $70–90^\circ$ slope of a pockmark. The mirroring of the metabolite concentrations and their isotopic composition in the Acre location could be explained by two scenarios: a horizontal sampling through the extension of a vertical hydrocarbon-rich vein or a hydrocarbon enrichment of defined volume at this location, such as a buried carcass. The spatial span of sulfate concentration minimum and the maximum values of $\delta^{34}$S$_{SO4}$ was only c. 2 cm, while $\delta^{13}$C$_{CH4}$ had a minimum not larger than 1 cm; hence, the cross-section of the hydrocarbon enrichment is probably < 2 cm in diameter. We note that this core was pushed fully into the sediment by the ROV; therefore, there can be an offset in the profile, and the hydrocarbon enrichment peak can be actually c. 1 cm deeper. This is also a reason for the absence of surface water isotopic data in the Acre location.

In the Acre sediments, three major clusters of ANME were present. Beside potential ANME group B present in the deeper sections of the sample, one cluster was identified as ANME-3, while the cluster named potential ANME group A was also related to ANME-3 and Methanococoides genus. ANME-3 and potential ANME group A constitute together up to 44% of total archaeal sequences in Acre core, compared with < 1% in all Palmachim sections. Several SEEP-SRB groups were identified previously as ANME-3 synthrophs: Desulfobulbaceae (DBB)-related cluster including SEEP-SRB4 (Niemann et al., 2006a, b; Lösekann et al., 2007) and SEEP-SRB1a (Schreiber et al., 2010), both found within Acre sediments. ANME-3/DBB aggregates were described in the first 1–3 cm of Haakon Mosby Mud Volcano sediments underlying Beggiatoa sp. mats (Lösekann et al., 2007); here, we find SEEP-SRB4

<table>
<thead>
<tr>
<th></th>
<th>SEEP-SRB clam patch</th>
<th>Desulfobacteraceae</th>
<th>SEEP-SRB-1a</th>
<th>SEEP-SRB4</th>
<th>Desulfobulbus related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unidentified group A</td>
<td>0.959 (1.13 x 10$^{-2}$)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unidentified group B</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unidentified group C</td>
<td>–</td>
<td>–</td>
<td>0.900 (3.85 x 10$^{-4}$)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MBGD-related A</td>
<td>0.849 (1.83 x 10$^{-2}$)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MBGD-related B</td>
<td>0.937 (6.43 x 10$^{-2}$)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Potential ANME B</td>
<td>–</td>
<td>0.777 (8.12 x 10$^{-3}$)</td>
<td>0.951 (2.42 x 10$^{-5}$)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Potential ANME A</td>
<td>–</td>
<td>0.872 (1.01 x 10$^{-3}$)</td>
<td>0.651 (4.13 x 10$^{-2}$)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ANME-2c</td>
<td>0.919 (1.72 x 10$^{-4}$)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ANME-3</td>
<td>–</td>
<td>0.955 (1.74 x 10$^{-5}$)</td>
<td>0.856 (1.59 x 10$^{-2}$)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
mainly in 1–2 cm sections where they play potentially similar roles. The DBB bacteria were also present in 1–3 cm sections of Acre sediment, indicative of their affinity to a niche with higher sulfate concentrations. Within the deeper sediment sections, SEEP-SRB1a replaces SEEP-SRB4. The most abundant SRB OTU 2 did not cluster with previously identified SEEP-SRB sequences, but was related to Desulfobacter and Desulfobacula genera, facultative anaerobes that can utilize various electron donors, including H₂ (Widdel, 1987; Wöhlbrand et al., 2013), acting as electron shuttle between ANME and SRB (Alperin & Hoehler, 2010). The abundance of this group is most significantly correlated with the relative abundance of ANME-3, hinting at their syntrophy in this niche.

Yet, the ability of ANME–SRB complexes to solely oxidize methane was questioned, and methanogenesis by the latter microbiota was suggested (Alperin & Hoehler, 2009a, b, 2010). ANME-3 sequences are highly similar phylogenetically to methylamine/trimethylamine utilizing methanotrophic genera Methanococoides and Methanothrix bus (Sowers & Ferry, 1983; Singh et al., 2005; Mochimaru et al., 2009; Antony et al., 2011). Trimethylamine is a product of choline degradation, often found in decomposing organic matter (Hippe et al., 1979). The trimethylamine consuming methanogenesis does not compete with otherwise inhibitory sulfur reduction for intermediates such as acetate and H₂ (Garcia et al., 2000; Antony et al., 2011; Siegert et al., 2011); hence, it can coexist with SRB, resulting in a peak of both methane and sulfide production in the same sediment section. Hypothesizing that Acre sediment can be enriched in more complex hydrocarbons, it is possible that such process can induce local methanogenesis in a sulfide-rich environment and provide an explanation for the secondary methane δ¹³C peak in shallow Acre sediment (Fig. 2e).

A different archaeal consortium populates the Palmachim sediment core. The dominant unidentified group A, diverged by 22% from the nearest cultured Methanococcus maripaludis, is not related to any known ANME sequence, and due to its high abundance yields an important novel methanogenic/methanotrophic cluster. Three other groups were related to MBG-D [including unidentified group B that was related to previously described MBG-D Archaea (Dang et al., 2010)]. This group has a potential to degrade detrital proteins (Lloyd et al., 2013), although methanogenic Methanomassiliicoccus spp. (Dridi et al., 2012) are also affiliated with this cluster. Unidentified groups A and B were also present as minor fraction of Acre sediments, representing the small non-ANME euryarchaeal population. The AOM potential of the Palmachim sediment microbial community is inferred from the presence of ANME-2c sequences, although their maximum abundance did not exceed 5%, significantly lower than in Acre samples. This aspect is interesting, as methane reaches the surface of Palmachim sediments, as indicated by ebullition, supposedly placing the main AOM niche SMTZ next to the SWI (Niemann et al., 2006a, b; Sommer et al., 2006). Yet, sulfate concentrations and its isotopic composition change only below 4.5 cm, defining a deeper SMTZ that creates a niche for most abundant euryarchaeal population. Moreover, the relative abundance of potential SEEP-SRB was lower in Palmachim sediments compared with Acre, and the major group was present mainly in the 4–5 cm sections. This was reflected in lower sulfide concentrations that correlated linearly with SRB abundance (R² = 0.65; Fig. S7). The clam field SEEP-SRB group, most abundant at 4–5 cm SMTZ, had most significant covariance with the relative abundance of unidentified group A pyrotags, potentially identifying these microorganisms as syntrophs. Moreover, the small fraction of SEEP-SRB 1a, a potential partner of ANME-2 (Schreiber et al., 2010), was also present in Palmachim sediments, and its relative abundance covaried with the relative abundance of ANME-2c only in Palmachim sediment, suggesting that ANME-2c additionally metabolize syntrophically.

The marked difference between the sampled locations is the TOC wt.%, doubled in Acre and Acre control sediments, compared with Palmachim sediments. The relatively low TOC fraction within Palmachim sample is comparable to the value of 0.4 wt.%, previously described as biologically refractory organic matter, yielding maximum sulfate-dependent AOM and limiting degradation of nonmethane organic matter (Pohlman et al., 2013). TOC values were significantly higher within Acre sediments: in the control sample, the deeper sections had lower TOC content as a result of diagenetic processes (Burdige, 2006). On the other hand, TOC enrichment occurred in deeper euxinic sediments that can be caused by flux of buried hydrocarbons. Although the geological process forming the pockmark field in Acre location is unknown, previous affiliation of pockmark fields with hydrocarbon seepage (Hovland et al., 2002, 2010; Judd & Hovland, 2007; Cathles et al., 2010) implies that hydrocarbon seepage in this location is possible. Moreover, not only methane, but larger hydrocarbons/oil seepage can cause such large TOC enrichments (Hovland, 2007; Cathles et al., 2010). The dominance of ANME-3 in Acre sediments may be a result of the presence of larger hydrocarbons, as this group is mainly restricted to submarine mud volcanoes (Knittel & Boetius, 2009). Beside the CH₄ ebullition, mud volcanoes are defined by the efflux of brines with complex organic materials (Milkov, 2000). Thaumarcheal groups 3 and 4 that phylogenetically cluster with mud volcano and seep sediment sequences were also affiliated with high TOC concentrations.
Marine Thaumarchaeota are abundant autotrophic nitrifiers in the oxygenated water column (Könneke et al., 2005; Treusch et al., 2005; Francis et al., 2007), although their role in anoxic environments is still unclear (Pester et al., 2011). Some thaumarcheal specimens from marine sediments are able to degrade detrital proteins (Lloyd et al., 2013) and provide metabolites to anammox bacteria (Lam et al., 2007; Woebken et al., 2007). Moreover, mixotrophic and heterotrophic metabolism was demonstrated in Thaumarchaeota (Herndl et al., 2005; Agogué et al., 2008; La Cono et al., 2011; Mussmann et al., 2011; Xu et al., 2012). The ability of Thaumarchaeota to compete with methylo trophs for substrates was speculated, based on the ability of ammonia-oxidizing bacteria to oxidize methane (Ettwig et al., 2008; Pester et al., 2011) and sequestration of methane emission from soils following fertilizer addition (Bodelier, 2011). Interestingly, bacterial ammonia oxidizers can catabolize polycyclic aromatic hydrocarbons, products of organic matter pyrolysis (Chang et al., 2002) that can be present in hydrocarbon-enriched sediments. Hence, thaumarcheal groups 3 and 4 may be involved in reworking of organic compounds and proteins in high TOC sediments. Thaumarchaeal group 1 most closely related to water column nitrifying Nitrosopumilus genus and more abundant close to SWI. Several sediment and seep sequences are also included in this group, including symbiotic Giganthauma genus, found in sulfidic marine habitats (Muller et al., 2010). Nitrification products can inhibit methane oxidation (Bodelier, 2011); hence, association of this group with minimal AOM sections hints that it indeed oxidizes NH₄. Conversely, nitrate-dependent AOM was suggested for shallow sediments with low organic matter concentration and high nitrate concentration (Thauer & Shima, 2006) that can be provided by Thaumarchaeota. Such conditions may exist in 2 cm section of Palmachim sediments. Group 2 was also affiliated with various anoxic environments and constitutes the majority of thaumarcheal sequences in Acre cores. In the SMTZ of Palmachim sediment, Thaumarchaeota virtually disappear, lacking substrates or unable to compete with methanotrophy/methanogenesis/SRB.

Several other bacterial groups shall be noted to better understand the microbial processes at the deep SWI. High abundance of fermenters, virtually identical phylogenetically to Lactobacillus, Bifidobacterium, and Gilliamella genera found in the honeybee gut microbiota was uncovered at the SWI of the control core. The main property of this distinctive microbiota is the ability to digest the nutrient-rich pollen cytoplasm, refractory to most digestive systems due to protection by a carbohydrate exine (Martinson et al., 2011). We hypothesize that the sediment–water interface microbiota specializes in digestion of the refractory fraction of marine snow that is unoxidized by the water column microorganisms. These bacteria were absent in the SWI sections of both Palmachim and Acre hydrocarbon-enriched sediments, probably as the fermenters were unable to compete with the microorganisms that utilize other hydrocarbon sources and electron acceptors that provide higher energetic yields. We note that these bacteria were found in additional locations in the deep Mediterranean sediments, all associated with abundant refractory organic matter (data not shown).

Epsilon proteobacterial pyrotags, mainly clustered with sulfide-oxidizing bacteria, were minor within control sediments, indicative of insufficient sulfide supply. In contrast, alpha protobacterial pyrotags, indicative of non-seep-related productive systems (Polymenakou et al., 2009), were abundant by an order of magnitude in control sediments compared with euxinic sediments. Caldithrix-related OTUs were abundant in deeper sections of Acre euxinic sample. Caldithrix reduces nitrate under anaerobic conditions (Miroshnichenko, 2003), potentially providing NH₄ for nitrifiers.

Conclusions

We conducted high-resolution study of microbial communities in two locations in the Levantine basin, the methane seeps in scarp at the toe of Palmachim disturbance and the hydrocarbon-enriched sediments within the pockmark area offshore Acre. TOC fraction and hydrocarbon source are the main factors dictating the microbial community structure in these locations. Based on geochemical and TEFAP analysis, we determined that AOM, carried out by ANME and SRB syntrophs, occur in the shallow sediments, although different ANME and SRB groups are involved in AOM at both locations. Moreover, four main clusters of Thaumarchaeota were identified. Thaumarchaeota are the key players in the nitrogen cycling in both euxinic and 'normal' sediments, although the exact function of these microbes in the hydrocarbon-rich sediments is still unclear. We provide a snapshot of the deep-sea microbial community that is highly complex and variant over short spatial gradients and fuels the cycling of C, N, S, and other metabolites next to the SWI.

References


Hydrocarbon microorganisms – Levantine basin


Takai K & Horikoshi K (2000) Rapid detection and quantification of members of the archaeal community by


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

*Fig. S1.* Maximum-likelihood phylogenetic tree showing the position of sediment main thaumarchaeal 16S rDNA OTUs.

*Fig. S2.* Covariance of TOC wt. % and relative abundance of major thaumarchaeal groups.

*Fig. S3.* Maximum-likelihood phylogenetic tree showing the position of sediment main euryarchaeal 16S rDNA OTUs.

*Fig. S4.* The relative abundance of bacterial genera along the profiles in the sampled sediments deduced from the similarity of pyrotags to sequences from Greengenes database.

*Fig. S5.* Maximum-likelihood phylogenetic tree showing the position of sediment representative bacterial 16S rDNA OTUs.

*Fig. S6.* Maximum-likelihood phylogenetic tree showing the position of sediment most abundant delta-proteobacterial 16S rDNA OTUs.

*Fig. S7.* Covariance of reduced sulfur concentration and relative abundance of SRB.