

# Microbial diversity in deep hypersaline anoxic basins

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## 1 Introduction

Deep hypersaline anoxic basins (DHABs) have been discovered on the seafloor in different oceanic regions, such as the Gulf of Mexico (Shokes et al. 1977), the Red Sea (Pautot et al. 1984) and the Eastern Mediterranean Sea (Jongsma et al. 1983; Dupré et al. 2007). DHABs contain brines that due to their temperature and/or salinity do not mix with the upper seawater layers and lead to the stratification of the water column. Gradients of oxygen, sulphide, ammonium and other electron acceptors/donors can occur, quite often contributed by microbial activity (Sass et al. 2001). The chemoclines at the interface between the hypersaline waters of the brines and the overlaying seawater have been recognized as hot spot for microbial abundance and activity (van der Wielen et al. 2005; Daffonchio et al. 2006). Environmental gradients represent high turnover spots in the biosphere and have to be taken into account to model global biogeochemical cycles and their response to environmental changes (Brune et al. 2000).

All the DHABs studied so far have been found to be colonized by microbial communities active and rich in biodiversity. Microorganisms living in these environments have to cope with multiple stresses that limit the activity of organisms thriving in conventional environments. Hypersalinity, 5–10 times higher than seawater, lacks of oxygen and highly reducing conditions, high pressure and absence of light make microorganisms of DHABs true extremophiles.

## 2 Localization and origin of DHABs

DHABs have been discovered in different geographical regions, generated in different geological conditions. Brine pools are described on the caldera of active and ancient mud volcanoes, whilst brine basins (or lakes) originated by the dissolution of buried evaporitic rocks.

## 2.1 The anoxic brine basins in the Red Sea: the Shaban and Kebrit Deep

During the past 50 years several deep-sea brine pools have been identified in the Red Sea, that can be defined as an ocean *in statu nascendi*, forming during the past 20 million years due to the divergent movement of the Arabian and African continental plates (Degens and Ross 1969; Backer and Schoell 1972; Girdler and Styles 1974; Pautot et al. 1984). This movement is associated with the genesis of new oceanic crust, which can be observed in the southern Red Sea as a 1500–2000 m deep axial graben. Towards the north the graben narrows up to 21°N, where isolated deeps can be found, approximately 25 of which are filled with extreme saline water, formed by the leaching of subbottom Miocene evaporite. Recent volcanic intrusions in proximity of some of the brines are responsible for their high temperatures (Hartmann et al. 1998). Two of the most studied deep-sea brine pools in the Red Sea are the Shaban and the Kebrit Deep. The Shaban Deep consists of four depressions where the brine-seawater interface can be found at a depth of  $1325 \pm 3$  m and has remained constant since its discovery. The Kebrit Deep is a roundish basin of approximately 1 km in diameter and a maximum depth of 1549 m, filled by 84-m-thick anaerobic brine (Eder et al. 1999).

## 2.2 The Orca basin and other seafloor anoxic brines in the Gulf of Mexico

In the Gulf of Mexico several mud volcanoes, brine pools and brine basins have been described. This marine area is an economically relevant hydrocarbon basin containing late Jurassic age oil and gas (Macgregor 1983). The bathymetry of the Gulf of Mexico was shaped by the tectonics of an early Jurassic salt deposit which underlies the northwestern margin of the basin (Joye et al. 2005). The discovery of the Orca basin, a bathymetric depression on the northern slope of the Gulf of Mexico was reported in the late 1970s by Shokes et al. (1977). The Orca basin is elbow-shaped; it covers about 400 km<sup>2</sup> and it has a maximum depth of 2400 m. Several lines of evidence suggest that the extreme saline brine contained in the Orca basin originated from the dissolution of nearby salt deposits (LaRock et al. 1979; van Cappellen et al. 1998).

More recently shallower hypersaline habitats in the northern Gulf of Mexico have attracted the attention of many researchers and their ecosystems have been investigated. A quiescent brine pool (site GC233, 27°43.4'N, 91°16.8'W) covering 190 m<sup>2</sup> is located at a depth of 650 m and is considered the remaining of an ancient mud volcano. A second larger brine lake is constituted by an active mud volcano (site GB425, 27°33.2'N, 92°32.4'W) with a strong fluid flow through its crater emitting high rates of gas and fluidized hypersaline mud formed by a fine clay suspension (MacDonald et al. 2000; Joye et al. 2005).

### 2.3 Eastern Mediterranean Sea: DHABs and the Chefren mud volcano

Five DHAB lakes have been discovered and studied since the 1980s in the Eastern Mediterranean Sea. The basins Bannock, Tyro, Urania, L'Atalante and Discovery represent extreme and largely unexplored habitats. These lakes are the deepest known DHABs, far below the photic zone (3200–3500 m deep) and are located in the Mediterranean Ridge in the Eastern Mediterranean Sea, an accretionary complex subjected to continental collision. The origin of their brines has been attributed to the dissolution of the 5–8-million-year-old Messinian evaporites or to diagenetic relics of evaporated seawater entrapped as interstitial water in Messinian sediments released by tectonic deformation of the sediments on the Mediterranean Ridge. These waters have been subsequently modified from the original composition by geochemical events such as dolomitization and gypsum precipitation, as well as biological activities such as sulphate reduction and decomposition of sediment organic matter (Camerlenghi 1990; Vengosh et al. 1998). The downward flow of the dense brines into local depressions was followed by the progressive development of anoxia in the brine lakes (Wallmann et al. 1997). The haloclines established at the seawater-brine interfaces act as density barriers and limit the mixing of the upper oxygenated water columns with the underlying brines. This implies that DHABs have been physically isolated from other habitats on Earth for thousand of years (Ferrer et al. 2005), thus potentially leading to the selection of new very peculiar extremophiles.

In the Eastern Mediterranean Sea, one of the most oligotrophic marine water bodies, active mud volcanism associated with diverse ecosystems has recently been detected on the Nile Deep Sea Fan (Dupré et al. 2007; Omoregie et al. 2008). In 2000 the bathymetry surveys of this area allowed the discovery of the Chefren mud volcano of the Menes Caldera (Masclé et al. 2006). The Menes Caldera is a circular depression with a diameter of 8 km that is approximately 50–100 m deep, at a water depth of about 3000 m in the western area of the Nile Deep Sea Fan. The caldera contains three mud volcanoes named Chefren, Cheops and Mykerinos. Chefren is about 500 m in diameter and rises about 60 m above the bottom of the caldera. Omoregie et al. (2008) discovered that the centre of the Chefren volcano is filled by a brine and mud lake.

### 3 Geochemical features of DHABs

The different origins and locations make each DHABs a specific environment with a peculiar geochemical setting. Common traits are hypersalinity, anoxia, high pressure and absence of light.

All DHABs present a transition zone at the interface between upper seawater and the underlying brines. This layer is a few to ten metres thick, contains an oxic/anoxic

boundary and constitutes a halocline/chemocline where chemical species are distributed in a gradient of concentrations. The profiles of concentration of the different chemical species allow evaluating if and how they are produced and/or consumed. Changes of concentrations can reflect the occurrence of chemical or biological processes, assuming that the concentration profiles are not significantly affected by lateral transport or changes of the transport properties over the considered range of depth. The strategy commonly used to eliminate the effects of variable vertical eddy diffusivity on the concentration distribution of solutes in the transition zone consists in plotting the concentration versus salinity. The concentration of a conservative dissolved species (e.g. NaCl) in such a diagram will appear as a straight line between the seawater and brine concentration values. In contrast, when the plot shows a curvature it indicates production (concave-up) or consumption (convex-down).

### 3.1 DHABs of the Red Sea

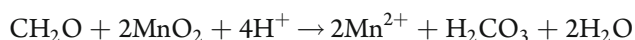
The Shaban Deep is formed by four depressions filled with anaerobic hydrogen sulphide-free brines. The brines in the north and west basins have a temperature of 22.7°C and a pH of 5.7 whereas in the south and east basins both the temperature (24.0–25.2°C) and pH value (6.0–6.2) of the brines are higher. The brines collected from the south and east basins are enriched in Na and Cl (4.0–4.4 M and 4.4–4.5 M, respectively) compared with the upper seawater. Suboxic condition within the brine and the lower part of the interface make possible an increase of the concentration of dissolved Fe (122–130 µM) and Mn (35.5–37.6 µM) with depth (Eder et al. 2002). In addition, many other elements show a relevant increase moving from the seawater to the brine (i.e. Mg, K, Ca, Li and Ba).

Similarly to the Shaban Deep, the Kebrit Deep exhibits a steep increase of NaCl concentration (from 4 to 26%, w/v) within only 3 m at the seawater-brine interface, but the brine chemistry is different. Within about 7 m the temperature increases from 21.6 up to 23.4°C, the CH<sub>4</sub> concentration increases from 50 nl/l to 22 ml/l and H<sub>2</sub>S content rises up to 12–14 mg of S/l. In the same depth range, pH values decrease from 8.1 to 5.5 and O<sub>2</sub> concentration drops from 3.2 ml/l to zero. The high gas content of the brine is constituted mainly of CO<sub>2</sub> and H<sub>2</sub>S and small amounts of N<sub>2</sub>, CH<sub>4</sub> and C<sub>2</sub>H<sub>6</sub> (Eder et al. 1999, 2001).

### 3.2 DHABs of the Gulf of Mexico

In correspondence with the oxic–anoxic interface of the Orca basin, the oxygen is depleted, the temperature increases slightly from 4.45 up to 5.35°C, the phosphorous concentration raises and the water salinity reaches saturation for NaCl (Shokes et al. 1977; LaRock et al. 1979) in 13 m, a larger interval than in DHABs of the Red

Sea and the Eastern Mediterranean (van Cappellen et al. 1998). The ammonium concentration follows a near-linear trend in correspondence to the 6–24.5% salinity range, indicating a conservative mixing of the ammonium-enriched brine with seawater along with depth (van Cappellen et al. 1998). Below 2200 m manganese and iron oxides have been indicated as the energetically most favourable terminal electron acceptors for heterotrophic bacteria (Stumm and Morgan 1996). Reductive dissolution of manganese oxides in the intermediate salinity range (6–18%) can be gathered from the concave-up curve of the diagram reporting dissolved  $\text{Mn}^{2+}$  concentration versus salinity (van Cappellen et al. 1998). Both alkalinity, which can be used as a tracer of pathways of organic matter breakdown, and  $\text{Mn}^{2+}$  concentrations show peaks at salinities around 15%. The data are in agreement with the stoichiometric oxidation of organic matter by  $\text{MnO}_2$ , according to the following reaction:



Below a depth of 2225 m, the presence of detectable dissolved sulphide that can react with Fe(II) and Fe(III) is resulting in the formation of iron sulphide in the water column. This reaction is consistent with the high concentration of particulate iron detected in the brine and the sediments rich in iron sulphides observed in the Orca basin (van Cappellen et al. 1998).

The brines recovered from the active mud volcano (site GB425) and the brine pool (site GC233) were constituted via halite dissolution. In both sites the brines showed the same  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations (1800 and 2100 mM, respectively), the absence of sulphate and a high concentration of dissolved hydrogen (Joye et al. 2005). Hydrogen concentrations range from hundreds of nanomolar in the mud volcano brines up to about 6  $\mu\text{M}$  in the brine pool. The pH of the brine collected from the brine pool is about 6.4–6.8 whereas at the mud volcano the pH value is higher (7.4). In both brines the most abundant alkane is methane and the dissolved organic carbon (DOC) increases along with depth, indicating the presence of a subsurface DOC sink (Joye et al. 2009).

### 3.3 DHABs of the Eastern Mediterranean Sea

The chemical composition of Urania, L'Atalante, Bannock and Discovery basins has been investigated extensively. All the hypersaline lakes show a distinct geochemistry, though Bannock, L'Atalante and Urania basins are more similar and significantly different from Discovery (van der Wielen et al. 2005).

The global salinity of Urania is lower than that of the other Mediterranean DHABs, with NaCl concentration 5.4–7 times higher than normal seawater.

Methane concentration in Urania reaches 5.56 mM, which is 10–100 times higher than in the other DHABs. Very high concentrations of sulphide (up to 16 mM in the brines) place Urania amongst the most sulphidic marine water bodies on Earth (Borin et al. 2009). Sulphide levels range between 0.7 and 3.0 mM in the other Mediterranean DHABs, whereas the seawater shows a concentration of  $2.6 \times 10^{-6}$  mM. In this lake two discontinuities in salinity and temperature have been found along the water column. The first constitutes a halocline of 2 m depth (interface I) where salinity strongly increases from 3.7% to 16.1% and temperature values change from 14°C to 16.5°C. After 67 m (brine I) a second chemocline is present (interface II) between body brines of different salinities and temperatures (Borin et al. 2009).

The main difference between the geochemistry of Discovery brine compared with the other three basins is an extremely high concentration of  $\text{Mg}^{2+}$  and the absence of  $\text{Na}^+$ . The Discovery lake contains a brine that shows the highest concentration of  $\text{MgCl}_2$  (around 5 M) that has ever been measured in a marine environment (Wallmann et al. 2002). The highest  $\text{MgCl}_2$  concentration previously reported (up to 2 M) in a natural environment is in the Dead Sea and Lake Bonney in Antarctica (Matsubaya et al. 1979; Oren 1999).

L'Atalante basin has a sharp 1.5-m-deep halocline showing a steep salinity gradient (from 3.9% at its upper part to 36.5% at the lower part). The decrease of the redox potential to negative values in the initial part of the halocline indicates a complete depletion of oxygen in the underlying brine lake. Along the halocline a progressive increase of ammonia from 5.5 up to 3000  $\mu\text{M}$  has been described (Yakimov et al. 2007), making the Eastern Mediterranean DHABs the marine environments with the highest ammonia concentrations. The brine of the L'Atalante basin is characterized by anoxic, high sulphidic (2.9 mM) conditions and salt concentration to almost saturation (411 g/l NaCl).

In the Bannock basin the halocline is about 2.5 m deep. Down the halocline the nitrate concentration decreases from 6.6 to 0.3  $\mu\text{M}$  and the ammonium concentration increases from 5 to 3450  $\mu\text{M}$  (De Lange et al. 1990), a concentration that is 1–5 order of magnitudes greater than those observed in the open ocean and coastal waters (Könneke et al. 2005). The concentration of sulphate, the most abundant electron acceptor along the Bannock chemocline, increases from 31 to 84 mM. Manganese increases from 0.4 to 8.3  $\mu\text{M}$  and redox potential drops from 210 mV to very negative values, as a result of total oxygen depletion in the lower part of the halocline. Dissolved manganese, nitrate, ammonium and sulphate concentrations have non-linear slopes, indicating a non-conservative behaviour, possibly due to biologically mediated processes (Daffonchio et al. 2006).

### 3.4 DHABs of the Mediterranean Sea: the Chefren mud volcano (Nile Deep Sea Fan)

During a dive with the submersible *Nautilie* at the bottom of the Chefren mud volcano two kinds of closely associated microbial mats were discovered on top of the bottom sediments. The mats showed different colours (white and orange) and covered about 25 m<sup>2</sup>. The surface of the cores collected from the white mat included thick white precipitates constituted by filamentous sulphur aggregates. On the surface of the cores recorded from orange mat a thick layer of fluffy yellow material and flaky, orange particles resembling Fe<sup>3+</sup> (hydr)oxides were observed. Fluids from the brine pool at Chefren mud volcano are characterized by a salinity value of around 15%, relatively high methane (2.4 mM) and sulphide (7.2 mM) concentrations and sulphate concentration of 50 mM (Omeregiet et al. 2008). In both the white and orange mats, the concentrations of Na<sup>+</sup> and Cl<sup>-</sup> are higher than those of the bottom water, indicating the presence of upward brine flow through the sediments. About 6 cm below the white mat it is possible to observe a black, reduced sediment layer characterized by high iron and sulphur concentrations of the solid phase, consistent with the high content of FeS and pyrite. The sulphate concentration decreases from the surface layer (28 mM) moving towards the bottom of the core (5 mM at 15 cm depth), whereas the methane concentration increases by one order of magnitude from 0.01 up to 0.1 mM.

## 4 Microbial life in the DHABs: biodiversity and adaptation

More than 70% of the Earth's surface is covered by oceans and about 60% of this area is represented by water more than 2000 m deep. Nevertheless the oceans are the last environment to be investigated for their microbiology on our planet (Bull et al. 2000). Microscopy and biochemical investigations (i.e. lipid analysis, measurements of ATP and CH<sub>4</sub>) indicated the existence of microbes in hypersaline brines (LaRock et al. 1979; Dickins and Van Vleet 1992). However only in the past two decades, thanks to the development of powerful cultivation-independent molecular surveying techniques, a more careful analysis of the prokaryotes became possible and a surprisingly high degree of biodiversity has been discovered in marine microorganisms representing all domains and viruses. Bacteria and Archaea belonging to new taxonomic lineages were discovered in high abundance in DHABs by the screening of 16S rRNA libraries and fluorescent in situ hybridization (FISH). In some cases, they were considered salt-adapted and responsible for key microbial activities that were detected in the brines, without parallel detection of microbial taxa of known physiology.

#### 4.1 Prokaryotic biodiversity of the Kebrit and Shaban Deeps, Red Sea

Bacterial and archaeal 16 S rRNA gene libraries were obtained from an extreme saline brine sediment of the Kebrit Deep. The phylogenetic investigation of these sequences showed that most of them had only low sequence similarity with cultivated Bacteria or Archaea.

The bacterial sequences grouped together forming a novel branch named KB1 in the 16 S rRNA tree, distantly related to *Aquificales* and *Thermotogales*. All the archaeal 16 S rRNA sequences retrieved from the Kebrit Deep belonged to the phylum *Euryarchaeota* and were only distantly related to sequences detected in other marine habitats (Eder et al. 1999). Many clone sequences belonging to the KB1 cluster were obtained also from the higher salinity (24%) part of the seawater-brine interface of the Shaban Deep and in the Mediterranean DHABs Bannock and Urania, showing that the KB1 group is ubiquitous within the brines of the Red Sea and the Mediterranean Sea. These lines of evidence confirm that the KB1 lineage comprises bacteria specifically adapted to anoxic highly hypersaline conditions, unfortunately still not obtained in culture. Only from the lower part of the halocline at the Shaban Deep a second well separated group of sequences, named SB1, was detected that did not exhibit high similarity with any of the cultivated Bacteria. The screening of 16 S rRNA gene libraries revealed that the Shaban basin differs from the Kebrit Deep also in the archaeal community, represented not only by *Euryarchaeota*, but also by *Crenarchaeota*.

The assessment of the microbiota diversity at the deep basins of the Red Sea has been carried out also through the application of cultivation methods. The first halophilic bacteria cultivated from DHABs were obtained from the Kebrit Deep. These strains are Gram-negative, motile rods with rounded ends that grow singly or in pairs. They are strongly related to members of the genus *Halanaerobium* comprising anaerobic, halophilic and fermentative bacteria. The same species was also detected in 16 S rRNA libraries of the seawater-brine interface of the Kebrit Deep, indicating its adaptation to the interface habitat (Eder et al. 2001). Recently Antunes et al. (2008) were able to obtain in pure culture a novel obligate anaerobic bacterium from the brine-sediment interface of the Shaban Deep. The isolate SSD-17B<sup>T</sup> is Gram-negative, able to grow in a medium containing NaCl concentration that ranges from 1.5 to 18%; it exhibits denitrifying and fermentative metabolisms and it is characterized by pleomorphic cells without cell walls. It has 'tentacle-like' protrusions with motility function. Phylogenetic studies indicated that strain SSB-17B<sup>T</sup> is a representative of a new lineage between the phyla Firmicutes and Mollicutes. SSB-17B<sup>T</sup> isolate belongs to the novel species *Haloplasma contractile*, order *Haloplasmatales* (Antunes et al. 2008).



#### 4.2 Phylogenetic analyses of the microbial communities colonizing different seafloor hypersaline brines of the Gulf of Mexico

The presence of an active microbial population at the seawater-brine interface of the Orca basin is suggested by several lines of evidence. Direct cell counts showed a peak of total cells in correspondence with the seawater-brine interface, the only portion of the basin in which the uptake of [ $^3\text{H}$ ]-uridine was detected (LaRock et al. 1988). The highest ATP level (20 ng/l) was measured in the upper interface and the presence of Archaea has been demonstrated through the isolation of specific isoprenyl ether-linked lipids (Dickins and Van Vleet 1992). The seawater-brine interface of the Orca basin hosts a redoxcline essentially dominated by manganese. Iron- and manganese-reducing bacteria were isolated on selective media and the plate counts indicated that the cell number of these two functional groups decreases along seawater up to a depth of 2170 m, whereas they significantly increase between 2170 and 2225 m (van Cappellen et al. 1998). Despite the detection of high numbers of iron- and manganese-reducing bacteria at the intermediate salinity region of the interface, only manganese oxide resulted to be depleted at these depths, whereas the iron oxides showed a conservative profile. To explain the same pattern of distribution of iron- and manganese-reducing bacteria, van Cappellen et al. (1998) hypothesized the presence of bacteria able to use both iron and manganese oxides as electron acceptors.

Cell plate counts from samples of the mud volcano (site GB425) and the brine pool (site GC233) showed that prokaryotes are two orders of magnitude more abundant in the brines than in the seawater. The microbial diversity was evaluated through metabolic activity assays, 16 S rRNA and functional gene libraries. According to the different distribution of chemical species and activity assays, different metabolic processes seemed to prevail in the brine of sites GB425 and GC233. In the brine pool sulphate reduction was the dominating metabolic pathway, acetoclastic methanogenesis rates were lower and both hydrogenoclastic and anaerobic methane oxidation (AOM) were below the detection limit (Joye et al. 2009). In contrast, the mud volcano showed higher rates of acetoclastic methanogenesis and also hydrogenoclastic methanogenesis was detected. The absence of AOM was registered also at the mud volcano site, though high methane fluxes were observed at these two sites. In both brines, 16 S rRNA gene libraries showed the presence of sulphate-reducing bacteria (SRB). SRB diversity, described by dissimilatory sulphite reductase (*dsrAB*) gene libraries, was higher in the quiescent pool than in the mud volcano brines, in accordance with activity data. Moreover, the presence of *Epsilonproteobacteria*, able to oxidize sulphide and hydrogen, in the brine pool suggested that an active sulphur cycle occurs in this deep hypersaline habitat (Joye et al. 2009). Despite the low rates of acetoclastic and hydrogenoclastic methanogenesis

measured at the brine pool, methyl coenzyme M reductase (*mcrA*) sequences taxonomically linked to acetoclastic, methylophilic, hydrogenotrophic and methane-consuming microorganisms were retrieved at this site. The *mcrA* gene sequences obtained from the mud volcano were mainly represented by acetoclastic species whereas hydrogenoclastic methanogens were not identified, possibly due to the presence of novel unknown salt-adapted hydrogenotrophs (Joye et al. 2009). The diverse microbiota composition observed at the brine pool and mud volcano sites can be correlated with their different fluid flows and organic matter inputs.

#### 4.3 Investigation of the microbiota composition inhabiting the Chefren mud volcano (Nile Deep Sea Fan)

The composition of the prokaryotic community that colonizes morphologically different microbial mats on the sediment surface at the Chefren mud volcano was analyzed by Omoregie et al. (2008) by the application of FISH and 16 S rRNA gene libraries. Significant differences were found comparing bacterial 16SrRNA gene libraries obtained from white and orange mats collected in this habitat, as expected from their different geochemistry. In both mats many clones were identified as SRB belonging to the *Deltaproteobacteria* group, similar to genera commonly found in cold seeps, such as *Desulfobacter*, *Desulfosarcina*, *Desulfocapsa* and *Desulfobulbus* (Knittel et al. 2005). *Gammaproteobacteria* were present in both the mats and prevalent in the orange one, where they represent up to 74% of the total bacterial community. Most of the *Gammaproteobacteria* sequences were correlated to bacteria capable of iron, sulphide or methane oxidation. A high number of sequences of sulphur-oxidizing *Epsilonproteobacteria* were also detected in the white mats, according to their chemical composition (Omoregie et al. 2008). FISH and 16 S rRNA libraries showed that up to 24% of the total cells within the white mat were targeted by an oligonucleotide probe specific for '*Candidatus Arcobacter sulfidicus*'. This species produces long sulphur filaments, resulting from sulphide oxidation (Sievert et al. 2006).

The orange mat is composed of iron (hydr)oxide encrusted sheaths. Known iron-oxidizing bacteria were not recovered in 16 S rRNA gene libraries from this mat, but unexpectedly many 16 S rRNA sequences of species involved in the reductive portion of the iron cycle were retrieved. These sequences cluster close to *Sulfurospirillum deleyianum*, known to perform reduction of iron via sulphur cycling, and they could likely enhance sulphur and iron cycling by oxidizing sulphur compounds using  $\text{Fe}^{3+}$ .

Archaeal 16 S rRNA gene clones retrieved from the mats were mainly represented by anaerobic methane oxidizers (ANMEs) belonging to the ANME-2 and ANME-3 groups, constituting respectively 55% and 74% of the archaeal sequences in the white and orange mats, respectively. ANMEs are responsible of AOM, a

process that occurs at the sediments underneath of both of the mats, as demonstrated by activity measurements and the distribution of methane concentration (Omoregie et al. 2008). The known stoichiometry of AOM to sulphate reduction (1 : 1) is very different from what was observed at the Chefren mud volcano (>28:1). The specific geochemical parameters that dominate this mud volcano possibly resulted in the selection of AOM and SRB consortia capable to utilize, in addition to methane, higher hydrocarbon compounds that have been measured within the pore waters of the Chefren volcano.

#### **4.4 Stratified microbial communities at the deep seawater-brine interface of hypersaline anoxic basins of the Eastern Mediterranean Sea**

The microbial diversity of brine-water interfaces and water columns of the DHABs of the Eastern Mediterranean are the most carefully studied cases by taking advantage of a simple sampling strategy that allowed to sample 5–10 cm wide water layers in the chemoclines (Daffonchio et al. 2006; Borin et al. 2009). Such a high resolution sampling allowed describing the stratification of the microbial groups within the chemoclines in response to changes of the concentration of electron donors and acceptors.

Total cell counts showed that the chemoclines of the DHABs are strongly enriched in comparison to overlying seawater and underlying brine. FISH allowed to determine the Bacteria/Archaea ratio and revealed that only the Urania basin is dominated by Archaea, whereas Bannock, L'Atalante and Discovery are dominated by Bacteria. Overall the bacterial diversity is higher than the archaeal diversity. Cluster analysis of the archaeal and bacterial 16 S rRNA sequences recovered from the four basins showed that they are colonized by different microbial communities, according to the differences in geochemistry. The prokaryotic composition of the Discovery lake is rather diverse from the others, whilst Bannock and L'Atalante show the highest similarity (van der Wielen et al. 2005). These data are in accordance with the cluster analysis performed on the geochemical parameters of the four basins. The distribution of the operational taxonomic units (OTUs; groups of sequences sharing >97% 16 S rRNA gene sequence similarity) indicated that the bacterial community of the Discovery interface is significantly different from those inhabiting the seawater and brine. Many OTUs were specific for the Discovery brine, belonging to microbial species not present in the other DHABs. In particular, sequences belonging to the archaeal species *Halorhabdus utahensis* were not detected in seawater but strongly increased along the chemocline moving towards the brine, where they constituted up to the 33% of the total archaeal community. This heterotrophic archaeon, able to grow performing a fermentative metabolism, tolerates up to 0.8 M MgCl<sub>2</sub> (Waino et al. 2000). The abundance of *H. utahensis* together with the high ectoenzymatic

activities and the low sulphate reduction and methane production occurring in Discovery brines, indicated that heterotrophic prokaryotes might play a major role than just that of being responsible for methanogenesis and sulphate reduction. MSBL-1 is a new archaeal candidate division retrieved only in all of the Eastern Mediterranean DHABs, belonging to the archaeal phylum *Euryarchaeota*, which is phylogenetically distantly related to methanogens. Since only few sequences related to known methanogens were detected in the DHABs, the methane production measured in the brines is likely due to the activity of this new archaeal halophilic taxon (van der Wielen et al. 2005).

Differently from what was observed in Discovery, sulphur cycling and methanogenesis shaped the prokaryotic communities colonizing the Urania DHAB. In Urania, methanogenesis and methane concentration were higher than in the other DHABs (Borin et al. 2009), along with dominance of the group MSBL-1 in the archaeal clone libraries of brines and the most saline layers of the chemocline. Sulphate reduction rates and sulphide concentrations were greater in Urania than in the other DHABs in agreement with the high sulphide concentration. The distribution of bacterial phylogenetic groups observed along the Urania water-brine interface showed bacteria of taxonomic groups having a sulphur-based metabolism. Whereas the bacterial community of seawater was mainly represented by *Alphaproteobacteria* and *Fibrobacteres* (van der Wielen et al. 2005), the Urania chemoclines and brines were dominated by *Deltaproteobacteria* and *Epsilonproteobacteria*. *Deltaproteobacteria*, mostly represented by *Desulfobulbaceae* and *Desulfobacteraceae*, formed up to 22.2–30.8% of the total bacterial community along the Urania chemoclines (Borin et al. 2009) and included most of the known SRB (Castro et al. 2000). *Epsilonproteobacteria* (11.7–47.8% of the bacterial clones) comprised different species capable of sulphide and sulphur oxidation (Campbell et al. 2006).

16 S rRNA gene libraries were constructed and isolation of different bacterial strains was performed also with samples from the Bannock basin. Eighty-four isolates were obtained from the Bannock interface belonging to the *Firmicutes*, *Bacteroidetes*, *Alpha*-, *Gamma*- and *Epsilon-proteobacteria* taxa. The 16 S rRNA gene libraries showed nevertheless a higher bacterial taxonomic richness and permitted the identification of new candidate divisions (MSBL 2–6). The relatedness of MSBL-2 to the candidate division SB1, detected for the first time at the interface of Shaban Deep (Red Sea) and its absence from seawater and brine samples point out to the specific adaptation of MSBL-2 division to the seawater-brine interfaces (Daffonchio et al. 2006). The detection of the MSBL 3–6 candidate divisions only in the deeper part of the seawater-brine interface of Bannock basin suggested that they are specifically adapted to high NaCl concentrations. In the deeper part of Bannock and L'Atalante halocline sequences belonging to KB1 group, previously identified in

the Kebrit Deep, were found (Daffonchio et al. 2006). The presence of KB1 and SB1 taxa demonstrated analogies between the microbiota colonizing DHABs of the Eastern Mediterranean and the Red Sea. All the metabolically active bacteria detected in the seawater overlying the L'Atalante brine belong to the class *Gammaproteobacteria*, 20% of which were represented by the genus *Thiomicrospira*. This sulphur-oxidizing bacterium dominated the upper part of the halocline and represented 100% of *Gammaproteobacteria* detectable at salinity values in the range 13–20‰ (Yakimov et al. 2007). Salinity affected also the distribution of *Epsilonproteobacteria* in the deepest part of the haloclines, where the *Deltaproteobacteria* seemed to be the most dominant class.

Autotrophic bacteria, responsible for energy production in the dark and contributing to the primary production of the DHAB ecosystems, were found along the water-brine chemoclines. Chemoautotrophic activity and the detection of the expression of functional enzymes have been documented for L'Atalante and Discovery (Yakimov et al. 2007). At the oxic–anoxic boundary of Urania and L'Atalante 16S rRNA signature sequences of aerobic sulphur oxidizers were retrieved (Yakimov et al. 2007; Borin et al. 2009) and along the Urania chemocline, signatures of anaerobic ammonium oxidizers (Borin et al. 2009).

## 5 Concluding remarks

DHABs were shown to be colonized by highly diverse microbial communities, whose diversity is shaped by the specific geochemical context. Studying the diversity of the microbial communities inhabiting hypersaline isolated environments can facilitate the understanding of adaptation mechanisms and the evolution of biogeochemical cycles and life on the early Earth or on extraterrestrial bodies. Evolution and adaptation of microbes in hypersaline habitats can be advantageous for the horizontal transfer of genetic information. In all the brines from DHABs Urania, Bannock, L'Atalante and Discovery, dissolved plasmid DNA was demonstrated to be substantially preserved for a period of 32 days in axenic conditions, maintaining the ability to transform naturally competent bacteria (Borin et al. 2008). These results indicate the potential role of dissolved extracellular DNA in hypersaline environments as a reservoir of genetic information that can be spread by HGT mediated by natural transformation.

Hypersaline habitats represent a huge and still largely unknown source of biodiversity exploitable for biotechnological applications. Halophilic microorganisms produce a wide set of enzymes stable under conditions which would lead to denaturation of most proteins and which are commonly used during many industrial processes (Ventosa et al. 1998; DasSarma and Priya 2001). Esterases which exhibit halophilic and additional innovative properties and are active in hypersaline

conditions, have been expressed from the metagenome of Urania DHAB brines (Ferrer et al. 2005), confirming that a wide biochemical potential is available for biotechnological exploitation.

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