Inter-comparison of the potentially active prokaryotic communities in the halocline sediments of Mediterranean deep-sea hypersaline basins

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ABSTRACT

The sediment microbiota of the Mediterranean deep-sea anoxic hypersaline basins (DHABs) are understudied relative to communities in the brines and halocline waters. In this study, the active fraction of the prokaryotic community in the halocline sediments of L' Atalante, Urania and Discovery DHABs was investigated based on extracted total RNA and 454 pyrosequencing of the 16S rRNA gene. Bacterial and archaeal communities were different in the sediments underlying the halocline waters of the three habitats, reflecting the unique chemical settings of each basin. The relative abundance of unique operational taxonomic units (OTUs) was also different between deep-sea control sediments and sediments underlying DHAB haloclines, suggesting adaptation to the steep DHAB chemical gradients. Only a few OTUs were affiliated to known bacterial halophilic and/or aerobic groups. Many OTUs, including some of the dominant ones, were related to aerobic taxa. Archaea were detected only in few halocline samples, with lower OTU richness relative to Bacteria, and were dominated by taxa associated with methane cycling. This study suggests that while metabolically active prokaryotic communities appear to be present in sediments underlying the three DHABs investigated, their diversity and activity are likely to be more reduced in sediments underlying the brines.

INTRODUCTION

Among described extreme environments on Earth, the Mediterranean deep hypersaline anoxic basins (DHABs) are considered some of the most extreme since they are characterized by several physico-chemical stress factors known to be hostile to most organisms from all three domains of life. These include high NaCl (thalassohaline) or high non-NaCl (athalassohaline) salinity leading to low water activity, high hydrostatic pressure, and dissolved oxygen concentrations ranging from micro-oxic to anoxic or anoxic with high concentrations of hydrgogen sulfide. The specific geochemical features of different DHABs are associated with their different origins. Most were thought to originate several thousand years ago due to the dissolution of outcropped subterranean deposits of mixed salts laid down during the late Miocene period (ca. > 5 million years ago; Camerlenghi 1990) or the release of entrapped brines during tectonic activity, and their entrapment in sea-floor depressions (Cita 2006 and references therein). The composition of the source salt deposits can vary significantly depending on the stage in the evaporation series that led to its deposition. Due to minimal mixing of these dense brines with overlying seawater, they have likely remained geochemically stable since their accumulation in seafloor depressions.

Species diversity is considered a key feature of any biological community. The structuring of this diversity (composition and relative abundance) is widely accepted as a chief determinant of the functioning and the dynamics of ecological communities (Loreau et al. 2001; Ives and Carpenter 2007; Loreau 2010). Explorations of biological diversity in DHAB water-columns led to the discovery of microorganisms with novel structures and metabolic/physiological capabilities, and to an increased understanding of the adaptability of different taxonomic groups and of the physico-chemical limits for life on Earth. Although there is some evidence that living metazoa occur in the DHABs (Danovaro et al. 2010), only unicellular organisms are expected to endure in these habitats, as more complex organisms are not likely to cope with these harsh conditions for more than short periods of exposure (e.g., during feeding excursions into haloclines). Indeed, microbial life from all three domains has been shown to occur in such aquatic systems (e.g. van der Wielen et al. 2005; Daffonchio et al. 2006; Alexander et al. 2009; Borin et al. 2009; Edgcomb et al. 2011; Yakimov et al. 2007a). While new cell morphologies and ecophysiological traits have been assigned to some of these microorganisms, only recently a more specific picture of the metabolic activities of DHAB microorganisms has started to emerge, e.g. S-oxidizing chemolithotrophy, microaerophilic autotrophy, heterotrophic sulfate reduction, methanogenesis and anaerobic methane oxidation (Borin et al. 2009; La Cono et al. 2011; Ferrer et al. 2012; Pachiadaki et al. 2014; Yakimov et al. 2013; Alcaide et al. 2015). In spite of these recent advances, we still know little about how these microorganisms survive and grow under the prevailing conditions of the DHABs. For example, although microbial metabolism has been reported at high hydrostatic pressures –several orders of MPa– (Picard 2014), very little is known about the combined effect of anoxia and high pressure, and even less is known about the combined effects of anoxia, high pressure, and high ionic strength.

Existing knowledge about microbial life in Mediterranean DHABs comes primarily from studies of planktonic prokaryotes (e.g. Daffonchio et al. 2006; Yakimov et al. 2007a,b; La Cono et al. 2011) and unicellular eukaryotes (e.g. Edgcomb et al. 2009; Pachiadaki et al. 2014) found in the seawater-brine interfaces and the brines of several of these systems.

There are only a few studies that investigated the occurrence of Bacteria and Archaea in DHAB sediments. One study examined diversity of Bacteria and Archaea in sediments of the Medee basin (Akoumianaki et al. 2012) and another study presented information on bacterial isolates generated from Mediterranean Sea DHAB sediments (Sass et al. 2008). A more recent study, conducted in parallel with the investigation presented in this manuscript, focused on protist and fungal assemblages present in the sediments underlying the haloclines of Discovery, Urania and L'Atalante basins (Bernhard et al. 2014). Here we show data on active bacterial and archaeal assemblages present in the surface sediments underlying the halocline water column of Urania, L' Atalante and Discovery basins based on analysis of reverse transcribed rRNA gene sequences.

MATERIALS AND METHODS

Sample collection and RNA extraction. Push-core sampling was visually guided by the ROV *Jason* deployed during R/V *Atlantis* cruise AT18-14 between 24 November and 06 December 2011. The top of the halocline also coincided with a whitish "beach" delineating the leading edge of the halocline as it impinged on the seafloor at each basin (Figure S1). The surface of the halocline was easily discernable by the schlieren effect produced by the density gradient as dense halocline water encountered normal salinity seawater. Sediment samples were collected with *Alvin*-type push cores (6.35-cm diameter; hereafter referred to as "cores") obtained from the Deep Submergence Lab at Woods Hole Oceanographic Institution (www.whoi.edu/groups/DSL/) and configured with a seal to prevent contamination during ascent from the seafloor of L' Atalante, Urania and Discovery basins (Figure 2 in Bernhard et al. 2014; Table S1). Control or normoxic, i.e. oxic and typical salinity values, sediment samples were collected approximately 5 m away from this "beach" at Urania basin. Three samples were retrieved from sediments underlying the halocline of each basin, and at L' Atalante and Discovery basins, samples were obtained from sediments under the lowest possible position along the halocline (limited by the buoyancy of the ROV) toward the brine (designated as mid- and lower-haloclines, Table S1). Since ROV *Jason* is neutrally buoyant in the brines of these basins, it was not possible to core deeper into the DHAB brine sediments, so sampling was targeted to visibly distinct zones at each DHAB corresponding to sediments underlying the upper (less hypersaline) and lower (more hypersaline) halocline. Cores from under the deeper halocline sediments were obtained by positioning the ROV *Jason* on the seafloor within the halocline, and reaching toward the brine with the ROV manipulator. From within the halocline the "ceiling" of the halocline was clearly visible. Positioned on the sediment surface within the halocline, the ROV lasers were not able to penetrate the brine. From this position, the ROV reached into the blackness of the lower halocline and retrieved cores that were only visible upon return to the ROV.

Upon retrieval of cores on deck R/V *Atlantis* (within 4-6 hrs), the cores were visually inspected for adequate sediment content, lack of disturbance, and texture and color. Based on these criteria, nine cores in total, were retained for further processing for these analyses: two from L' Atalante (under the upper and lower halocline; AUH, ALH, respectively), two from Urania (control, i.e. sediment underlying the normal salinity water column outside the DHAB, and from under the lower halocline; UC, ULH, respectively) and five from Discovery (two from under the upper halocline, one from under the middle halocline and two from under the lower halocline; DUH1, DUH2, DMH, DLH1, DLH2,

respectively). Salinities of the overlying water were measured using a refractometer, and oxygen profiles were performed on replicate cores from the same location (Table S1; Bernhard et al, 2014). Subsamples from each core were taken from the core center using a sterile 20-ml syringe (inner diameter of ca. 1.4 cm, length of ca. 10 cm) with the Luer end cut off. Sub-cored sediment was sectioned in 2-cm increments, placed in Falcon tubes and stored immediately at -80^{0}C until RNA extraction.

RNA from ca. 8 g of the top 2 cm from each core was extracted using an optimized protocol and the RNA Power Soil kit (MoBio, USA) after testing and assessing RNA extraction efficiencies of two other widely used extraction kits for sediment nucleic acids. Major modifications included introduction of 3 cycles of freeze-thaw (-80°C, 5 min., 65°C, 5 min.), bead beating with 2x 5 min. intervals on a horizontal vortexer, an overnight nucleic acid precipitation, and a one-hour centrifugation during the precipitation step. In addition, we introduced two DNAase treatments using TurboDNAase (Ambion, USA). Removal of DNA was confirmed by PCR using general bacterial and archaeal primers. One blank extraction was also included in the sample analysis.

Tag-pyrosequencing and data analysis. RNA was purified using the MEGAclear kit (Ambion, USA). Reverse transcription of the purified RNA samples was performed using the QuantiTect kit (Qiagen, USA). Tag-pyrosequencing of the 16S rRNA gene was performed using PCR amplification of the V4-V6 region of the 16S rRNA gene and the primer pair S-DBact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') for Bacteria (Klindworth et al. 2012), and archaea349F (5'-GYGCASCAGKCGMGAAW-3') and archaea806R (5'-GGACTACVSGGGTATCTAAT-3') for Archaea (Takai & Horikoshi 2000), as described in Dowd et al., (2008). A one-step, 30-cycle PCR reaction was performed using HotStarTaq Plus Master Mix Kit (Qiagen, USA). PCR conditions included: 94°C for 3 minutes, followed by 28 cycles of 94^{0}C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 5 minutes. Following PCR, all amplicon products (ca. 450 bp) from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, USA). Nine distinct tags (multiplex identifiers, or MIDs) were incorporated into the primers used for each of the nine samples. Samples were sequenced utilizing the Roche 454 FLX Titanium platform and reagents according to the guidelines at the MRDNA Ltd. (Shallowater, TX, USA) sequencing facility. Processing of the resulting sequences, i.e. trimming and quality control, was performed with the MOTHUR software (v 1.30) (Schloss et al., 2009) including denoising of the flowgrams using PyroNoise (Quince et al. 2009) and data normalization to the smallest number of sequences in the resulting libraries. Sequences ≥250 bp with no ambiguous base assignments and no homopolymers ≥8 bp were included in downstream analyses. Single singletons, i.e. sequences that appeared only once in the whole dataset, were excluded from further analysis. The remaining sequences were aligned using the SILVA SSU database (release 108, Pruesse et al., 2007). All sequences were binned into Operational Taxonomic Units (OTUs) and were clustered (average neighbor algorithm) at 97% sequence similarity (Stackebrandt and Goebel, 1994, Kunin et al., 2010). Coverage values were calculated with MOTHUR (v 1.30). The batch of sequences from this study has been submitted to the Short Reads Archive (http://www.ncbi.nlm.nih.gov/sra) with BioProject PRJNA270764.

RESULTS AND DISCUSSION

The structure of microbial communities is important for revealing the potential metabolic capacities that may occur in specific microbial habitats (Konopka 2009; Magurran & McGill 2011). In microbial habitats, i.e. ecosystems where only microorganisms occur such as the deep subsurface (Whitmann 1998) or certain extreme environments (Sekbach 2006), microbial diversity plays a significant role, as resident microbial communities are entirely responsible for ecological functioning in these environments that are too harsh to support other forms of life. Within microbial communities, responses to environmental variations occur first at the cellular level, and if these conditions persist long enough and are intense enough, then responses can be monitored at the population level. Moreover, although prokaryotes are characterized by tremendous observed but yet –largely– undetermined structural diversity, this results in overlapping metabolic traits, especially regarding core metabolism even between highly divergent species (Chubukov et al. 2014). Since DNA is preserved far longer in marine sediments in comparison to RNA (e.g. Dell' Anno et al. 2002), its use for describing community structure might result in erroneous conclusions by including in the portrait of *in situ* communities phylotypes that originate from extracellular DNA or inactive cells (e.g. Corinaldesi et al. 2014). Based on these considerations, we elected to more closely target the metabolically-active fraction of the prokaryotic communities inhabiting the sediments of three DHABs by investigating the diversity of bulk rRNA as a general marker of metabolic activity (Campbell et al. 2009; Kamke et al. 2010; Jones & Lennon 2011; Blazewicz et al. 2013; Kang et al. 2013). We recognize that this approach runs the risk of missing taxa that are alive, but whose activity levels are so low that their signatures may not appear in the rRNA libraries. Due to a lack of information on the relative relationship between rRNA abundance and growth/metabolic activity (Blazewicz et al. 2013), we focus our analysis only on the most abundant ribotypes, as these are most likely to be well represented in the original samples, and we discuss operational taxonomic unit (OTU) presence/absence rather than relative abundance.

Bacteria. Bacterial cDNA was successfully produced from all nine samples (Table 1). The highest number of unique OTUs (Table 1) were retrieved from under the L' Atalante upper halocline and from the Urania control samples. However, in the Urania control sample, the most abundant OTU (Table 1) represented only 14.3% of the total sequences while in the rest of the samples this percentage ranged between 19.9% and 37.5%. Moreover, in the Urania control sample a total of 116 OTUs comprised ca. 75% of the total sequences, while in the rest of the samples, 75% of total sequences were comprised of only 6 – 26 OTUs (Table 1). This suggests that the sediments under haloclines harbor only a few dominant Bacteria, a typical picture in a community dominated by site-specific species (Konopka 2009). The 116 most dominant OTUs in the control sample (Table 1) belonged to the Chloroflexi, Gammaproteobacteria, Firmicutes, Alphaproteobacteria, Deltaproteobacteria, Gemmatimonadetes, Planctomycetes, Actinobacteria, Nitrospirae, Acidobacteria, Betaproteobacteria, Bacteroidetes and unafilliated Proteobacteria. The Chloroflexi were represented by 38 OTUs, all of them falling in the yet-uncultivated phylogenetic clade SAR202. These Bacteria are common in meso- and bathypelagic ocean waters (Morris et al. 2004; Schattenhofer et al. 2009) but also in oxygen minimum zones (Stevens & Ulloa 2008). The 30 Gammaproteobacterial OTUs recovered from sediments at the control site were related to known bacterial genera in deep-sea sediments such as *Pseudomonas, Pseudolateromonas, Psychrobacter* and *Aliivibrio*. We also detected

Nitrosococcus-related OTUs in our Urania control and in one of the sediment samples from under the Discovery upper halocline, indicative of ammonia-oxidizing taxa (Ward & O'Mullan 2002).

The brines of the Mediterranean DHABs are geochemically distinct (van den Wielen et al. 2005, Table S2) and contrast with the rather uniform and homogeneous overlying oligotrophic water column (Huertas et al. 2012). These differences are most likely shaping prokaryotic community structure in the waters of DHABs (Kerhervé et al. 1999). Differing DHAB chemistries have been shown to shape the protistan assemblages of DHAB haloclines, which share only a few common eukaryotic phylotypes (Bernhard et al. 2014; Stoeck et al. 2014). This leads us to speculate that the geochemical features of DHAB sediments (Table S2) would shape prokaryotic communities as well. Our results show distinctly different dominant prokaryotic assemblages (Figure 2) in the sediments of the three DHABs. By examining the three most abundant OTUs in each sample (Figure 2), these communities were comprised of distinct phylogenetic groups in the sediment samples from under the different DHAB haloclines (Table S3). Taxonomic groups detected in our samples under the haloclines included Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, Actinobacteria and the Bacteroidetes/Chlorobi group. In most cases, the OTUs that showed at least a 5% increase in relative abundance towards the lower halocline, were related either to taxa known to tolerate high salt concentrations or to phylotypes described from high salt concentration and/or low water-activity habitats. Conversely, the ones that decreased in relative abundance by at least 5% from the upper to the lower halocline sediments were in most cases related to typical low salinity environments (Table S3).

A very low number of shared OTUs was found when comparing results between the control sample and upper halocline sediment samples. This was also observed when comparing results for sediments under upper vs. lower haloclines (Figure 3). Only two bacterial OTUs (OTU554 and OTU1288) were shared between the Urania control and sediments under the upper haloclines of L' Atalante and Discovery (Figure 3). OTU554, related to *Sphingomonas* spp. (Table S3), is not only among the most abundant OTUs (Figure 2) but its relative abundance also increased ca. 30% along the transition from the upper to the lower halocline in Discovery (Figure 4). Moreover, OTU1288 is very closely associated (Table S3) to a *Clostridium*-like phylotype, found previously in the L'Atalante basin (Sass et al. 2008). These observations suggest that these two OTUs may represent potentially active populations in the halocline sediments.

Moving from the upper to the lower halocline sediments in L'Atalante, the number of OTUs is reduced by almost one half (Table 1). However, the number of shared OTUs between the two habitats is far greater (131) than between the control sediments and those under the upper halocline (2) (Figure 3). In contrast, the OTU composition in sediments along the upper-to-lower halocline transition in Discovery, resulted in only 10 shared OTUs. Two of these OTUs, (OTU554 and 2940) were grouped among the most abundant ones (Figure 2) and showed an increase of >5% in sediments along the Discovery upper to lower halocline transition (Figure 4). These differences in shared OTUs between sediments under the upper and lower halocline in Discovery and L'Atalante DHABs could be related to the thalassohaline vs. the athalassohaline character of L'Atalante and Discovery, respectively (van der Wielen et al. 2005).

In the L' Atalante upper halocline sediments, among the most abundant OTUs, the Gammaproteobacteria prevailed, represented by *Pseudoalteromonas, Halomonas* and

Pseudomonas spp., suggesting a mixed assemblage of halophilic and halotolerant microorganisms, as expected in this transition zone. Other relatively important OTUs belonged to the spore-forming anaerobic families of Clostridiaceae and Lachnospiraceae. Spore formation is known to be induced under salt stress (Krumbein et al. 2004) and so spore-forming taxa are likely to be detected under these conditions. The transitional nature of this sample was also reflected in the rest of the OTUs, which were affiliated to the aerobic genera *Maribacter* (Nedashkovskaya et al. 2004), *Mesonia* (Nedashkovskaya et al. 2006) and *Sulfitobacter* (Sorokin et al. 1995). *Maribacter* is a halotolerant taxon (Nedahskovskaya et al. 2004) and it has been reported to occur in evaporitic habitats (Dorador et al. 2009) while members of the *Sulfitobacter* genus can grow at 10% NaCl salinity (Park et al. 2007), suggesting these OTUs could represent metabolically active bacteria in these samples.

Sediments under Urania's lower halocline hosted different dominant OTUs than those under the lower halocline of L' Atalante's DHAB. These 26 dominant OTUs found in the lower haloclines (Table 1) belonged to the Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. These OTUs were not related to strict halophilic or even explicitly marine Bacteria, suggesting their allochthonous origin from the overlying water column, perhaps delivered to the sediments via sinking particles. While human contamination during sample handling is unlikely given all the necessary precautions taken, this cannot be ruled out. For example, since the family Staphylococcaceae contains only one halophilic genus *Salinicoccus* (de la Haba et al. 2011), the *Staphylococcus*-like OTU3692 is not likely to be indigenous. Although it is plausible that novel halophilic or halotolerant taxa could be found in DHABs, another interpretation is that inactive cells may retain some amount of intact rRNA in more extreme environments. This idea is supported by reports of the survivability of *Streptococcus faecalis* and *Clostridium perfringens* in the deep-sea (Baross et al. 1975), and strains of *Clostridium* spp. that have been isolated from deep-sea sediments (Lauro et al. 2004). The possible allochthonous origin of some of the Bacteria we detected is also supported by recovery of three OTUs related to plastids, suggesting deposition of cell material from the overlying water-column possibly through rapid sedimentation of large particles. Such transport of cells via sinking particles is not only known to occur in the deep-sea (e.g. Eloe et al. 2011; Lomas & Moran 2011) but recent evidence suggests that some unicellular eukaryotes can survive deep-sea conditions after being transported there from their surface-water habitat (Morgan-Smith et al. 2013).

Several of the dominant OTUs (Table S1) in the DHABs sediments are not typically found in marine sediments, e.g. the Burkholderiales, but are mostly associated with soils. Representatives of some bacteria not frequently found in typical marine environments that have been found in other DHAB waters include sequences affiliated with the genus *Bacillus* from the Urania chemocline and brine water column (Sass et al. 2008). Surface sediments underlying DHAB brines, at least in terms of water activity and water potential (i.e. the amount of water that is needed for microbial activity), may be better described as dry soils rather than marine sediments. In such low water potential environments several microorganisms are known to be metabolically-active (Orchard & Cook 1983) and thus their occurrence in DHAB sediments cannot be excluded.

Despite all possible limitations of the cDNA approach as a proxy for metabolic activity (Blazewicz et al. 2013), we observed some OTUs with increased relative abundance of >5% in samples from sediments underlying lower haloclines relative to upper halocline

or control sites (Figure 4, Table S3). Transitioning to some kind of resting stage is a likely scenario for survival of many taxa under these harsh conditions (Krumbein et al. 2004). However, none of the OTUs that showed a considerable increase in their relative abundance in sediments under lower haloclines (Table S3), represent Bacteria that form spores –at least at the genus level. Many of the bacterial genera we detected with increased representation, i.e. *Burkholderia, Sphingomonas, Lactobacillus, Corynebacterium* and *Pseudomonas*, include mostly obligate aerobic or oxygen-tolerant species. There may be undescribed anaerobic species within these genera, as well as halotolerant or halophilic species. However, based on the aerobic nature of described taxa and the fact that we did not detect known obligate halophiles (see above), we speculate that many of the relatively abundant bacterial OTUs we detected from rRNA in sediments under haloclines may not be active in the sediments under the brines of these DHABs.

Archaea. Archaeal cDNA was successfully retrieved only from sediments under the L' Atalante upper halocline (413 OTUs), the Urania lower halocline (122 OTUs) and the Discovery upper halocline (98 OTUs) (Figure 2). No archaeal rRNA amplification was achievable from control sediments, or sediments under the L' Atalante upper halocline, or Discovery middle or lower haloclines. The apparent OTU richness of Archaea was much lower than for Bacteria, as is the case in most sediment habitats (Smeti et al. 2013 and references therein). The most abundant archaeal OTU contributed between 30.5% (L' Atalante) and 51.6% (Urania) of total reads. L' Atalante's unique character was also reflected in the high number (146) of unique archaeal OTUs compared to the low number of unique archaeal OTUs (29) found in Urania lower halocline (Figure 3). Despite the low number of shared OTUs (3) between the three samples, the shared OTUs were all affiliated to taxa known to be involved in methane metabolism (Table S3), reflecting the distinct prevailing geochemical conditions in each basin (Table S2). Although no sequences affiliating with the candidate division MSBL-1 (originally described from anoxic DHAB brines and thought to have a methanogenic metabolism; van der Wielen et al. 2005) were detected, the archaeal OTUs we detected in our cDNA libraries are believed to have a putative methanogenic metabolism based on their phylogeny (van der Wielen et al. 2005). Representatives of the archaeal ANME-1 group have also been suggested to be halotolerant as they have been found in high-salt concentration environments (Lloyd et al. 2006; Maignien et al. 2013; Pachiadaki et al. 2014). Our results indicate that there may be additional important archaeal taxa involved in DHAB methane cycling aside from MBSL-1, and possibly ANME-1.

Possible metabolic activity. Despite findings suggesting that chemolithoautotrophy is the dominant metabolic trait in halocline waters of Mediterranean DHABs (Yakimov et al. 2013), the OTUs detected in our study represent prokaryotes that need organic substrates for growth. Available organic carbon that reaches the seafloor of Mediterranean DHABs is expected to be relatively recalcitrant, compared to newer, more labile carbon sources in the overlying water column, but there is no information on available electron donors in DHAB sediments. Furthermore, sinking carbon has a tendency to accumulate in the halocline due to the steep density gradient (Sass et al. 2001). This reduces fluxes to the sediment surface inside haloclines and brines. In the Mediterranean Sea DHAB Medee, measurements of enzymatic activity suggest preservation of sediment organic matter rather than its utilization, where only a single *Pseudomonas* DNA phylotype was detected in the center of

Medee basin (Akoumianaki et al. 2012). This suggests that if prokaryotic growth takes place in these poly-extreme sediments, it is expected to be at low rates.

Overall, this study showed that ribosomal RNA profiles of bacterial and archeael communities were largely unique in sediments underlying L'Atalante, Discovery, and Urania haloclines, likely reflecting the unique chemistries of each of these basins. Some of the dominant OTUs in individual sediment samples were represented at significantly higher or lower relative abundance between upper and lower haloclines, and between halocline and control samples, further supporting the notion of adaptation of near-surface sediment communities to the steep DHAB chemical gradients that occur in the overlying waters of these haloclines relative to our control site. While some OTUs were affiliated to known halophilic groups of bacteria, many were not, and some of the most dominant OTUs were affiliated to genera comprised largely of aerobic taxa. Archaeal OTUs were only detected in a few (3) of the sediment samples underlying haloclines, and in those they were far less diverse than bacterial OTUs, appearing to be dominated by sequences affiliated to putative methane cycling taxa. Collectively, our results suggest it is unlikely that diverse active bacterial and archaeal populations exist in the sediments under the haloclines of these DHABs.

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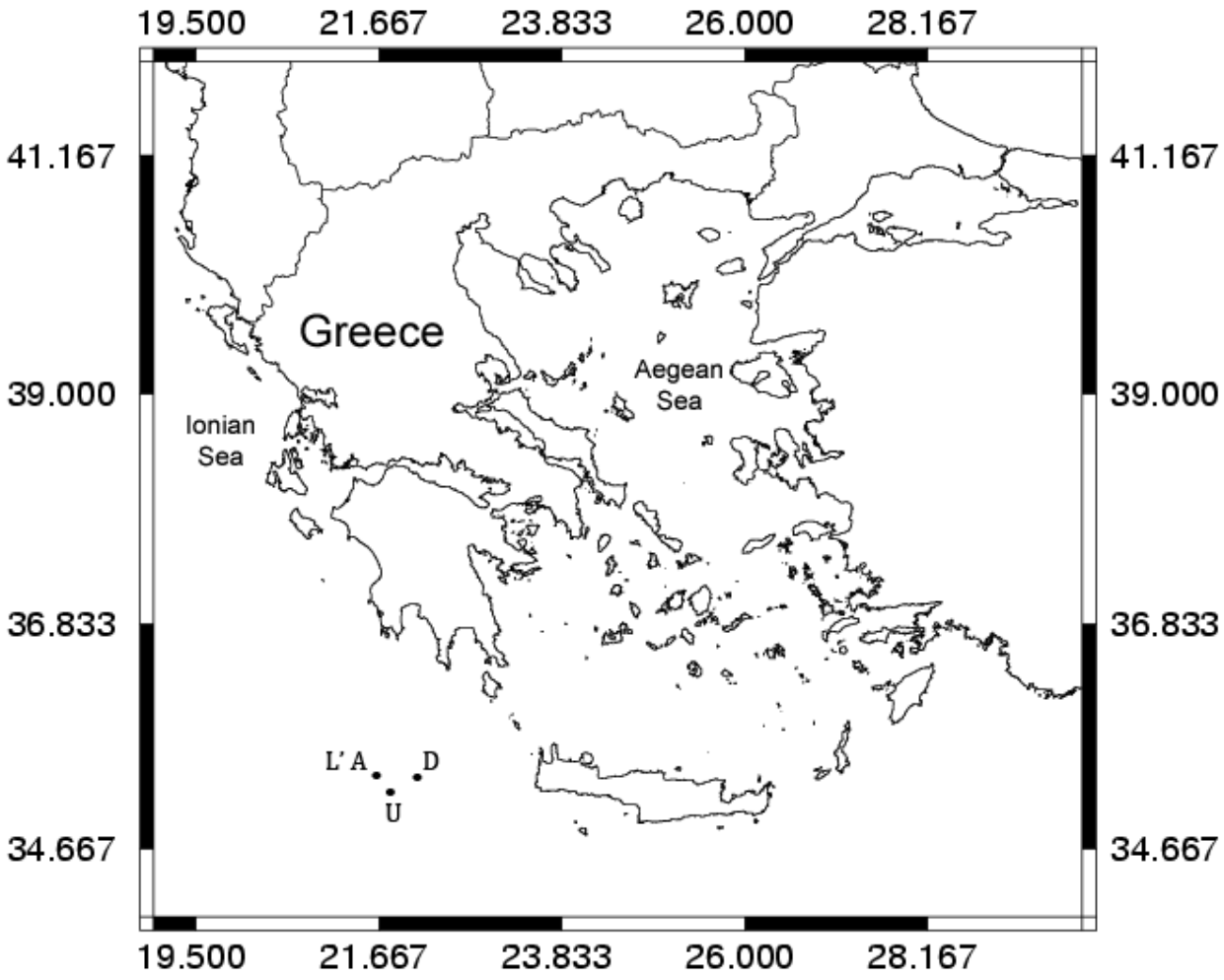

Figure 1. Geographical positions of the investigated basins. L'A: L' Atalante, U: Urania, D: Discovery.

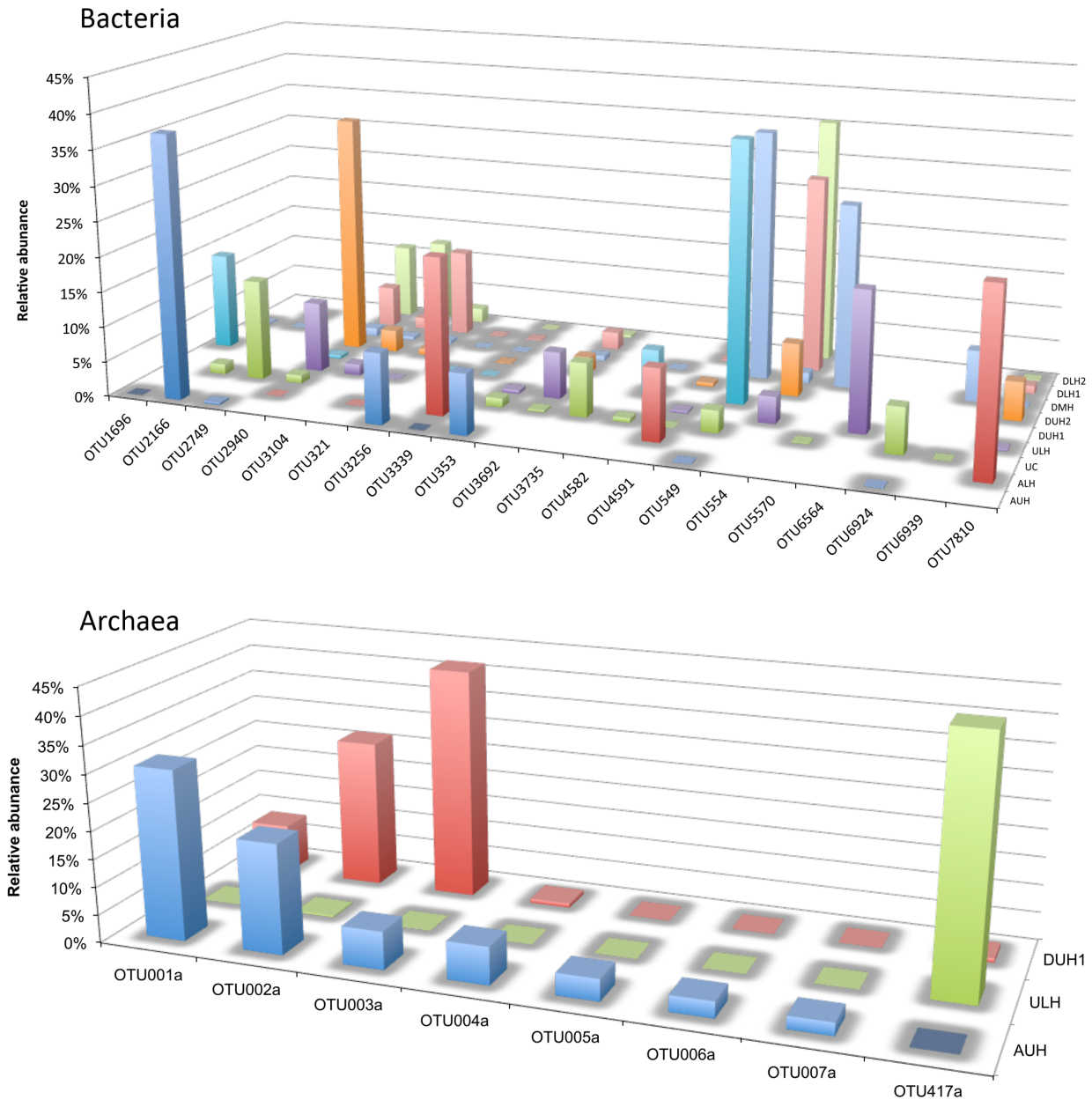

Figure 2. The three most abundant operational taxonomic units (OTU) in the sediments of the Urania (U), L'Atalante (A) and Discovery (D) control (C), upper halocline (UH), middle halocline (MH) and lower halocline (LH).

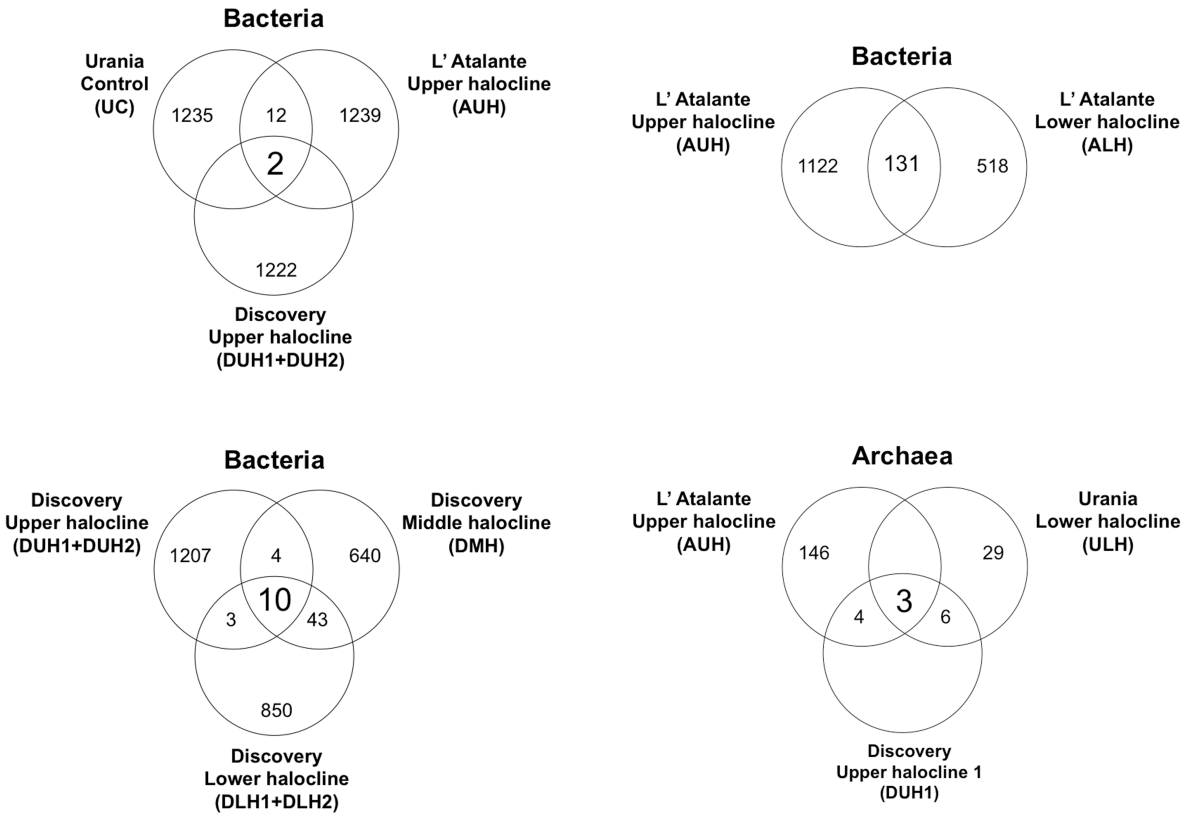

Figure 3. Venn diagrams showing the shared and unique operational taxonomic units (OTU) in the sediments of the Urania (U), L'Atalante (A) and Discovery (D) control (C), upper halocline (UH), middle halocline (MH) and lower halocline (LH).

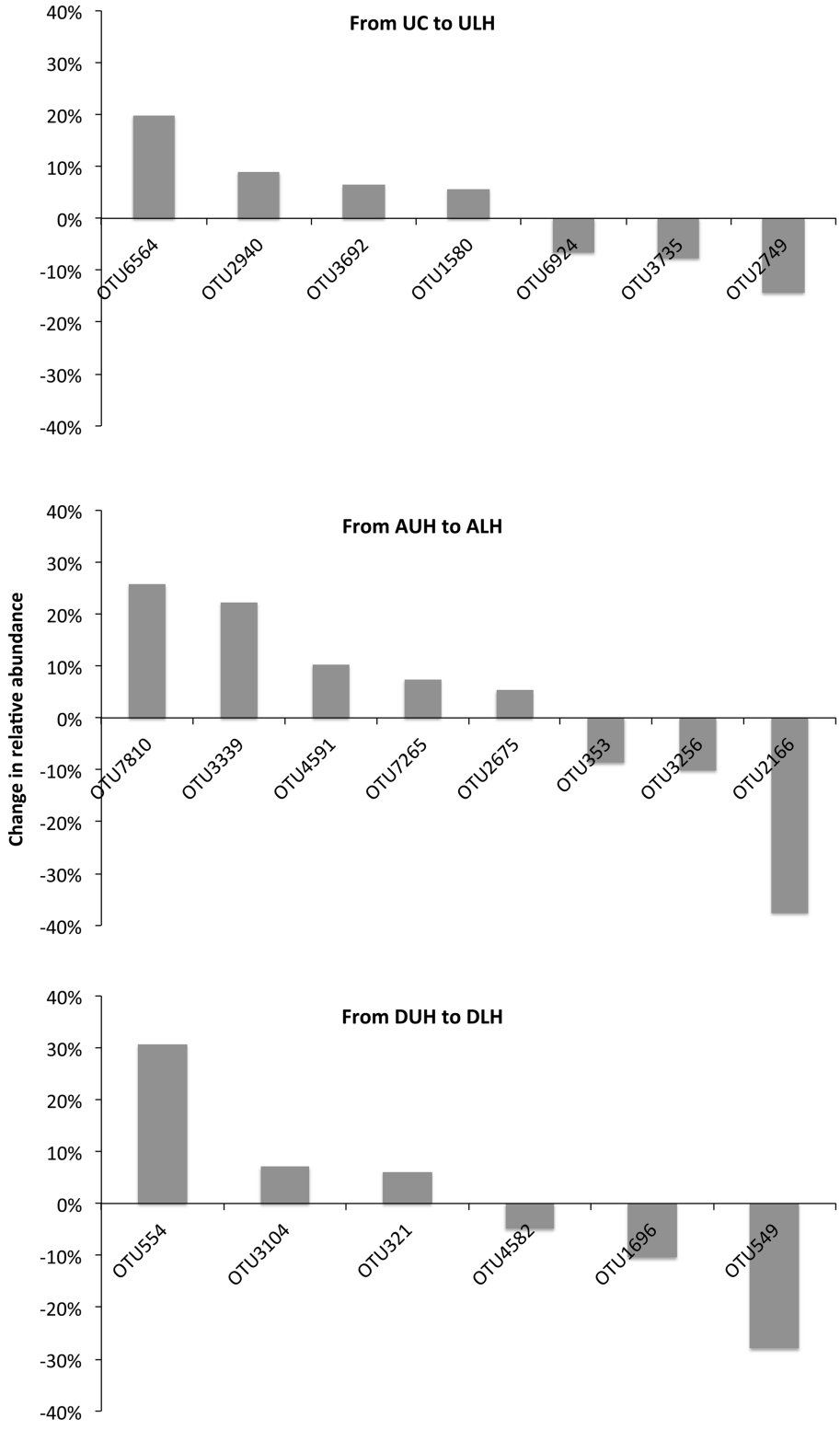

Figure 4. Bacterial operational taxonomic units (OTU) which showed a difference of ≥5 % increase or ≤5 % decrease in their relative abundance moving towards halocline sediments in the Urania (U), L'Atalante (A) and Discovery (D) basins.

Supplementary material

Table S1. Physico-chemical data of the brines and haloclines above Urania, Discovery and L'Atalante Basins of the current study and previous studies.

Sample	Sediment core code (Dive # - Push Core #)	Coordinates	Depth (m)	Total salinity (PSU)	Oxygen (µM)
L'Atalante Upper Halocline	611-6	35°18'N 21°23'E	3499	41[a]	0-50
L'Atalante Lower Halocline	611-13	35°18'N 21°23'E	3501	100[a]	0
Urania control	608-1	35°13'N 21°28'E	3460	38[b]	250-260
Urania Lower Halocline	609-9	35°13'N 21°28'E	3470	172[b]	0
Discovery Upper Halocline 1	609-12	35°19'N 21°41'E	3582	ND/70[c]	20-100
Discovery Upper Halocline 2	610-15	35°19'N 21°41'E	3583	ND	10-75
Discovery Middle Halocline	609-17	35°19'N 21°41'E	3584	102[a]	0-35
Discovery Lower Halocline 1	610-15	35°19'N 21°41'E	3585	125[a]	0-2.3
Discovery Lower Halocline 2	610-9	35°19'N 21°41'E	3586	ND	0-0.6

[a]Data from Bernhard et al. (2014). Data based on refractometer readings or standard conductivity sensors, which are not reliable for athalassohaline brines enriched in divalent cations.
[b]Ranges provided for oxygen concentrations through the top 2cm used for analyses; from Bernhard et al. (2014).
[c]Data from Edgcomb et al. (2011). Discovery upper halocline value for ~1m into halocline.

Edgcomb, V.P., W. Orsi, H.-W. Breiner, A. Stock, S. Filker, M.M. Yakimov, and T. Stoeck. 2011, Novel kinetoplastids associated with hypersaline anoxic lakes in the Eastern Mediterranean deep-sea. Deep-Sea Res I 58::1040-1048

Table S2. Geochemical characteristics of the surrounding sea water, brines and sediments of Mediterrnaean deep-sea hypersaline anoxic basins from various studies.

	Urania Control Seawater	Urania Control Sediments	Urania Brine	Urania Lower Halocline	Urania Lower Halocline Sediments	Discovery Brine	Discovery Upper Halocline Sediments	Discovery Lower Halocline Sediments	L'Atalante Brine	L'Atalante Lower Halocline Sediments	L'Atalante Brine Sediments
Depth (m)	3350	3460	3607	3470	3470	3600	3583	3586	3550	3501	[6]3600
Salinity %	[3]~3.7	3.8	[8]24.0	[3]~17	17.2	[5]9.5	7	>12.5	[2]>30%	10	
Temperature °C	14.02	14.5	[8]18.3	16.9	17.2	[1]14.5	14.05	14.07	[8]14.3	14.1	
Oxygen (µM)	260	200-250	0	[5]53.6	0	0	10-75	0-0.6	0	0	[6]0
pH	~8.0		[10]6.8			[4]~4.5, [9]6.2			[11]6.4-6.7		
Na^+ (mM)	[1]528		[1]3503	[5]876		[1]68			[1]4674		
Cl^- (mM)	[1]616		[1]3729			[1]9491		[4]~9000	[1]5289		
Mg^{2+} (mM)	[1]60		[1]316	[5]79		[1]4995		[4]~4600	[1]410		
SO_4^{2-} (mM)	[1]31.8		[1]107	[5]42		[1]96			[1]397		
HS^- (mM)	[1]2.6x10^{-6}		[1]16	[5]0.66		[1]0.7			[1]2.9		[6]2.9
CH_4 (mM)	[1]1.5x10^{-6}		[1]5.56			[1]0.031			[1]0.52		
NO_3^- (µM)	[3]~3		[11]<161	[3]0.5-nd					[11]322-484		
NH_4^+(mM)	[3]nd		[8]2.87	[3]~2.8					[7]3000		
Mn^{2+} (µM)				[3]~3.2		[9]0.8					
Sulfate reduction rate (µM H_2S day^{-1})	[1]0.236		[1]29.82			[1]23.91			[1]82.15		
Methane production rate (µM CH_4 day^{-1})	[1]0		[1]85.79			[1]2.65			[1]16.93		[6]520

sediments=porewater
nd=not detected

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11. M. Yakimov pers comm.

Table S3. Changes in relative abundance of bacterial and archaeal operational taxonomic units (OTU) along the pelagic-halocline sediments in the Urania, L'Atalante and Discovery Mediterranean deep-sea hypersaline basins. Abundant: among the top three most abundant in at least one sample. Increase/Decrease: ≤5%≥ increase/decrease in relative abundance moving towards lower halocline sediments.

	Notes	**Closest relative**	**Similarity (%)**	**GenBank accession No.**	**Habitat of origin/Information**	**Reference**
Bacteria						
OTU321	Abundant; Increase	*Variovorax paradoxus* BD18-E04	100.0	HF584859	grapevine root system	Marasco et al. (2013)
OTU353	Abundant; Decrease	Uncultured *Halomonas* sp. clone HA_82	100.0	KF859623	Deep-sea sediment	Unpublished
OTU549	Abundant; Decrease	Bacillus sp. DV9-31	100.0	GQ407176	Badwater salt pan, Death Valley, CA, USA	Unpublished
OTU554	Abundant; Increase	*Sphingomonas* sp. Lor54	100.0	KJ016215	Endolichenic (*Lobaria retigera*)	Unpublished
OTU766	Abundant	Clone G250WV301A2KT0 – *Burkholderia* related	98.0	KF338255	Shrimp intestine	Rungrassamee et al. (2014)
OTU1288	Common in Urania control and L'Atalante and Discovery upper haloclines	*Clostridium* sp. AN-AS8	99.2	FR872934	L'Atalante Basin	Sass et al. (2001)
OTU1580	Increase	*Cellulomonas chitinilytica* strain X.bu-b	98.8	NR_041511	cattle farm compost	Yoon et al. (2008)
OTU1696	Abundant; Decrease	Uncultured *Salegentibacter* sp. clone HAHS13.027	98.6	HQ397002	Haloalkaline soil	Unpublished, but see Dorador et al. (2009)
OTU1792	Abundant	clone G250WV301BL5R6 – *Sphingomonas* related	97.2	KF338845	Shrimp intestine	Rungrassamee et al. (2014)
OTU2166	Abundant; Decrease	*Alteromonas* sp. ECSMB57	100.0	KM369862	nature biofilms formed in the coastal seawater at	Unpublished

					Gouqi Island	
OTU2570	Abundant	*Pseudomonas geniculata* OTU-a9	100.0	KJ147059	Fungus *Pandora neoaphidis* endosymbiont	Unpublished
OTU2675	Increase	*Serratia proteamaculans* strain ai2	100.0	KJ194992	"Succession of Lignocelulolytic Facultative-Anaerobic Bacterial Consortia bred from Lake Sediment"	Unpublished
OTU2749	Abundant; Decrease	*Pseudomonas stutzeri* strain NM2E7	100.0	KM874453	Root; Isolation and characterization of endophytic bacteria associated with heavy metals tolerant plants on mine tailings in Villa de la Paz (Mexico)	Unpublished
OTU2940	Abundant; Increase	*Burkholderia stabilis* SPP-21	99.3	KF836499		Unpublished
OTU3104	Abundant; Increase	Uncultured bacterium clone Espejo_9_20_11_Pumice.67702	98.7	KM157737	Pumice	Unpublished
OTU3256	Abundant; Decrease	Uncultured bacterium clone 3051bac1-55	99.5	GU982762	Pacific deep-sea sediment	Unpublished
OTU3339	Abundant; Increase	*Enterobacter hormaechei* D10	100.0	KJ123711	Vinegar fermentation starter	Li et al. (2014)
OTU3507		*Lactobacillus delbrueckii* subsp. *bulgaricus*	100.0	KJ026672	Fermented dairy product	Unpublished
OTU3692	Abundant; Increase	*Staphylococcus epidermidis* voucher RIFA 1117	100.0	KF624759	ant mound	Unpublished
OTU3735	Abundant; Decrease	Uncultured bacterium clone RS-B32	98.8	JF809742	Medee DHAB sediment	Akoumianaki et al. (2012)
OTU4467		*Corynebacterium aurimucosum* H2456	98.60	NR_115262	Genomic DNA	Daneshvar et al. (2004)
OTU4582	Abundant; Decrease	*Firmicutes* bacterium M71_D94	98.6	FM992835	Eastern Mediterranean Sea	Gartner et al. (2011)

OTU4591	Abundant; Increase	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: smkt_B-pro_001_031	100.0	AB806525	Shimokita Penninsula offshore drilling core sample	Unpublished
OTU5570	Abundant	*Novosphingobium* sp. 2P1G10	100.0	HF936984	River sediment	Unpublished
OTU6564	Abundant; Increase	Uncultured cyanobacterium clone C_13	97.3	FJ490250	Antarctica Dry Valleys	Pointing et al. (2009)
OTU6924	Abundant; Decrease	*Psychrobacter nivimaris* strain XH236	100.0	KF424830	deep-sea sidiments	Unpublished
OTU6939	Abundant	Uncultured bacterium clone LNH_12_1_11_Water.267033	98.3	KM146672	Pumice	Unpublished
OTU7265	Increase	*Pedomicrobium ferrugineum* strain ATCC 33119	100.0	NR_104840	Temperature range is 10–40^{0}C. pH range is 3.5–10.0. Survival, but no growth, range 1–5% NaCl	Brenner et al. (2005)
OTU7810	Abundant; Increase	*Bradyrhizobium elkanii* OTU-c63	100.0	KJ147076	Fungus *Pandora nouryi* endosymbiont	Unpublished
Archaea						
OTU001a	Most abundant, shared among all three samples	Clone NZ_78_Arch_33	100.0	JN884897	Deep-sea methane seep	Ruff et al. (2013)
OTU002a	Most abundant, shared among all three samples	Clone T13M-A21, Thaumarchaeota MGI	98.2	JN798493	Low temperature hydrothermal oxides	Unpublished
OTU003a	Most abundant, shared among all three samples	Clone SB11_H10, Thaumarchaeota	99.5	KF176700	Sponge *Tethya aurantia*	Unpublished
OTU004a	Most abundant	Clone 62.64_c5, Crenarchaeota	98.6	HE579761	Low temperature deep-sea vent	Perner et al. (2011)
OTU005a	Most abundant	Clone 48H-0S-18	99.5	GU270206	Deep-sea methane seep surface sediments	Dang et al. (2010)
OTU006a	Most abundant	Clone MC118_36A17	99.5	HM601383	Deep-sea hydrocarbon seep	Unpublished

					sediment	
OTU007a	Most abundant	Clone Zeebrugge_A84	98.6	HM598534	Brackish sediments contaminated with hydrocarbons and heavy metals (3 m water depth)	Siegert et al. (2011)
OTU417a	Most abundant	clone NapMat-0_4-rtB10b	98.2	HQ443430	Napoli mud volcano hypersaline marine sediment, 0-4 cmbsf	Lazar et al. (2011)

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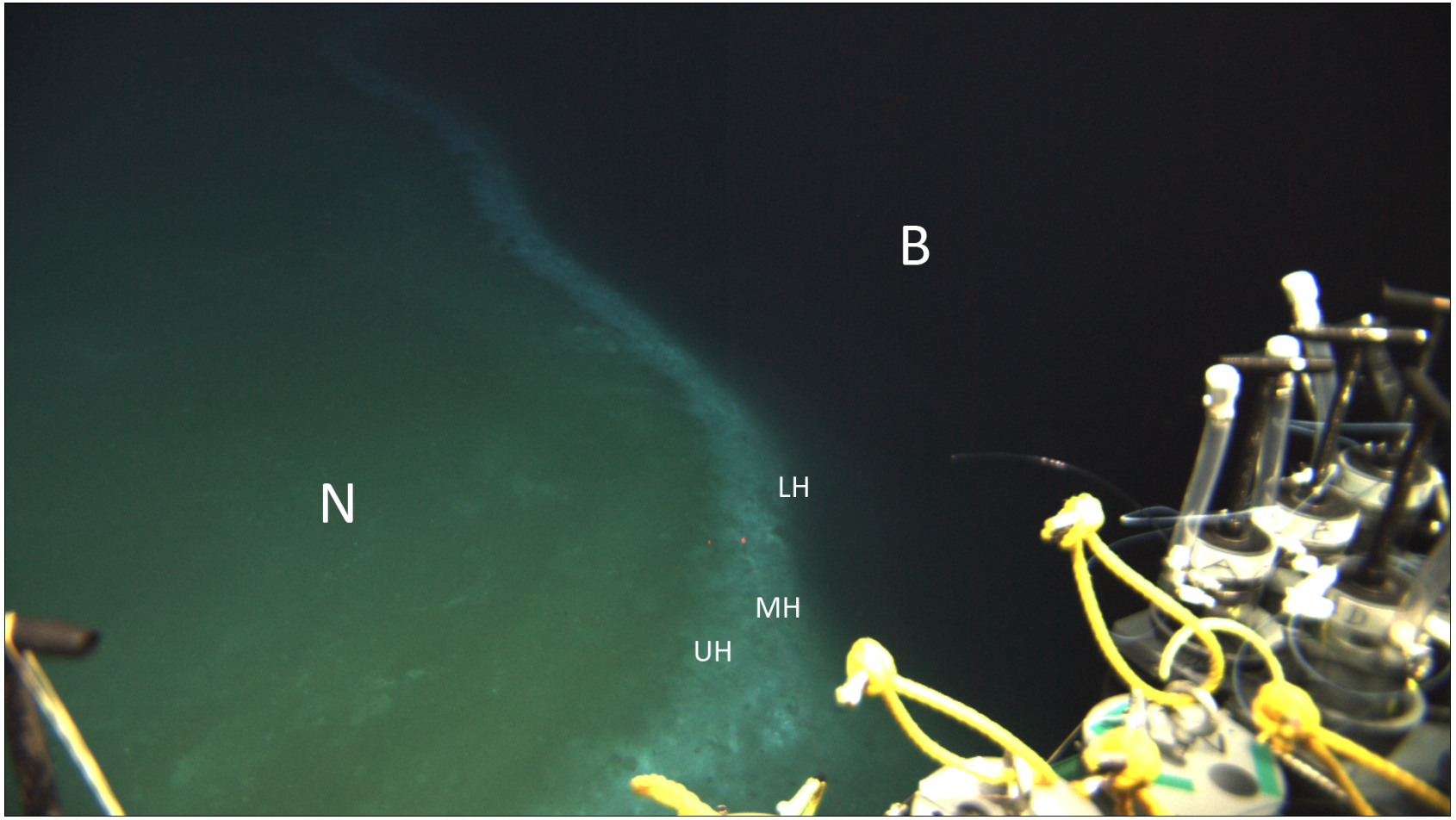

Figure S1. Bathymetry of the L' Atalante halocline zone (white zone). N = normoxic control region, UH=upper halocline, MH=middle halocline, LH=lower halocline, B=brine. Image © Woods Hole Oceanographic Institution, USA.