Diversity and spatial distribution of prokaryotic communities along a sediment vertical profile of a deep-sea mud volcano

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ABSTRACT

We investigated the top 30 cm sediment prokaryotic community structure in 5 cm spatial resolution, at an active site of the Amsterdam mud volcano, East Mediterranean Sea, based on the 16S rRNA gene diversity. A total of 339 and 526 sequences were retrieved, corresponding to 25 and 213 unique (≥98% similarity) phylotypes of Archaea and Bacteria, respectively, in all depths. The Shannon-Wiener diversity index H was higher for Bacteria (1.92-4.03) than for Archaea (0.99-1.91) and varied differently between the two groups. Archaea were dominated by ANME-1, -2 and -3 groups and were related to phylotypes involved in anaerobic oxidation of methane from similar habitats. The much more complex Bacteria community consisted of 20 phylogenetic groups at the phylum/candidate division level. Proteobacteria, in particular δ-Proteobacteria, was the dominant group. In most sediment layers, the dominant phylotypes of both the Archaea and Bacteria communities were found in neighbouring layers, suggesting some overlap in species richness. The similarity of certain prokaryotic communities was also depicted by using four different similarity indices. The direct comparison of the retrieved phylotypes with those from the Kazan mud volcano of the same field, revealed that 40.0% of the Archaea and 16.9% of the Bacteria phylotypes are common between the two systems. The majority of these phylotypes are closely related to phylotypes originating from other mud volcanoes, implying a degree of endemicity in these systems.

INTRODUCTION

Mud volcanoes (MV) are areas of active fluid seepage occurring both on land and at the seafloor [50]. Submarine MV are often associated with the presence of gas hydrates. During the past years, many studies have attempted to address the fate of methane originating from submarine MV, its impact on the global carbon cycle and the role of the in situ microbial communities on the production and the consumption of this potent greenhouse gas [7, 31, 38 and reference there in, 55]. Active marine MV could be considered as analogue habitats of fluidized or mobile muds (sensu Aller et al. [1]) since they consist of a mixture of fine-grained, creamy textured sediment and fluids, expanding down to 1 m or deeper, although these sediments can be remobilized due to irregular and unpredictable eruptions. Such habitats serve as efficient methane and sulfur reactors, hosting characteristically high bacterial and low archaeal diversity. It is expected that MV undergo temporal and spatial changes due to changes in gas/fluid fluxes, eruptions and gas hydrate association-dissociation, probably ensuing successive stages in microbial community structure and functioning.

In the eastern Mediterranean Sea, MV and cold seep areas were discovered during the late 1970s [13] while ongoing research has revealed several such formations on the accretionary prism of the Hellenic Arc (Mediterranean Ridge) and within the Anaximander Mountains [47, 85]. Amsterdam MV (AMSMV) has 'mud pie-like' topography and appears as a circular structure with a flat-topped mound at a water depth on its summit of 2025 m [46, 87]. Detailed morphological analysis of the AMSMV indicated that there are two discrete craters the "external" and the "internal" that merge to the southeast. Both are ellipses with dimensions of 6x5 km and 4x3.3 km, respectively, slightly elongated in an N–S direction. One common morphological characteristic of the craters is that they are both open in the southernmost part and directly connected to the slope with a 400 m wide canyon extending down to a depth of 2250 m [46].

AMSMV is considered as the largest and most active MV investigated in the Anaximander Mountains field based on the occurring mud breccia. It has been related to moderate to fast ascending fluidized mud and/or a relatively wide conduit [46, 87]. Seismic profiling and side scan sonar surveys across the mud volcanic structure revealed features attributed to recent mud extrusion in the crater and outflow downslope to the south [46, 85, 87]. Support for recent mud volcanic activity is provided from other data as well, such as the rough microtopography, the absence of extended areas with carbonate crusts on the seafloor, the observation of dewatering chimneys and the elevated concentrations of methane in the water column [12, 46, 53, 61, 87]. Active fluid seepage and gas bubble release are common at the summit, particularly in the eastern central part [87].

Considerable near-surface gas hydrate accumulations have been reported for several sites at the AMSMV, particularly close to the MV centre and its eastern sector [46, 61, 87]. High hydrostatic pressure, moderate bottom water temperatures (14.0°C), comparably high water activity and methane oversaturation generally favour gas hydrates to precipitate in shallow deposits of the AMSMV centre [61]. During eruptive phases, however, fluids from below might transport additional heat, leading to increases in temperatures exceeding hydrate dissociation temperatures in deposits covering the investigated depth interval. Compelling evidence support the theory of hydrate destabilization at shallow sub bottom depths, mainly ^{18}O enrichments of authigenic carbonates [2] and the high methane values in water above the AMSMV [12].

Although AMSMV is the largest MV in the Anaximander field, the microbial communities living in its active sediments have not yet been thoroughly characterised. However, there are a few studies providing evidence for the occurrence of AOM in AMSMV, based on the presence of specific lipid biomarkers and carbon isotopic signatures in carbonate crusts [2, 8] and in sediments [59, 60]. Only one study has used the construction of 16S rRNA gene libraries to reveal the inhabitants of AMSMV [29] but the studied sediment was away from the active site of the MV. We aimed at investigating the degree of vertical stratification or overlap between prokaryotic communities occurring in the active site of the AMSMV and how these communities are related to the prevailing geochemistry. To do so, we analysed the communities of Bacteria and Archaea at the

top 30 cm sediment of an active site in the AMSMV. Subsamples were taken every 5 cm and features of community structure based on 16S rRNA gene diversity were analysed. Moreover, we investigated possible spatial similarities between similar, but distant to each other, environments, and whether there are common phylotypes which are specific to mud volcanoes habitats.

MATERIALS AND METHODS

Sampling site. Box-core AN05BC05 with gas hydrate-containing sediment was collected from the AMSMV (35°20'02" N, 30°16'18" E) of the Anaximander Mountains, eastern Mediterranean Sea, with the R/V AEGEO in May 2003. Sediment sampling was conducted as described in [41]. In the current work the prokaryotic diversity at 0, 5, 10, 15, 20, 25 and 30 cm below sea floor (cm.b.s.f.) was analyzed. The detailed description of the geological setting and the geological parameters of the sampled site are reported in [46] while the concentrations of methane and sulfate were determined as described in [41]. In brief, samples for porewater extraction of box core sediments were taken immediately upon recovery into Greiner centrifuge tubes and were centrifuged for 20 minutes at 4000 rpm. Subsequently, the tightly-closed centrifuge tubes were transferred immediately in an oxygen-free glovebox, for filtering (0.2 um) and subsampling of the porewater samples [for details, see 26]. The subsamples intended for major elements and sulphate analyses were acidified using 25μl of a 5M HNO_3 solution per ml of porewater.

DNA extraction, amplification and cloning. DNA was extracted from 0.5 – 1 g of sediment from each depth using the UltraClean Soil DNA kit (MoBio Laboratories Inc., USA) following the manufacturer's protocol with minor modifications: bead beating was reduced from 10 to 5 min, and this step was immediately followed by three cycles of freeze-and-thaw (–80°C for 3 min and then immediately in 65 °C water bath for 5 min) after addition of the inhibitor removal solution. Bacterial 16S rDNA was amplified using the bacterial primers B8f-B1492r [74]. The PCR included an initial denaturation step at 94 °C for 1 min followed by 27 to 31 cycles consisting of denaturation at 94 °C for 45 s, annealing at 52.5 °C for 45 s, and elongation at 72 °C for 2 min; a final 7-min elongation step at 72 °C was added. Archaea 16S rDNA was amplified using the primer combination A8f and A1492r [74]. An initial denaturation step at 94 °C for 1 min was followed by 25 to 29 cycles consisting of denaturation at 94 °C for 45 s, annealing at 52.5 °C for 45 s, and elongation at 72 °C for 2 min; a final 7-min elongation step at 72 °C was added. The number of cycles was determined for each sample after cycle optimisation. PCRs were repeated with different cycle numbers, and the lowest number of cycles that gave a positive signal was then used for cloning and sequencing in order to avoid differential representation of 16S rDNA genes with low and high copy numbers [70]. Eight tubes of PCR products were pooled to reduce the biases of each individual reaction.

Polymerase chain reaction products were visualized on a 1% agarose gel under ultraviolet light, bands were excised, and PCR products were extracted with the Wizard SV Gel and PCR Clean-up kit (Promega Inc., USA) following the manufacturer's protocol. The PCR products were cloned using the TOPO TA for sequencing cloning kit (Invitrogen Corporation, USA) using electrocompetent cells according to the manufacturer's specifications. For each sample and each gene, randomly picked clones with inserts of the expected length were analysed. Clones were grown in liquid LB medium with kanamycin and their plasmids were purified using the NucleoSpin Plasmid QuickPure kit (Macherey-Nagel GmbH & Co. KG, Germany) for DNA sequencing. A number of 33 – 58 and 52 – 121 of archaeal and bacterial clones (true positives), respectively, was analysed in each sample.

Sequencing and phylogenetic analysis. Sequence data were obtained by Macrogen Inc. (South Korea) using capillary electrophoresis and the BigDye Terminator kit (Applied Biosystems Inc., USA) with the primers M13F(-20) and M13R. Every sequence read was approximately 900 bp and for each individual clone, forward and reverse reads were assembled. The sequences were screened for chimeras by comparing neighbour-joining trees made of the first and second halves of all sequences. The sequences that had different groupings in the first and second halves were then

checked using the Pintail program [4] (http://www.bioinformatics-toolkit.org/Web-Pintail/). Bellerophon software from GreenGenes [17] (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) was also used to detect chimeric sequences. All putative chimeras were excluded from further analysis.

For the detection of closest relatives, all sequences were compared with the BLAST function [3] (www.ncbi.nlm.nih.gov/ BLAST/). The sequences were automatically aligned against sequences from their closest relatives using SILVA alignment utility [65] (http://www.arb-silva.de/aligner/) and revised by manual removal of ambiguously aligned regions. Phylotypes or Operational Taxonomic Units (OTUs) were defined as sequences showing ≥98% homology to each other.

Phylogenetic trees were constructed by the neighbour-joining method using the Kimura 2-parameter. Bootstrap analyses for 1000 replicates were performed to assign confidence levels to the tree topology using the MEGA4 software [73]. Sequences of unique phylotypes found in this study have GenBank numbers HQ588634-HQ588688 (Archaea) and HQ588358-HQ588633 (Bacteria).

Clone library coverage, diversity and similarity analyses. Clone coverage was calculated using the equation $C = [1 - (n_i/N)]x100$, where n_i is the number of phylotypes and N is the number of 16S rRNA sequences examined [23, 37]. The Shannon-Wiener index H' was used as a diversity index and was calculated as follows: $H' = -\sum p_i \cdot \ln p_i$, where the summation is over all phylotypes i, and p_i is the proportion of clones belonging to phylotype i relative to the sum of all clones. The Pielou evenness index J was calculated as $J = H'/\ln S$, where S is the total number of phylotypes [64, 69].

The similarity among the microbial communities in all sediment layers was determined using four different indices of similarity: Morisita-Horn [32], Morisita [52], Jaccard Abundance adjusted and Sorensen Abundance adjusted [11]. Morisita-Horn and Morisita indices were chosen because they are widely applied and unbiased for sample size [84]. The most newly developed indices Jaccard Abundance adjusted and Sorensen Abundance were used as they were proved to be less biased when a substantial proportion of species are missing from samples [11]. Since similarity is a qualitative human construct and has no precise mathematical definition, all indices used to measure it inevitably hold some bias. For this reason all the above mentioned indices were used. Correspondence analysis based on Morisita similarity index was used in order to depict the differences of the prokaryotic communities along the sediment core.

The clone names used in this work indicate the sample site and library identification. The names begin with the letters AMSMV (as in Amsterdam MV), followed by a number showing the layer used to construct the library (0, 5, 10, 15, 20, 25, 30 cm.b.s.f.). The A or B character designates Archaea and Bacteria, respectively. The surface sampling was actually conducted in the 0-1 cm layer and, for simplicity reasons it is coded as 0 in the phylotype codes but annotated as "surface layer" in the text.

RESULTS

Geochemistry. The porewater sulfate concentration (Fig. 1) exhibited a decrease from bottom water values of near 30 mM to background values of less than 5 mM at ~22cm sediment depth. The methane content was low in the topmost few centimetres, but rapidly increased with sediment depth from 13 to 27 cm.b.s.f. (Fig. 1).

Archaea. The archaeal clone libraries coverage (Fig S1) according to Good's C estimator [37] was satisfactory (>0.8). A total of 339 Archaea 16S rRNA gene sequences were retrieved which were attributed to 55 phylotypes distributed in all sediment layers and 25 of them were unique for the whole core. Phylotypes grouped within the *Euryarchaeota* were the most abundant overall and the most abundant detected at each depth (Fig. 2, 3, S2).

Phylotypes belonging to ANME-1 group were retrieved in high abundances from all but 30 cm.b.s.f. libraries constructed. One ANME-1 phylotype was present at 0 (AMSMV-0-A18, 8.3%) and the same phylotype was retrieved at 5 cm.b.s.f. (as AMSMV-5-A10, 11.8%, Fig. 3). At 5

cm.b.s.f., two more phylotypes affiliated with ANME-1, AMSMV-5-A2 (23.5%) and AMSMV-5-A30 (2.0%) were found. ANME-1 phylotype AMSMV-0-A18 (or equivalently AMSMV-10-A33) dominated the 10 cm.b.s.f. layer, wherein comprised 54.6% of all Archaea clones sequenced in this library and 15 cm.b.s.f. layer (as AMSMV-15-A8, 45.5%). The same ANME-1 phylotype (AMSMV-20-A12, 32.8%) co-dominated 20 cm.b.s.f. together with AMSMV-0-A45 (or equivalently AMSMV-20-2, 31.0%) belonging to ANME-2a subgroup, and 25 cm.b.s.f. together with the ANME-2c, AMSMV-0-A12 (as AMSMV-25-A2, 39.1%).

The ANME-2 was the dominant group in the surface layer with AMSMV-0-A1 (Fig. 3), of the ANME-2b subgroup, the most abundant phylotype (37.5%). Two more ANME-2 phylotypes were found in the surface layer, AMSMV-0-A12 (16.7%) of ANME-2c subgroup and AMSMV-0-A45 (2.1%) of ANME-2a. At 5 cm.b.s.f. layer AMSMV-0-A45 (as AMSMV-5-A3) became the dominate phylotype (31.4%) but AMSMV-0-A1 (as ANME-5-A7) was also present (19.6%). These phylotypes were retrieved from 10 cm.b.s.f. layer, as well (AMSMV-0-A1 as ANME-10-A31, 25.4% and AMSMV-0-A45 as ANME-10-A35, 10.9%). The ANME-2a phylotype AMSMV-0-A45 was found at 15 cm.b.s.f. (AMSMV-15-A3, 9.1%) and 20 cm.b.s.f. (AMSMV-20-A2, 31.0%). At 20 cm.b.s.f. phylotype AMSMV-20-A2 (as AMSMV-5-A12) affiliated with group GoM Arc I, Gulf of Mexico Archaea I [44], formerly known as ANME-2d subgroup [48, 51], had significant representation (24.1%).

In the clone library from 30 cm.b.s.f. the dominant phylotype (70.8%), AMSMV-0-A33 (as AMSMV-30-A2), clustered within ANME-3 Euryarchaeota group (Fig. 3). This phylotype was also retrieved at 0 (AMSMV-0-33), 10 (AMSMV-10-A44) and 25 cm.b.s.f. (AMSMV-25-A12, but only with low abundance).

Phylotypes that grouped within MBG-D, Marine Benthic Group-D, (synonymous with marine group III – MG-III, a lineage within the Thermoplasmatales) were found at 0 (AMSMV-0-A22, 2.1% and AMSMV-0-A48, 2.1%), 25 cm.b.s.f. (AMSMV-25-A3, 6.5%) and 30 cm.b.s.f. (AMSMV-30-A53, 2.1%) layers.

All Crenarchaeota clones fell within MBG-B, Marine Benthic Group-B, (synonymous with deep-sea Archaea group). MBG-B Archaea exhibited high abundance at the surface layer (AMSMV-0-A10, 18.8% and AMSMV-0-A16 2.1%). Phylotypes belonging to MBG-B were also retrieved from 10 cm.b.s.f. (AMSMV-10-A48, 3.6%), 15 cm.b.s.f. (AMSMV-15-A7, 6.1%), 20 cm.b.s.f. (AMSMV-20-A17, 1.7%) and 25 cm.b.s.f. (AMSMV-25-A17, 2.2% and AMSMV-25-A24, 2.2%).

Phylotypes affiliated with the Thaumarchaeota (until recently characterized as Crenarchaeota clade MG 1 – Marine Archaeal Group 1) were present only in the surface layer. Three phylotypes were retrieved (AMSMV-0-A2, 2.2%, AMSMV-0-A34, 2.2% and AMSMV-0-A54, 2.2%).

Bacteria. The bacterial clone libraries coverage (Fig S1) according to Good's C estimator [37] was lower (ca. 0.4 – 0.8) compared to that for Archaea. A total of 526 bacterial 16S rRNA gene sequences were analyzed and 276 phylotypes were identified in all sediment layers and 213 of them were unique. The majority of the retrieved clones were affiliated with Proteobacteria (Fig. 2, 4, S3, S4). The rest of the phylotypes were affiliated with: Acidobacteria, Actinobacteria, Bacteriodetes, Chlorobi, Chloroflexi, Deferribacteres, Firmicutes, Planctomycetes, Spirochaetes, Verrucomicrobia, candidate division JS1 [80], OD1/OP11 [27, 33], OP8 [33], WS1 [86], WS3 [19], WS6 [19], OP5, Desulfurella Group [9] and seven unaffiliated groups (Fig. S5a, b, c).

At the surface layer phylotypes of δ- (23.1%) and γ- (22.3%) Proteobacteria co-dominated (Fig. 2). Phylotypes affiliated with ε-Proteobacteria exhibited high abundance (14.9%) and AMSMV-0-B65 (Fig. S3), the most abundant phylotype in this layer clusters within ε-Proteobacteria and is related to a phylotype reported from Kazan MV sediments. Representatives of Actinobacteria and Chloroflexi comprised 5.8% (each) of the total bacterial clones of the surface.

At 5 cm.b.s.f. γ-Proteobacteria showed the highest relative abundance (17.9%) followed by δ- and ε- Proteobacteria, which were retrieved in equal percentages (13.4%). The most abundant phylotype for this layer AMSMV-0-B50 (as AMSMV-5-B2) clustered within ε-Proteobacteria, was

also present at the surface layer and has previously been found in Kazan MV sediments. Phylotypes belonging to Bacteriodetes comprised 9.0% of the clone library from the 5 cm.b.s.f. layer and phylotypes belonging to Chloroflexi and Firmicutes appeared with relative abundance of 6.0%, each.

Phylotypes attributed to Chloroflexi dominated at 10 cm.b.s.f. (42.3%). At this sediment layer Proteobacteria were found in a rather low relative abundance (7.0% for each one of the subdivision of γ- and δ- Proteobacteria). Phylotypes clustering within candidate division JS1 were present in all studied sediment layers. Based on the ≥98 similarity criterion they were found to represent the same phylotype. At the surface, AMSMV-0-B186 was retrieved at a relative abundance of 1.7%, and increased at 5 cm.b.s.f. (as AMSMV-5-B62, 4.5%). JS1 phylotype dominated at 10 cm.b.s.f. (as AMSMV-10-B11, 15.5%), 15 cm.b.s.f. (as AMSMV-15-B20, 34.8%) and 20 cm.b.s.f. (as AMSMV-20-B1, 28.8%). The relative abundance of this phylotype decreased at 25 cm.b.s.f. (as AMSMV-25-B55, 18.4%) and further at 30 cm.b.s.f. (as AMSMV-30-B5, 11.6%).

Phylotypes of δ-Proteobacteria were retrieved at 15 και 20 cm.b.s.f. in relative abundances of 24.6% και 27.1%, respectively. δ- Proteobacteria was the dominant group at 25 cm.b.s.f. (65.6%) with AMSMV-0-B66 (as AMSMV-25-B51) the most abundant phylotype. The representation of δ-Proteobacteria was decreased at 30 cm.b.s.f. (17.3%). In that layer, phylotypes belonging to Chloroflexi dominated (19.2%).

Diversity and similarity indices. Shannon-Wiener diversity index H at all examined depths was higher for Bacteria (1.92-4.03) than for Archaea (0.99-1.91) (Fig. 5). Changes in H with depth were not consistent between the Archaea and Bacteria clone libraries. The Archaea communities in AMSMV exhibited the lowest H value in the deepest studied sediment layer, 30 cm.b.s.f. and the highest in the surface layer. At 5 cm.b.s.f. H was 1.72, while the rest of the layers showed intermediate H values around 1.4. For Bacteria the highest H value appeared at the surface layer and H values for 5 cm.b.s.f. and 10 cm.b.s.f. were also quite high (3.85 and 3.45, respectively). The lowest value was found at 25 cm.b.s.f..

The evenness index J (Fig. 5), followed approximately the same vertical profile with the diversity index, H. For Archaea the highest J value was recorded at 5 cm.b.s.f. (0.829) and the lowest at 30 cm.b.s.f. (0.554). For Bacteria, J took high values (>0.9) at 0, 5 and 10 cm.b.s.f. and the lowest value at 25 cm.b.s.f. (0.639). The four different indices of similarity used are in agreement for most cases (Table S1). Setting the similarity threshold at 0.65 showed, for Bacteria, only one cluster of similar layers 15 and 20 cmbsf. The correspondence analysis (Fig. 6) revealed that these layers are also in close proximity to 25 and 30 cmbsf, while 0, 5 and 10 cmbsf are distantly placed. Archaeal communities shared a greater degree of similarity. The sediment layers of 10, 15 and 20 cmbsf were found to form a cluster. Moreover the 25 cmbsf layer seemed highly similar to the 15 cmbsf layer and 5 cmbsf to 20 cmbsf (Fig. 6 and Table S1). The 30 cmbsf layer was highly dissimilar.

Phylotype comparison between AMSMV and KZNMV revealed 12 and 36 common phylotypes for Archaea and Bacteria, respectively (Fig. S6). Eleven of the common Archaea phylotypes were exclusively found in mud volcanoes and related environments, with seven of those belonging to the ANME groups. Thirteen of the common Bacteria phylotypes belonged to sulfate reducing δ-Proteobacteria and were similar to phylotypes from cold seeps. Seven phylotypes were attributed to γ-Proteobacteria related with putative sulfur and sulfide oxidizers. One phylotype was affiliated to the candidate division JS1. The rest of the common phylotypes belonged to the α- and ε-Proteobacteria, Chloroflexi, Firmicutes, Bacteriodetes, Acidobacteria, Actinobacteria and Deferribacteres.

DISCUSSION

The clone library coverage indicated sampling saturation for the Archaea, implying that the majority of the Archaea phylotypes were revealed. The coverage for the Bacteria clone libraries was not as satisfactory as for the Archaea. Nevertheless, the most abundant bacterial phylotypes were

retrieved, i.e. putative key players at the community level, and several "rare" phylotypes (singletons and doubletons) as well. The use of the Shannon–Wiener diversity index H in prokaryotic communities is applicable and realistically informative [30, 37] especially when the clone library coverage is satisfactory. In our study, the Archaea clone libraries was satisfactory while for the Bacteria, although in most cases the coverage approached a plateau, the overall coverage was lower possibly due to the higher number of singletons and doubletons (Figures S1, 3, S3, S5).

Archaea. Communities of Archaea in all layers were related to groups involved in methane metabolism. Phylotypes belonging to all three so far known ANME groups were found in high relative abundances (>50% of all archaeal clones) in all depth layers. These yet-uncultivated Euryarchaeota, namely ANME-1, ANME-2 and ANME-3, are mediating anaerobic oxidation of methane (AOM) in marine environments, including other gas hydrate bearing mud volcanoes [38 and references therein], and were differentially distributed along the investigated sediment layers.

Phylotypes of ANME-1 and ANME-2 groups were found to co-occur in most of the examined sediment layers, which is a rather common finding in AOM related studies [18, 40, 51, 57] since it seems that, although phylogenetically distant, these groups perform AOM in a wide range of environmental settings. ANME-1 have been found to dominate hypersaline sediments [44] or "oily" sediments [54] from the Gulf of Mexico and microbial mats from the Black Sea [6, 49]. They can reach high abundances in deeper sediment layers and in microbial mats [28, 40, 55, 58] but in the sediment of AMSMV their presence was unexpectedly high even in the top layers.

Since both ANME-1 and ANME-2 have been found in habitats with a wide range of methane concentrations and fluxes [44] it is possible that methane concentration is not the crucial factor for the selection of different groups. Instead, methane is believed to control total ANME biomass [14]. Other factors, such as salinity and oxygen presence, cannot sufficiently explain the relative abundance of either group. AMSMV sediments had low salinity but exhibited high relative proportion of C_{2+} alkanes due to decomposition of structure II hydrates [61], which could fuel ANME-1 communities [54]. Moreover, ANME-1 and ANME-2 might perform AOM through different physiological mechanisms, with ANME-2 most likely to preferentially use sulfate as a mediator for AOM. ANME-1 cells, in some cases, have been observed in tight association with bacteria (e.g. microbial mats) but are most often found alone or in filaments, with no obvious syntrophic partnership as is the case for ANME-2 [40, 49, 56]. It is therefore also possible that they can mediate AOM in the absence of syntrophy. This has been calculated to be more energetically demanding [78] but methane oxidizers are believed to be adapted in chronic energy stress [77]. It is believed that ANME-1 might tolerate less favourable conditions for growth shaping more stable communities than ANME-2 [14].

ANME-3 is a less studied group. ANME-3 have been found to dominate in sediments of the Haakon Mosby MV and believed to perform AOM either without obligatory physical association with a sulfate reducing partner or with alternative partners [45]. The high abundance of ANME-3 at the 30 cm.b.s.f. horizon, where methane concentration is high (Fig. 1), might suggest that this group can effectively use high methane concentrations even without sulfate.

The presence of only a few Thaumarchaeota-related phylotypes, at the surface layer depicts a footprint of the overlying water column, as this group is known to play an important role in biogeochemical cycling in deep ocean waters [16, 36] and bathypelagic sediments [21]. The rest of the retrieved Archaea phylotypes, belonged to the GOM Arc I , MBG-D and MBG-C. These groups are generally associated with methane rich environments, but their ecophysiological functions are still unknown [18, 40, 44, 75].

Bacteria. Communities of Bacteria showed much higher species richness and more complex structure. In total, the number of Bacteria phylotypes retrieved was more than five times higher than Archaea and was related to over 20 phylogenetic groups.

δ-Proteobacteria exhibited high relative abundance in almost all sediment layers. Sulfate-reducing δ-Proteobacteria phylotypes dominated the 25 cm.b.s.f. layer. The dominant phylotype AMSMV-25-B51 clustered within the *Desulfosarcina/Desulfococcus* group (SEEP-1) of Desulfobacteriaceae, syntrophic partners of ANME-1 and ANME-2 Archaea [7, 39, 49, 57, 67].

According to the geochemical profile, the 25 cm.b.s.f. horizon seems to be at the base of the sulfate reduction zone and it is possible that sulfate reducing AOM takes place there. A few *Desulfobulbus*-like phylotypes were retrieved as well, implying versatility in bacterial partnership and AOM syntrophy [67].

High abundance (ca. 30%) of sulfate reducing δ-Proteobacteria was also observed at 15 and 20 cmbsf, as anticipated by the geochemical profile showing sulfate decrement. These layers showed high similarity implying that they share similar features which can shape similar Bacteria communities. Both of these layers showed high abundance of Bacteria groups that are not typically associated with AOM, such as the candidate division JS1 and Chloroflexi. JS1 was found in anoxic sedimentary habitats [80, 82] and is often associated with hydrate bearing and methane rich sediments [34, 58]. Chloroflexi is a widespread group of Bacteria found in a diverse range of environments, not only subsurface sediments [15, 79] but also hot springs, hydrothermal sediments, soils, wastewater and polluted sites [33, 68, 74]. Within these two subsurface-related bacterial phyla there are no cultured members of JS1 and few cultured members of the Chloroflexi. For subsurface-related Chloroflexi it has been suggested that they anaerobically degrade recalcitrant carbon sources with H_2 consumption [83]. An insight of the physiology of JS1 was demonstrated [83] providing evidence of presence and active under anaerobic sulfate-reducing conditions and ability of glucose (or glucose metabolites) and acetate utilization. In the present study, the presence of the same JS1 phylotype in all sediment layers examined (and the dominance in most of them) clearly suggests a "key-player" role for this phylotype in AMSMV sediments. The detection of a practically identical phylotype (>98% similarity) in sediments of Kazan MV (Fig 3) and other methane seep environments such as Hydrate Ridge and Gulf of Mexico sediments implies that its ecophysiology is related to sulfur cycle (see below).

The ε-Proteobacteria found in the AMSMV sediments belong to Marine Group I (MG I) [10], which includes phylotypes from deep sea sediments, hydrothermal vents, sulfidic cave waters and cold seeps, phylogenetically associated with *Sulfurovum lithotrophicum* [35]. In addition to carbon cycling (due to CO_2 fixation [10, 25]), this cluster is oxidizing sulfur and sulfidic compounds with nitrate or oxygen, thus playing important role in the speciation of sulfur/nitrogen within a habitat. The majority of the retrieved γ-Proteobacteria in AMSMV was affiliated with sulfur and sulfide reducers and are expected to have similar impact in the sulfur cycling The sulfur cycling is further corroborated by the high abundance of δ-Proteobacteria, since both groups can benefit from their complementary roles in sulfur cycling. Such co-occurrence has been reported before [62, 74]in hydrothermal sediments. Sulfate reducing δ-Proteobacteria can utilize a wide spectrum of substrates which are being oxidized completely or incompletely to acetate, supplying their habitat with different forms of organic material (as well as reduced sulfur compound). These metabolic end-products could be used as substrates for growth by other microorganisms. There is evidence that a broad range of organisms can be active in an acetate slurry and able to use acetate directly or in close association with acetate-utilizers [81]. Members of JS1, Actinobacteria, α-Proteobacteria, Bacteriodetes, Firmicutes and Acidobacteria have been associated with acetate incorporation in sulfate reducing environments and that could explain the high abundance of these groups in AMSMV sediments. Actinobacteria are also know for their role in immobilizing the dissolved organic matter and in degrading complex organic substrates such as lignin [71 and reference there in]. Actinomycetes, as well, have the ability to degrade recalcitrant organic matter [24]. Both of these groups may be critical players in controlling the degradation and mineralization of complex organic matter.

Planctomycetes can oxidize organic substrates through nitrate reduction [20], like *Pirellula* which is capable of heterolactic acid fermentation [22]. Moreover, members of Planctomycetes mediate anaerobic oxidation of ammonium [72], are abundant and important in the nitrogen cycle of Black Sea [42] and other suboxic marine waters and sediments [76]. The role of the deeply rooted Candidates Division OP8, WS3 and OD1/OP11 remains unknown. They have been exclusively found in marine ecosystems but their function remains doubtful. Representatives of OP8 in marine

sediments were abundant in the iron/sulfate transition zone [19], as well as in hydrothermal fields and similar environments [62], while OP11 might be involved in the sulfur cycle [33].

The complexity of the prokaryotic community revealed by this study indicates that the process of AOM might involve more partners, than just a methanotroph archaeon and a sulfate reducing bacterium. When there is adequate sulfate, methane is oxidized anaerobically by ANME acting in syntrophy with "seep-specific" sulfate reducing δ-Proteobacteria but the process could probably benefit from the participation of more groups that are able to use the products of AOM. Some of these groups (such as α-, β-, γ-, ε-Proteobacteria, Planctomycetes and Bacteroidetes) were found in association with the AOM consortium [63]. Sulfide oxidizers, members of γ- and ε-Proteobacteria can use the produced sulfide and refuel the sulfur cycle, while JS1 (and other groups) utilize acetate. Although we found several bacterial phylotypes which could be assigned only to the phylum level, it is possible that these unknown Bacteria could have active ecophysiological roles in AMSMV.

Diversity and similarity patterns. Archaea diversity was highest at the uppermost sediment layer, reflecting the contribution of the overlying water column to the Archaea community in the surface sediment, whilst diversity decreased throughout the depth of the core. At all sediment layers the observed Archaea diversity was lower than the Bacteria, which is a general pattern in methane-related environments [29, 51, 66]. Bacterial communities in all but 15 and 20 cm.b.s.f. layers, appeared highly dissimilar, implying that a unique bacterial assemblage was established in each layer. Archaea communities, shared a greater degree of similarity. Only the surface and 30 cm.b.s.f. layer were different (Fig 6). Although there is evidence of stratification (mainly for the bacterial communities), we observed continuous shifts of certain phylotype abundances for both Bacteria and Archaea implying that these communities are not totally isolated with each other and are partially overlapping in species composition.

Direct comparisons between analogue/similar habitats are seldom feasible due to different methodological approaches used, ranging from sampling to analysis of clone libraries, despite their usefulness especially in cases of unknown/uncultivated microorganisms prevail. The phylotypes found in the current study are directly comparable with those from the KZNMV [41, 58] since the methodologies used in both MV were exactly the same. Phylotype comparison (Fig. 3) between AMSMV and KZNMV revealed that in the two systems, 48.0% and 63.1% , respectively, of their Archaea phylotypes are common. Most of them belong to ANME or other groups metabolising methane and originate exclusively from MV and methane-rich sediment environments, where AOM is an active process. Bacteria shared a much lower number of common phylotypes between the two MV (16.7% and 19.2% for the AMSMV and KZNMV, respectively). Most of the common Bacteria phylotypes were sulfate reducing δ-Proteobacteria, other sulfate reducing partners for ANME performing AOM and the rest were affiliated with putative sulfur/sulfide oxidizers, groups that can metabolize intermediate products of sulfate mediated oxidation of methane or hydrocarbons (such as acetate utilizers), groups that can degrade complex organic material or groups with yet unknown ecophysiological role. Most of them exhibit significant similarity to clones from seep environments, associated bacterial phylotypes with AOM consortia [63] or alternative bacterial partners for AOM [5]. Six of the Archaea (AMSMV-0-A12, -0-A18, -0-A22, -0-A33, -0-A45, -0-A48) and nine of the Bacteria (AMSMV-15-B5, -0-B66, -0-B186, -5-B23, -20-B3, -0-B123, -10-B9, -15-B49, -0-B195) phylotypes found in this study, were also found in Amsterdam and Kazan MV sediments sampled in 1998 [29]. Although MV and other cold seep ecosystems are formed from dissimilar geological processes and fluid flow can vary considerably, resulting in temporal unpredictable variations in the concentrations of methane, sulfide and other porewater compounds [43], the occurrence of these common prokaryotic phylotypes (Fig. S6) suggests that these microorganisms could serve as "foundation" species for similar environments and implies a degree of endemicity and functional redundancy for methane-based systems, such as mud volcanoes.

In conclusion, the prokaryotic communities at the sediments of the active site of the Amsterdam MV showed a stratified structure, with Bacteria communities being much more diverse but with Archaea having significantly more redundant functional roles. The majority of the found

phylotypes were related to the expected AOM-related microorganisms but the found diversity of Bacteria indicated that other microorganisms with nitrogen and sulfur related metabolic pathways participate in these communities. A considerable fraction of the Archaea phylotypes, and a smaller number of Bacteria, was practically similar from the Kazan MV of the Anaximander MV, implying a degree of endemicity. At the scale of 5 cm sediment depth, it seems that the occurring communities share common phylotypes suggesting than MV communities should be seen as continua instead of isolated communities.

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Figure legends

Figure 1. Vertical profile of methane and sulfate concentrations in the sediments of the Amsterdam mud volcano, East Mediterranean Sea.

Figure 2. Relative abundance grouping of the found prokaryotic phylotypes from sediments of the Amsterdam mud volcano, East Mediterranean Sea. cm.b.s.f.: cm below sea floor.

Figure 3. Phylogenetic tree of the Archaea 16S rRNA gene phylotypes (ca. 1500 bp) in the sediments of the Amsterdam mud volcano, East Mediterranean Sea, based on the neighbour-joining method as determined by distance using Kimura's two-parameter correction. The found phylotypes (bold letters) are named after the sediment depth origin. Numbers of identical (≥98% sequence similarity) phylotypes of the total phylotype number in each sediment depth are shown in parentheses. One thousand bootstrap analyses (distance) were conducted, and percentages ≥50% are indicated at nodes. Numbers in brackets are GenBank accession numbers. Scale bar represents 2% estimated distance.

Figure 4. Phylogenetic tree of the δ-Proteobacteria 16S rRNA gene phylotypes (ca. 1500 bp) in the sediments of the Amsterdam mud volcano, East Mediterranean Sea, based on the neighbour-joining method as determined by distance using Kimura's two-parameter correction. The found phylotypes (bold letters) are named after the sediment depth origin. Numbers of identical (≥98% sequence similarity) phylotypes of the total phylotype number in each sediment depth are shown in parentheses. One thousand bootstrap analyses (distance) were conducted, and percentages ≥50% are indicated at nodes. Numbers in brackets are GenBank accession numbers. Scale bar represents 2% estimated distance.

Figure 5. Shannon diversity index H and Pielou evenness index J for the Bacteria and Archaea 16S rRNA gene clone libraries from sediments of the Amsterdam mud volcano, East Mediterranean Sea.

Figure 6. Correspondence analysis of the phylotype relative abundance for the Bacteria and Archaea 16S rRNA gene clone libraries from sediments of the Amsterdam mud volcano, East Mediterranean Sea. The unique phylotypes are shown in blue letters, where the sediment depth is depicted with the first number followed by A or B for Achaea and Bacteria, respectively, and the phylotype number; cm.b.s.f.: cm below sea floor
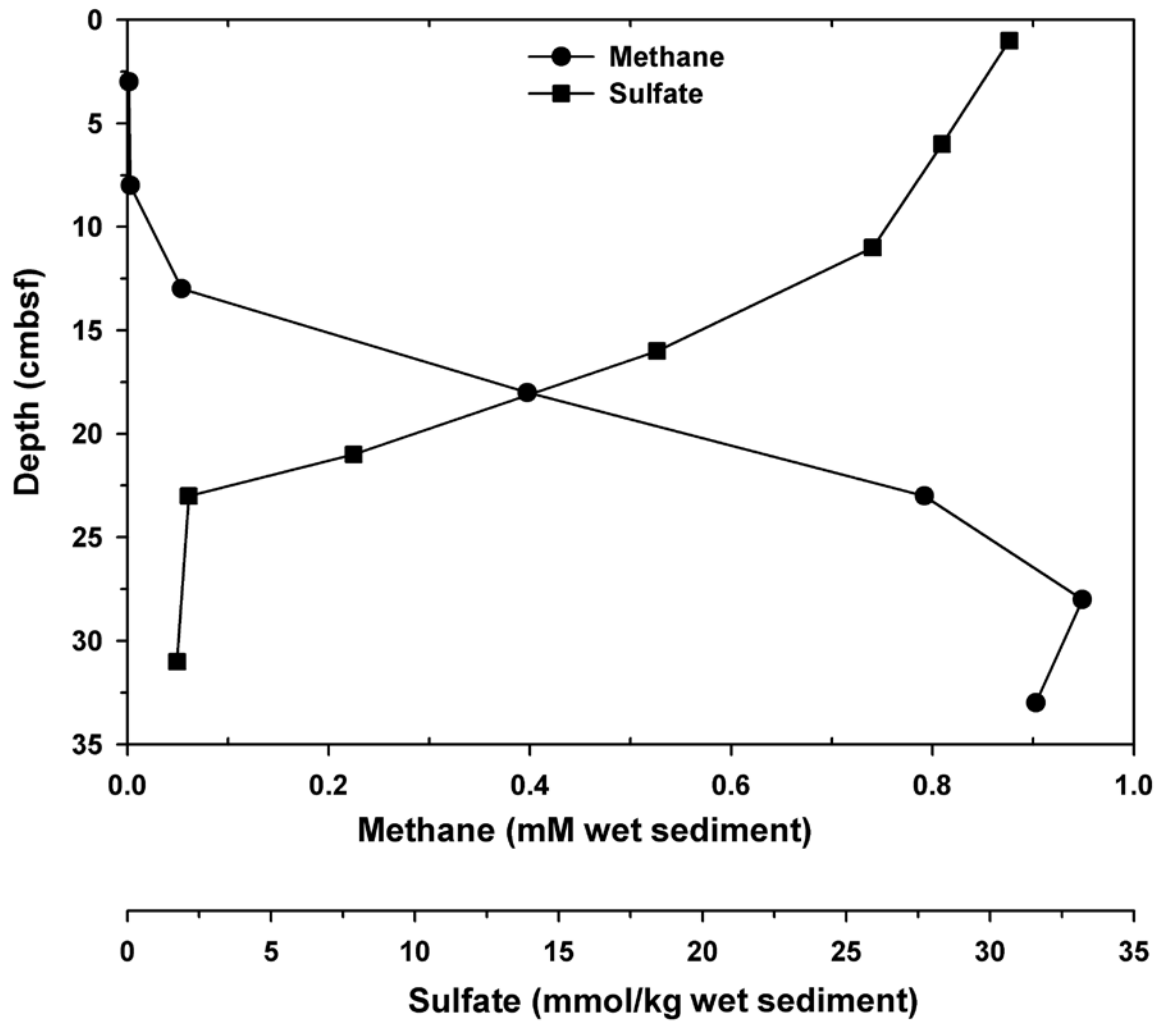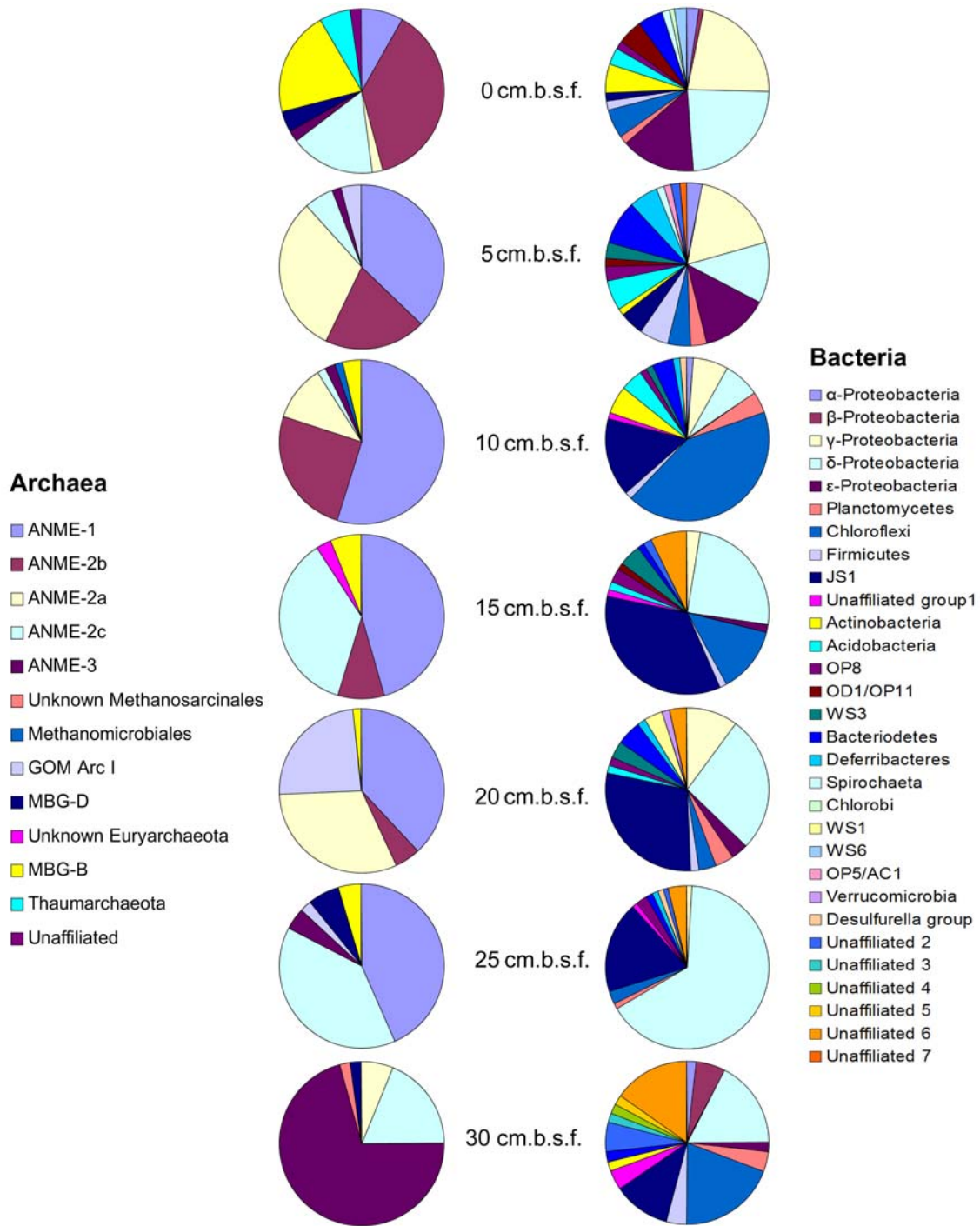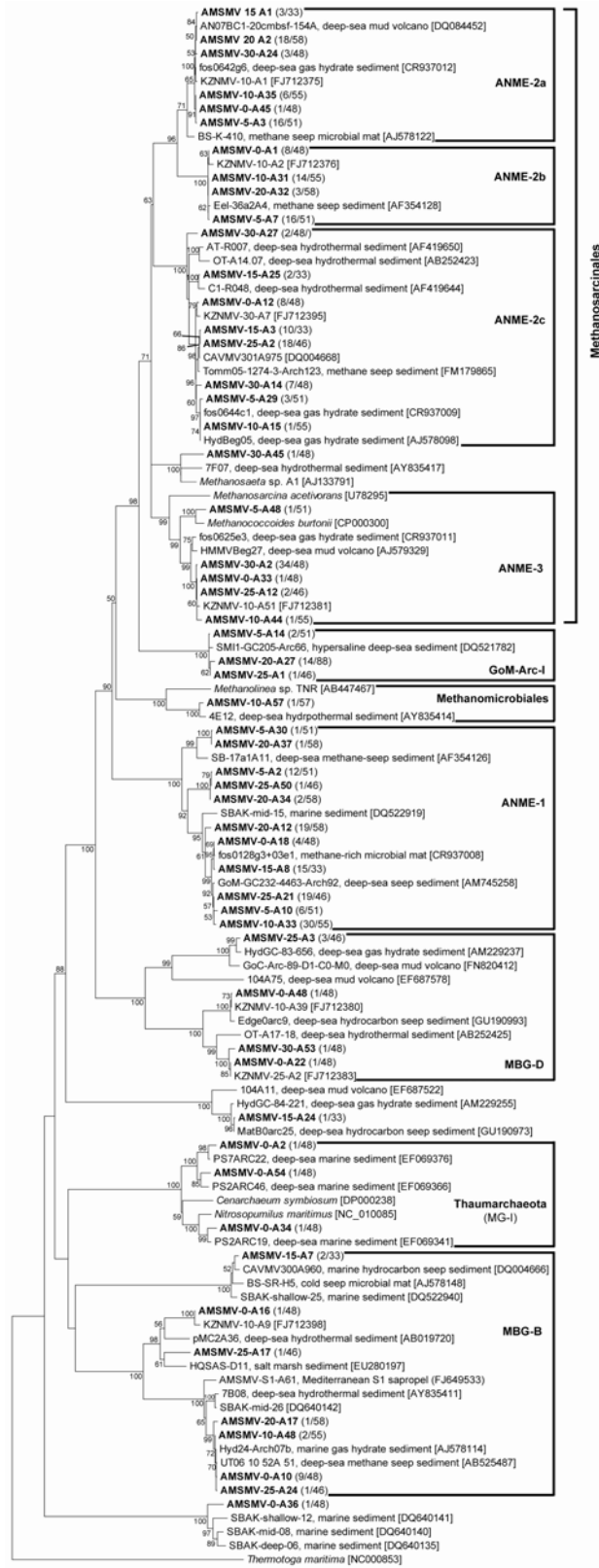
Figure 1.

Figure 2.

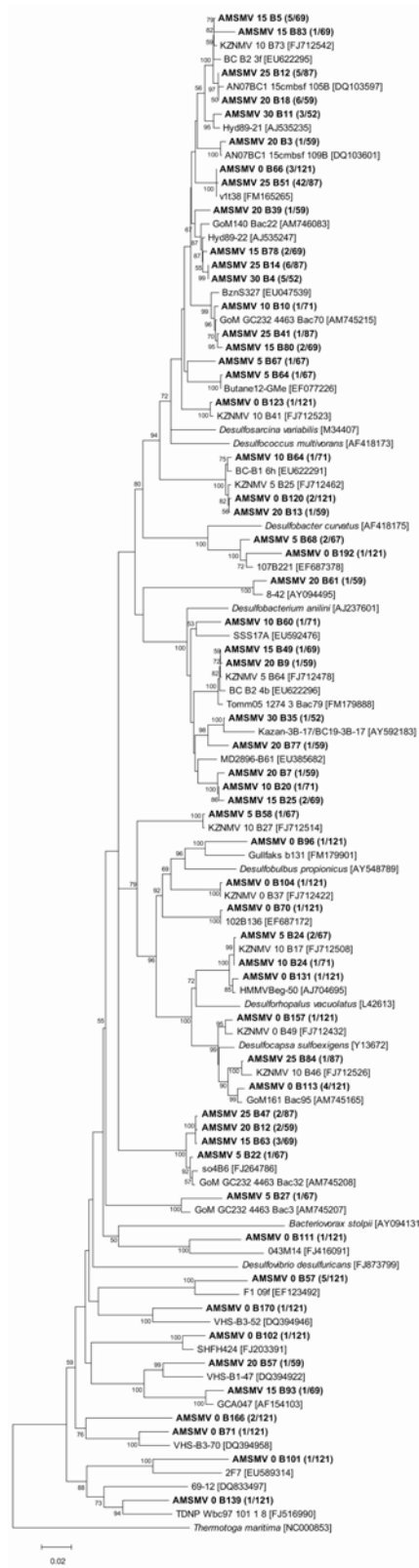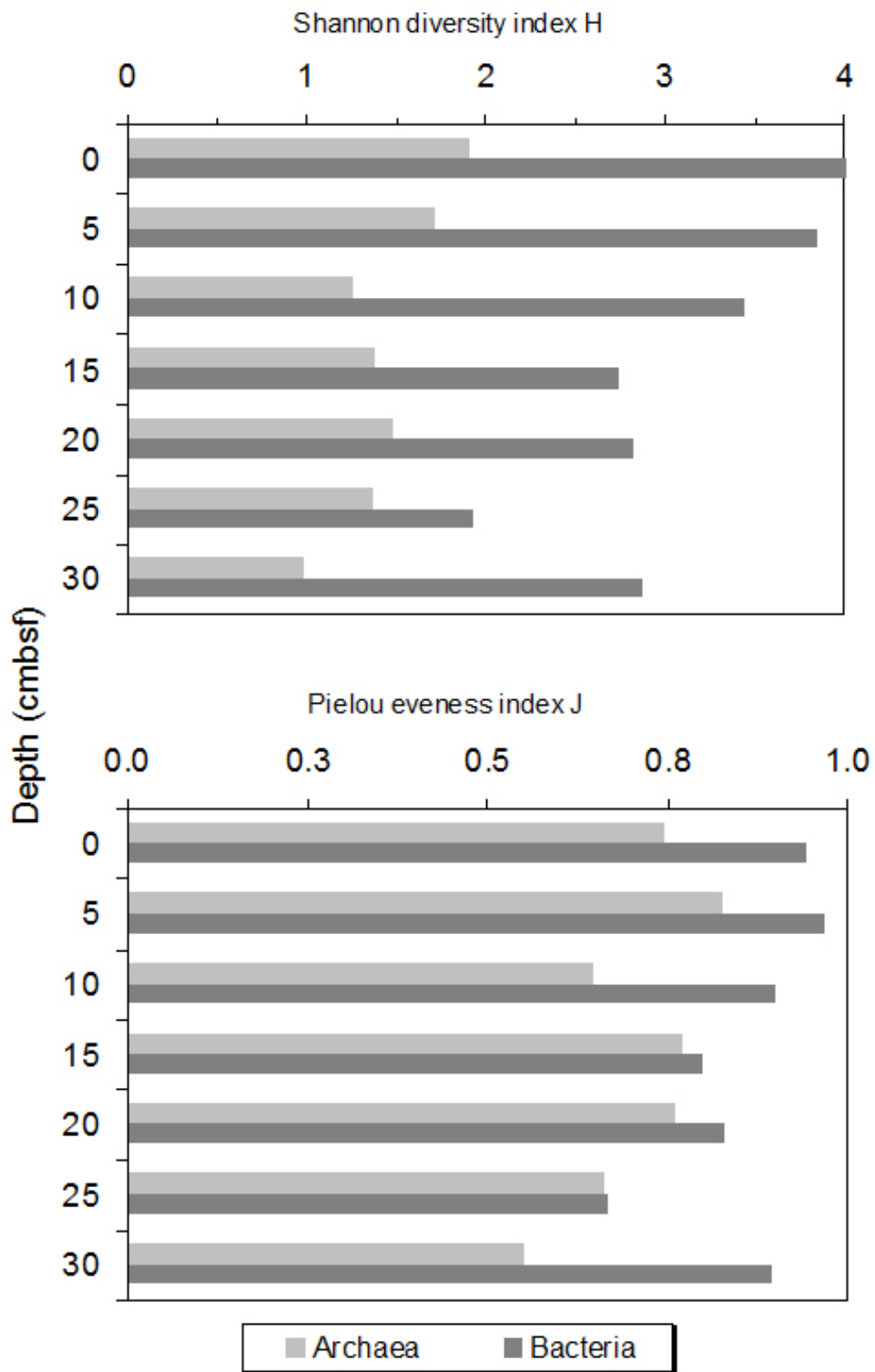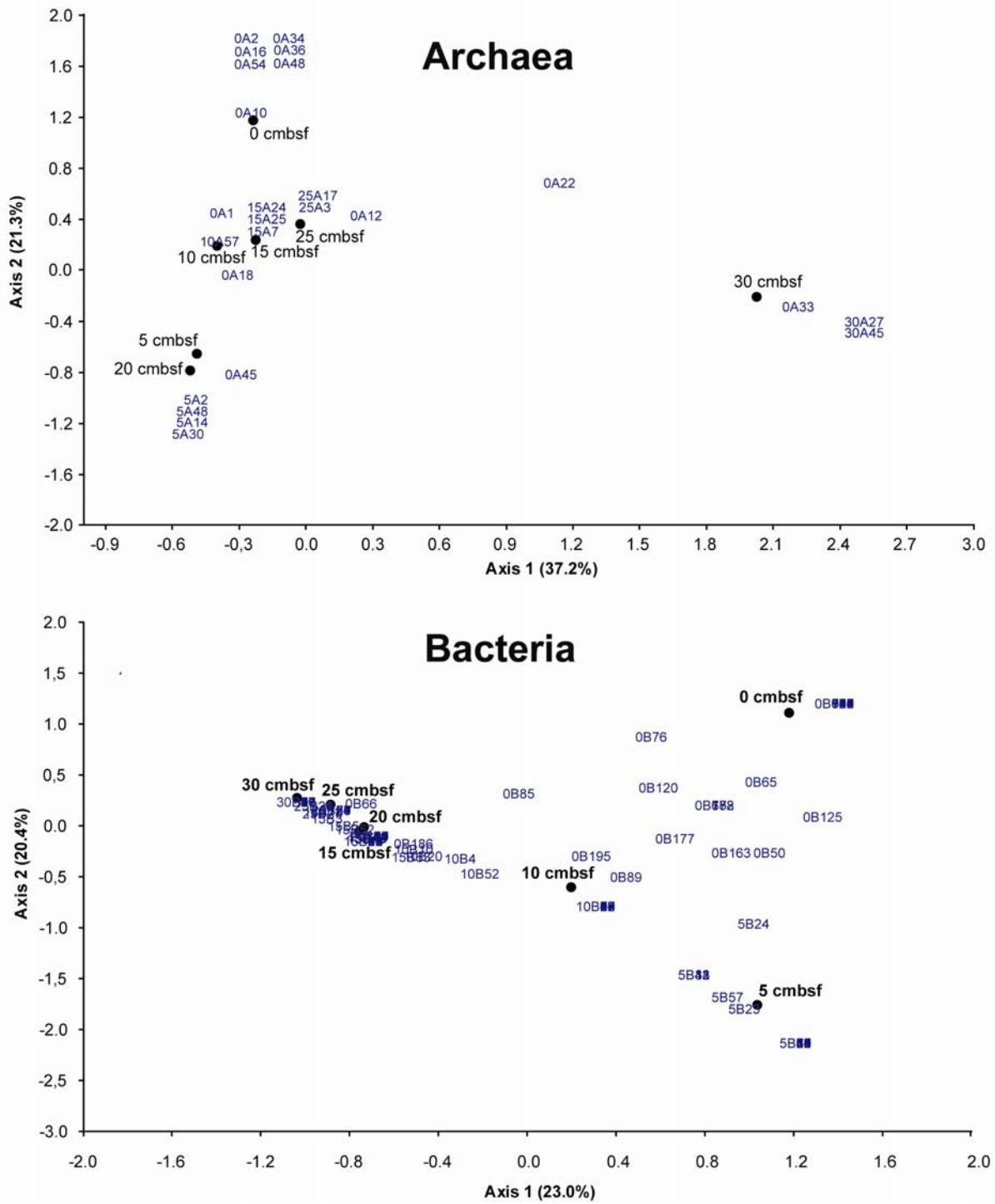**Figure 3.**

Figure 4.

Figure 5.

Figure 6.

SUPPLEMENTARY MATERIAL

Diversity and spatial distribution of prokaryotic communities along a sediment vertical profile of a deep-sea mud volcano

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Table S1. Similarity of Bacteria and Archaea sediment communities of the Amsterdam mud volcano, based on the indices of Morisita-Horn (first value), Morisita (second value), Jaccard adjusted (third value) and Sørensen adjusted (fourth value). Bold values indicate similar communities (≥0.650).

Bacteria

	0 cm b.s.f.	5 cm b.s.f.	10 cm b.s.f.	15 cm b.s.f.	20 cm b.s.f.	25 cm b.s.f.
0 cm b.s.f.						
5 cm b.s.f.	0.327 0.606 0.142 0.249					
10 cm b.s.f.	0.125 0.165 0.170 0.291	0.247 0.386 0.350 0.519				
15 cm b.s.f.	0.078 0.088 0.131 0.232	0.205 0.245 0.169 0.290	0.553 0.637 0.170 0.291			
20 cm b.s.f.	0.098 0.118 0.233 0.378	0.209 0.266 0.141 0.248	0.537 0.647 0.153 0.265	**0.890** **0.998** 0.628 **0.772**		
25 cm b.s.f.	0.099 0.105 0.041 0.078	0.057 0.061 0.049 0.094	0.173 0.186 0.124 0.220	0.356 0.374 0.357 0.526	0.311 0.331 0.235 0.380	
30 cm b.s.f.	0.073 0.096 0.101 0.184	0.112 0.161 0.065 0.122	0.260 0.337 0.071 0.132	0.511 0.590 0.335 0.520	0.445 0.535 0.238 0.385	0.203 0.219 0.316 0.480

	0 cm b.s.f.	5 cm b.s.f.	10 cm b.s.f.	15 cm b.s.f.	20 cm b.s.f.	25 cm b.s.f.	30 cm b.s.f.

Archaea

|	0 cm b.s.f.	5 cm b.s.f.	10 cm b.s.f.	15 cm b.s.f.	20 cm b.s.f.	25 cm b.s.f.	30 cm b.s.f.
0 cm b.s.f.							
5 cm b.s.f.	0.468 0.506 0.575 0.730						
10 cm b.s.f.	0.519 0.545 **1.000** **1.000**	0.508 0.533 **0.677** **0.808**					
15 cm b.s.f.	0.341 0.368 0.232 0.416	0.379 0.408 0.451 0.621	**0.762** **0.800** **0.672** **0.804**				
20 cm b.s.f.	0.235 0.250 **0.675** **0.806**	**0.686** **0.729** **0.924** **0.960**	**0.704** **0.732** **0.688** **0.815**	0.609 0.648 0.417 0.588			
25 cm b.s.f.	0.383 0.407 0.515 **0.680**	0.285 0.303 0.637 **0.778**	**0.661** **0.686** 0.636 **0.778**	0.947 1.004 0.640 **0.780**	0.474 0.497 0.489 **0.657**		
30 cm b.s.f.	0.110 0.114 0.239 0.386	0.076 0.079 0.154 0.267	0.049 0.051 0.146 0.254	0.118 0.123 0.158 0.273	0.049 0.050 0.055 0.104	0.204 0.210 0.405 0.576	
	0 cm b.s.f.	5 cm b.s.f.	10 cm b.s.f.	15 cm b.s.f.	20 cm b.s.f.	25 cm b.s.f.	30 cm b.s.f.
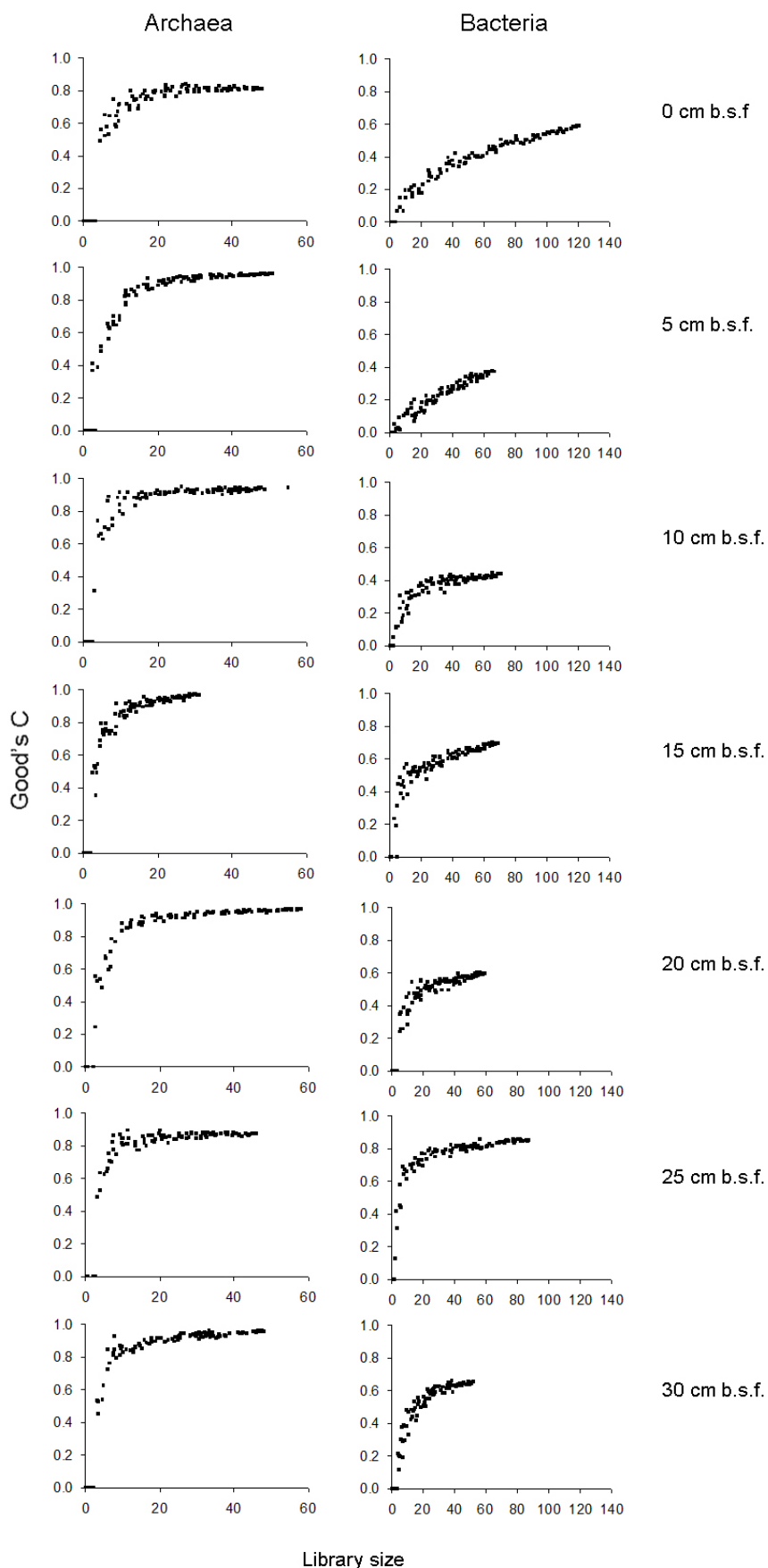
Figure S1. Clone library coverage based on Good's C estimator of the prokaryotic 16S rRNA gene libraries from the Amsterdam mud volcano, East Mediterranean Sea.
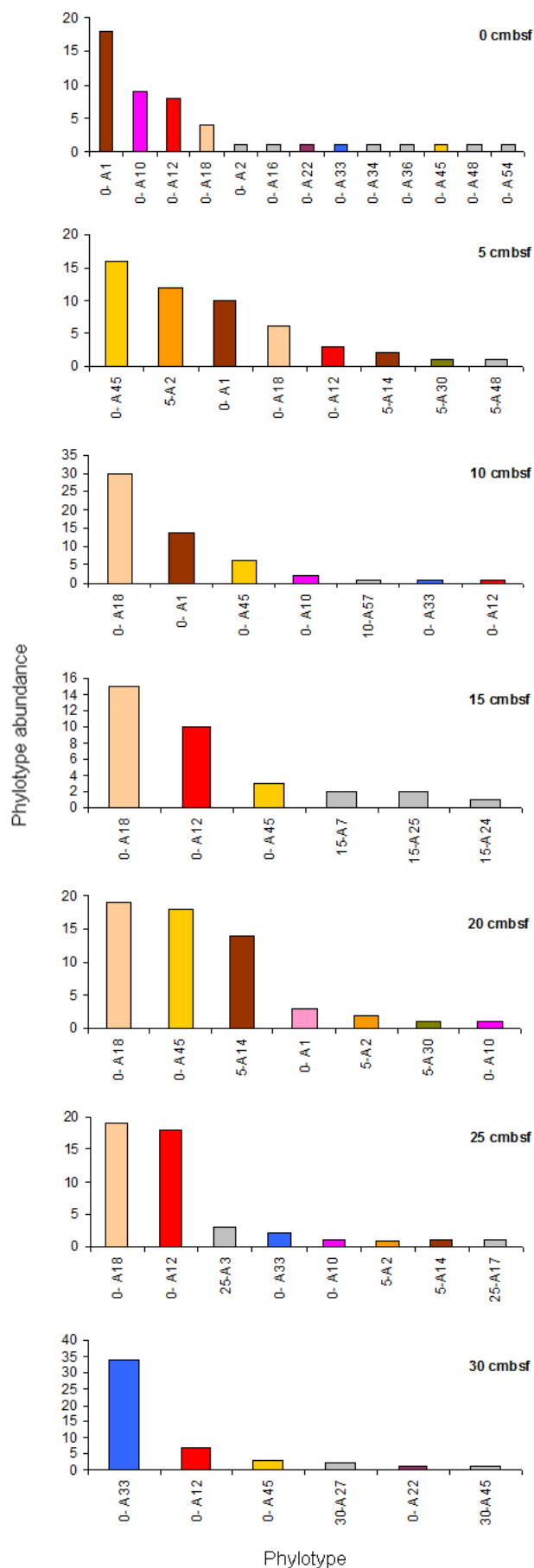
Figure S2. Occurrence of common Archaea phylotypes between the different sediment layers in the Amsterdam mud volcano, East Mediterranean Sea. The same colour corresponds to the same phylotype.
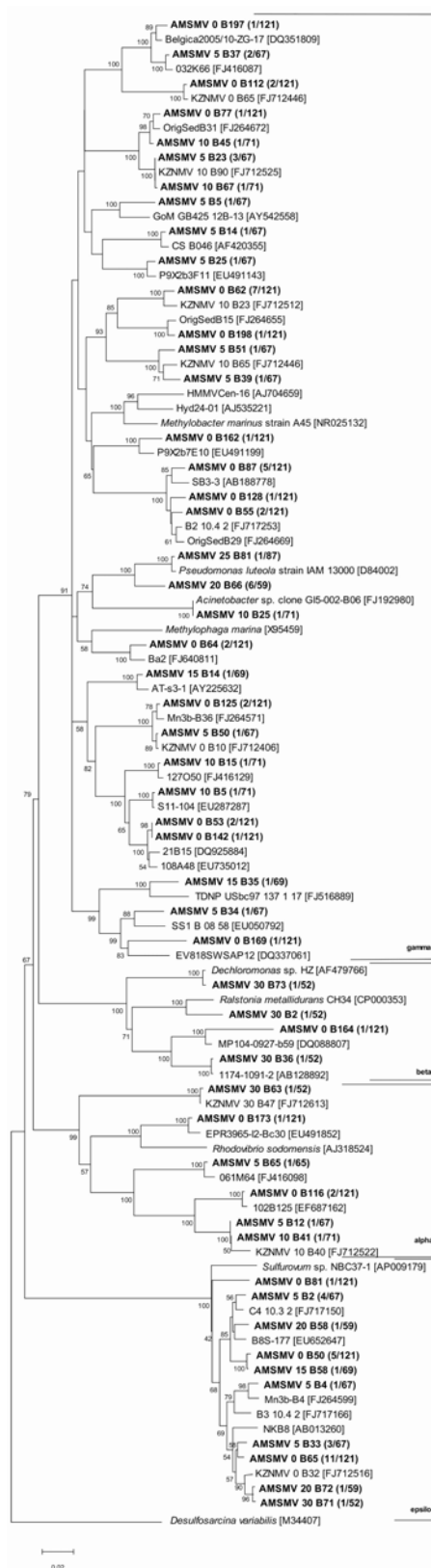
Figure S3. Phylogenetic tree of the α-, β-, γ- and ε-Proteobacteria 16S rRNA gene phylotypes (ca. 1500 bp) in the sediments of the Amsterdam mud volcano, East Mediterranean Sea, based on the neighbour-joining method as determined by distance using Kimura's two-parameter correction. The found phylotypes (bold letters) are named after the sediment depth origin. Numbers of identical (≥98% sequence similarity) phylotypes of the total phylotype number in each sediment depth are shown in parentheses. One thousand bootstrap analyses (distance) were conducted, and percentages ≥50% are indicated at nodes. Numbers in brackets are GenBank accession numbers. Scale bar represents 2% estimated distance.
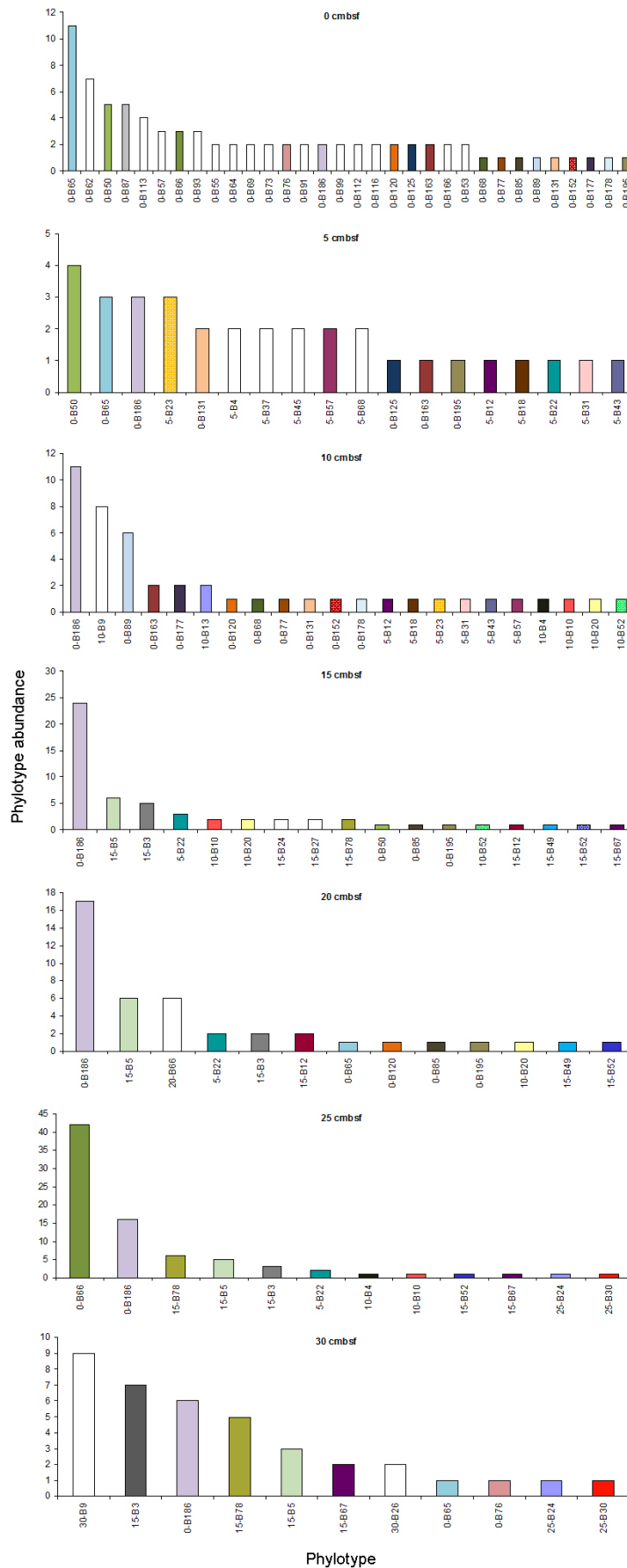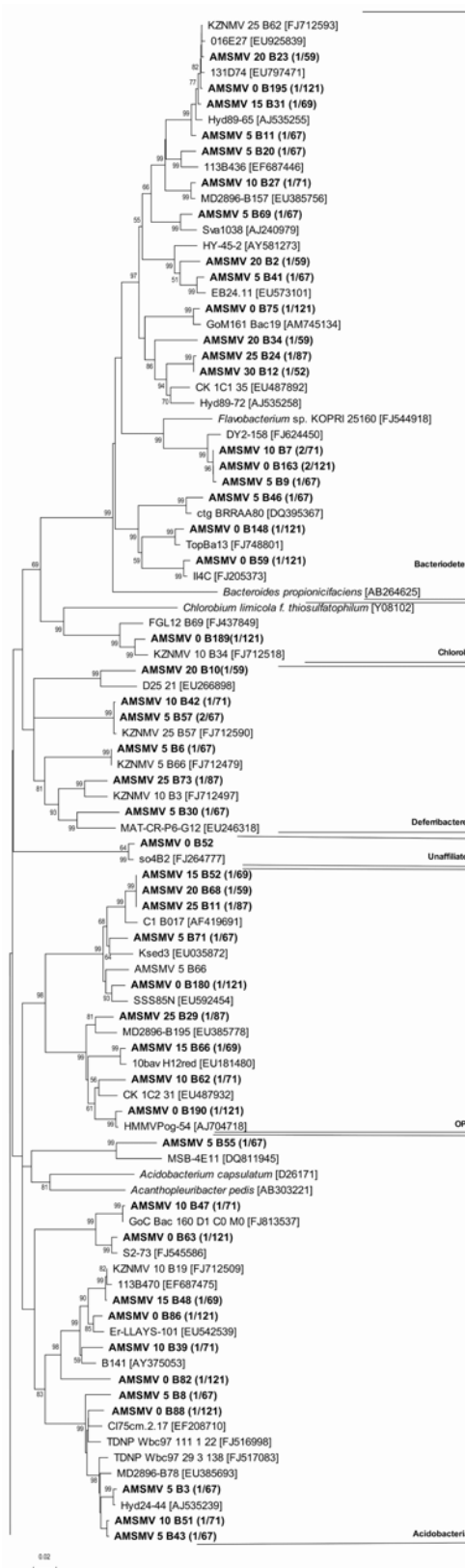
Figure S4. Occurrence of common Bacteria phylotypes between the different sediment layers in the Amsterdam mud volcano, East Mediterranean Sea. The same colour corresponds to the same phylotype, while blank columns depict phylotypes that have occurred only at one layer.

Figures S5a,b,c. Phylogenetic tree of the rest of the Bacteria 16S rRNA gene phylotypes (ca. 1500 bp) in the sediments of the Amsterdam mud volcano, East Mediterranean Sea, based on the neighbour-joining method as determined by distance using Kimura's two-parameter correction. The found phylotypes (bold letters) are named after the sediment depth origin. Numbers of identical (≥98% sequence similarity) phylotypes of the total phylotype number in each sediment depth are shown in parentheses. One thousand bootstrap analyses (distance) were conducted, and percentages ≥50% are indicated at nodes. Numbers in brackets are GenBank accession numbers. Scale bar represents 2% estimated distance.
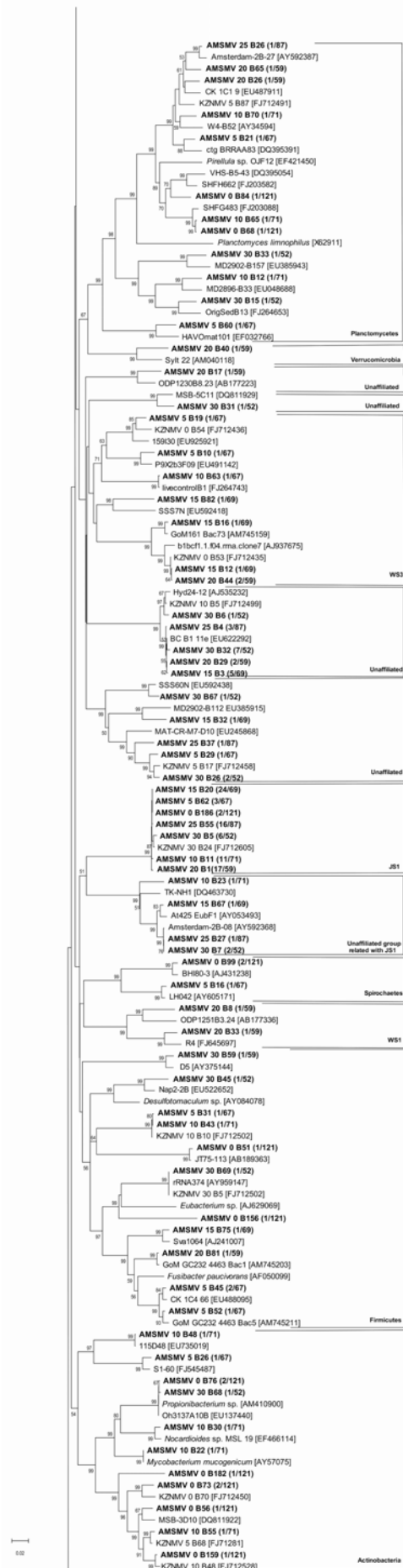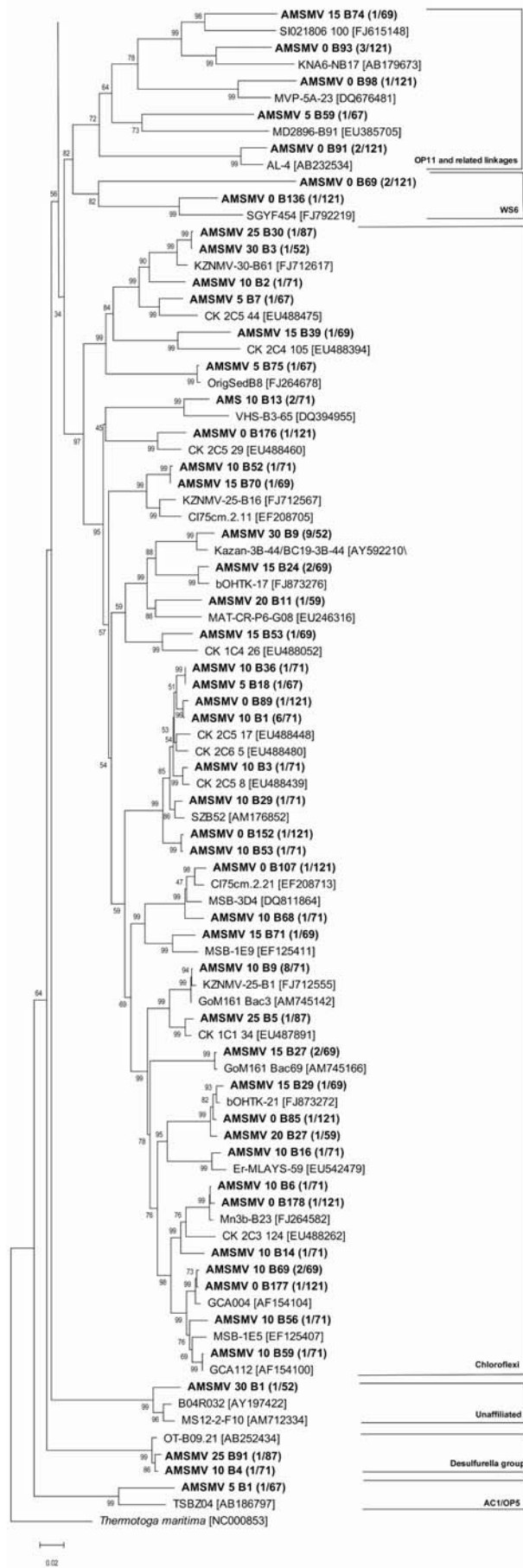
Figure S5b

Figure S5c

Archaea

ANME-1: AMSMV-5-A2, AMSMV-0-A18
ANME 2a: AMSMV-0-A45
ANME 2b: AMSMV-0-A1
ANME 2c: AMSMV-0-A12, AMSMV-30-A27
ANME 3: AMSMV-0-A33
MBG-B: AMSMV-0-A16, AMSMV-0-A22
MBG-D: AMSMV-0-A48
Methanomicrobiales: AMSMV-10-A57
Methanosaeta-related: AMSMV-30-A45

Bacteria

δ-Proteobacteria
AMSMV-0-B66, AMSMV-0-B120, AMSMV-0-B104, AMSMV-0-B123, AMSMV-0-B157, AMSMV-0-B131
AMSMV-5-B58, AMSMV-15-B5, AMSMV-15-B49, AMSMV-15-B78, AMSMV-20-B3, AMSMV-25-B84
γ-Proteobacteria
AMSMV-0-B112, AMSMV-0-B125, AMSMV-0-B197, AMSMV-5-B23, AMSMV-5-B25, AMSMV-5-B39,
AMSMV-10-B25
α-Proteobacteria
AMSMV-0-B116, AMSMV-5-B12, AMSMV-30-B63
Actinobacteria
AMSMV-0-B159, AMSMV-10-B55
Bacteriodetes
AMSMV-0-B195, AMSMV-10-B27
Deferribacteres
AMSMV-5-B6, AMSMV-5-B57
Firmicutes
AMSMV-5-B31, AMSMV-30-B69
Acidobacteria
AMSMV-15-B48
ε-Proteobacteria
AMSMV-0-B65
Chloroflexi
AMSMV-10-B9
JS1
AMSMV-0-B186
WS3
AMSMV-15-B12
Unaffiliated
AMSMV-30-B6

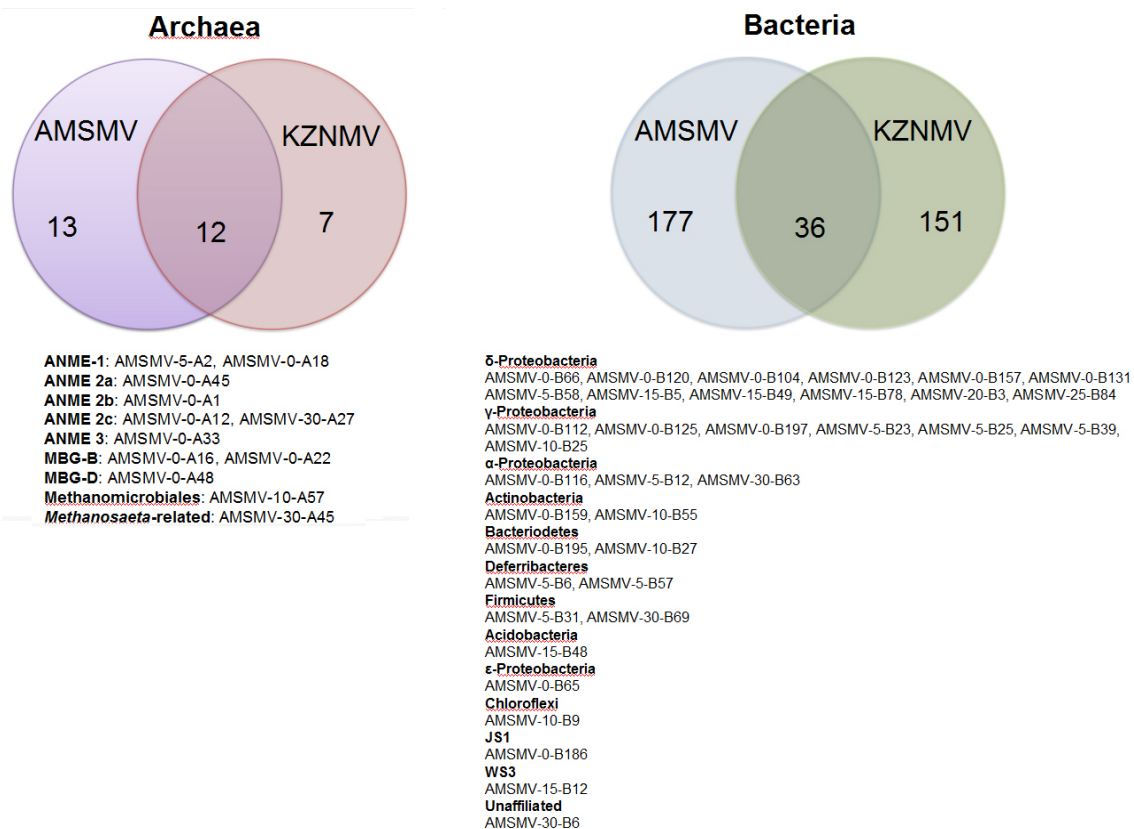**Figure S6.** Venn diagrams of the common phylotypes (≥98%) between Amsterdam (AMSMV, this study) and Kazan (KZNMV) mud volcanoes. Data for the KZNMV are from Kormas et al. (2008) and Pachiadaki et al. (2010). The code names of the common phylotypes from each phylum are shown.