Sulfur cycling and methanogenesis primarily drive microbial colonization of the highly sulfidic Urania deep hypersaline basin

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Urania basin in the deep Mediterranean Sea houses a lake that is >100 m deep, devoid of oxygen, 6 times more saline than seawater, and has very high levels of methane and particularly sulfide (up to 16 mM), making it among the most sulfidic water bodies on Earth. Along the depth profile there are 2 chemoclines, a steep one with the overlying oxic seawater, and another between anoxic brines of different density, where gradients of salinity, electron donors and acceptors occur. To identify and differentiate the microbes and processes contributing to the turnover of organic matter and sulfide along the water column, these chemoclines were sampled at a high resolution. Bacterial cell numbers increased up to a hundredfold in the chemoclines as a consequence of elevated nutrient availability, with higher numbers in the upper interface where redox gradient was steeper. Bacterial and archaeal communities, analyzed by DNA fingerprinting, 16S rRNA gene libraries, activity measurements, and cultivation, were highly stratified and metabolically more active along the chemoclines compared with seawater or the uniformly hypersaline brines. Detailed analysis of 16S rRNA gene sequences revealed that in both chemoclines δ- and ε-Proteobacteria, predominantly sulfate reducers and sulfur oxidizers, respectively, were the dominant bacteria. In the deepest layers of the basin MSBL1, putatively responsible for methanogenesis, dominated among archaea. The data suggest that the complex microbial community is adapted to the basin's extreme chemistry, and the elevated biomass is driven largely by sulfur cycling and methanogenesis.

deep anoxic hypersaline lake | element cycling | geosphere–biosphere interaction | Mediterranean Sea | microbial diversity

The Urania basin is one of the deep-sea hypersaline anoxic basins (DHABs) located in the eastern Mediterranean Sea. DHABs are far below the photic zone (3,200–3,600 m deep) and contain brines, the origin of which has been attributed to dissolution of 5.9- to 5.3-million-year-old Messinian evaporites (1). Urania is less saline than the other Mediterranean DHABs, with NaCl concentrations 5.4–7 times higher than normal seawater, but has higher concentrations of methane (5.56 mM) and exceptionally high levels of sulfide (up to 16 mM), making Urania basin among the most sulfidic marine water bodies on Earth (2–4).

Interfaces are considered to be hot spots for biological activity (2, 5), and environmental gradients represent an important part of the biosphere that must be accounted for in models of global biogeochemical cycles, especially in otherwise oligotrophic environments like the Eastern Mediterranean (6).

In the present study, we discovered 2 different environmental chemoclines within the Urania basin. We finely dissected the gradients and compared the oxic/anoxic upper interface of Urania basin with those found in chemically different DHABs. We concluded that the lower overall salinity but higher sulfide and methane concentrations in Urania DHAB are the primary factors determining the observed differences in microbial community composition and activity.

Results and Discussion

Fine-Scale Deep-Sea Sampling Reveals 2 Salinity and Temperature Discontinuities. The depth profile of the water column of the Urania DHAB showed 2 discontinuities in salinity and temperature [Fig. 1 and supporting information (SI) Fig. S1]. The first (interface 1) was a 2-m-deep halocline occurring >3,540 m with steep increases in salinity and temperature (from 3.7% to 16.1% and from 14 °C to 16.5 °C, respectively). In the following 67 m (designated brine 1), temperature and conductivity remained constant, until a second less pronounced chemocline (interface 2) separated brine 1 from a more dense brine (salinity 21.1%; temperature 18.1 °C), designated brine 2.

These haloclines act as density barriers, restricting mixing between the over- and underlying water bodies. In particular, the first chemocline between oxic deep seawater and brine 1 represents an oxic/anoxic boundary where we anticipated that the sharp redox potential changes would create favorable conditions for different types of metabolic processes. To address this, we carefully sampled, at the centimeter scale, the water column of the DHAB. The few meters-thick chemoclines have peculiar diffusion and sinking dynamics that make sampling particularly challenging (7) and contrast

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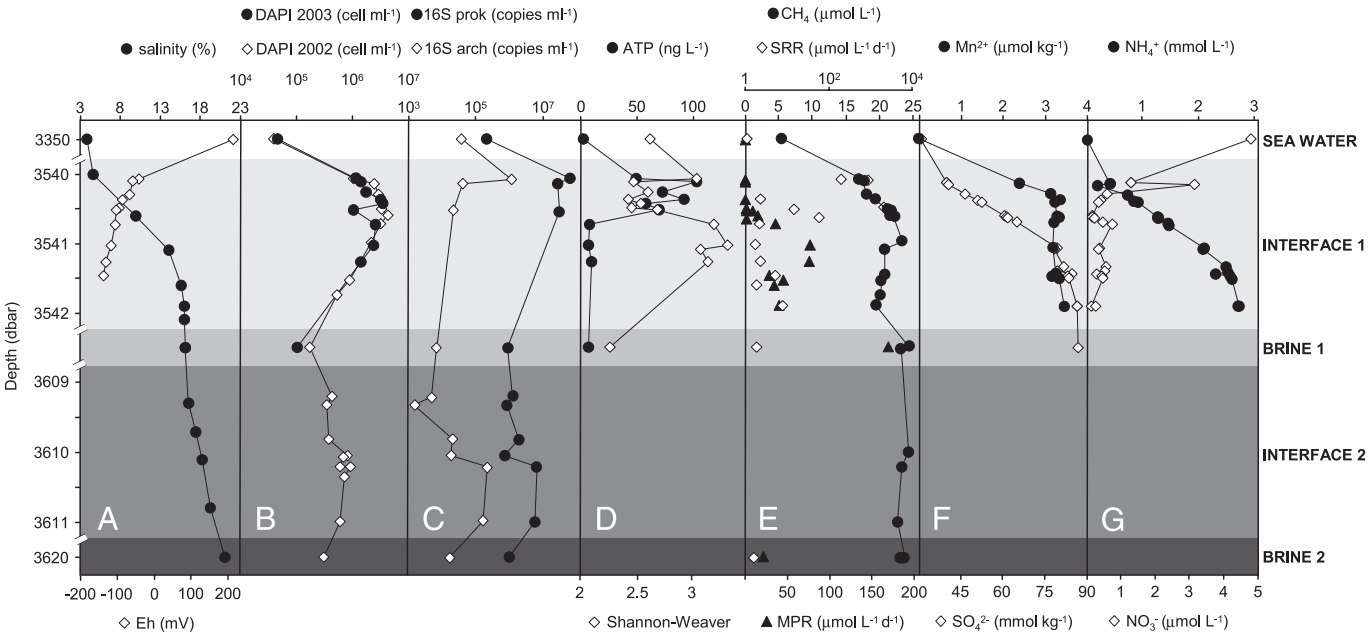

Fig. 1. Microbiological and geochemical profiling of the Urania water column. SW, seawater; I1,2, Interface 1,2; B1,2, brine 1, 2. (*A*) Filled circles, salinity profile measured by the CTD probe mounted on the SciPack system during deployment in the Urania water column; open diamonds, redox potential (Eh). (*B*) DAPI microbial counts; circles, data obtained in 2003 cruise; diamonds, data obtained in 2002 cruise. (*C*) 16S rRNA gene abundance; filled circles, prokaryote (prok); open diamonds, archaea (arch). (*D*) Filled circles, ATP; open diamonds, Shannon–Weaver index calculated from ARISA fingerprinting. (*E*) Open diamonds, sulfate reduction rates (SRR); filled triangles, methane production rates (MPR); filled circles, methane concentrations (upper *x* axis). (*F*) Open diamonds, sulfate concentrations; filled circles, dissolved Mn^{2+} concentrations. (*G*) Open diamonds, nitrates; filled circles ammonium. Error bars are within 4–48% of the reported values for DAPI counts; 3–5% for geochemical measurements; 1–55% for real-time 16S rRNA quantification; 7–32% for ATP quantification; 2–38% for SRR; and 2–59% for MPR.

with chemoclines that are tens of meters deep, found in other anoxic marine environments, like the Black and Baltic Seas or the Cariaco basin (8–10).

Sulfate Is the Main Terminal Electron Acceptor Throughout the Water Column, but Manganese IV, Nitrate, and Oxygen Are Important in the Upper Part of Interface 1. The redox potential decreased rapidly from +213 to −100 mV (Fig. 1*A*), indicating complete depletion of oxygen in the first 30 cm of interface 1. The increase in soluble manganese II (Mn^{2+}) from ≤0.01 to 3.47 μmol kg^{-1} positively correlated with a decrease in redox potential ($r = 0.89$; $P < 0.001$) (Fig. 1 *A* and *F*), whereas nitrate decreased from 4.85 μM to ≤0.3 μM in the first 30 cm of interface 1 (Fig. 1*G*). Ammonium concentration increased down the chemocline from 0.21 to 2696 μM. This is a peculiar feature of the Mediterranean DHABs, because the ammonia concentration was >2 orders of magnitude higher than in the chemocline of other marine anoxic environments (7, 9, 11). Sulfate was the most abundant electron acceptor in Urania DHAB, its concentration from 40 to 87 mmol kg^{-1}, correlating with salinity (Fig. 1*F*). The apparent conservative behavior of ammonia and sulfate, judged by a positive correlation with salinity, could on the one hand be viewed as the result of dissolution, but later we discuss evidence that it is probably due to production and consumption occurring in close proximity.

The increase in dissolved Mn^{2+} and decrease in nitrate concentration down interface 1 deviated from a linear correlation with salinity (Fig. 1 *F* and *G*), suggesting biologically mediated manganese oxide and nitrate reduction in the upper part of interface 1. A peak in nitrate concentration at a redox potential just below zero, also observed in Baltic and Black Seas' redoxclines (10, 12), could be putatively attributed to microaerophilic ammonium oxidizers (11). Urania interface 1 had a typical vertical sequence of the dominant electron acceptors, indicating that oxygen, nitrate, Mn IV, and sulfate are used in succession as oxidizing agents with increasing depth and salinity, as predicted by thermodynamics (13, 14).

Bacterial Abundance Increases >100-Fold in Interface 1 and at Least 10-Fold in Interface 2. Total microbial counts showed that both the chemoclines in the Urania DHAB are layers in the water column where microbial cells are remarkably enriched (Fig. 1*B*). Microbial abundance showed a rapid increase by 2 orders of magnitude from 3.9×10^4 cells mL^{-1} in the deep oxic seawater immediately above the basin, up to 4.3×10^6 cells mL^{-1} in the first half of interface 1. Although less pronounced than in the first chemocline, a second increase in microbial counts occurred in interface 2. Deceleration of falling particulate organic matter from the highly productive interface 1, is probably responsible for stimulating microbial growth and hence cell numbers in interface 2.

A similar trend in cell numbers measured in interface 1 during 2 different sampling cruises indicates temporal and spatial stability of microbes in the chemoclines (Fig. 1*B*). The enrichment in cell abundance within the chemoclines was confirmed by a similar increase in copy number of prokaryote 16S rRNA genes, measured by real-time PCR (Fig. 1*C*). Archaeal 16S rRNA gene copies (Fig. 1*C*) constituted a significant fraction (18.9%) of the prokaryotic communities in the deep seawater, as previously demonstrated (15). Interface 1 and brine 1 were largely dominated by Bacteria, with Archaea contributing <0.2% of the prokaryotic 16S rRNA gene copies as found in Bannock basin (7).

Microbial Community Is Highly Stratified Along Interface 1 and Has Elevated Diversity in the Lower Part. Cluster analysis of the amplified ribosomal intergenic spacer analysis (ARISA) fingerprints from Bacteria showed that each layer of different salinity and geochemistry has a distinct bacterial community, which is very different from that in the oxygenated deep seawater (Fig. S2). Bacterial diversity, as estimated by the Shannon–Weaver index of ARISA fingerprints, was relatively low in the upper part of interface 1, at the oxic–anoxic

Table 1. Percentage of clones in the different bacterial phylogenetic groups at different salinities within the deep oxic seawater (SW) above the Urania basin, the first (Interface 1) and the second (Interface 2) chemoclines, and the upper (Brine 1) and the lower (Brine 2) brines

	Clones, %						
Phylogenetic group	SW*	Interface 1			Brine 1	Interface 2	Brine 2
Salinity, %	n.d.	3.7–5.1	7–10.9	13.1–15.6	16.1	18.1	21.1
Proteobacteria	55.6	86.2	72.6	58.2	57.8	53.3	50.0
ε-		47.8	41.3	26.0	11.7	38.9	27.8
δ-	5.6	23.9	30.7	28.1	32.8	13.3	22.2
γ-	14.4	12.3	0.6	2.7	12.5	1.1	
α-	35.6	2.2	0.0	1.4	0.8		
KB1					20.3	25.6	40.0
Bacteroidetes		3.6	7.8	6.2	3.9	3.3	1.1
Firmicutes		1.4	2.2	6.8	2.3	8.9	1.1
Spirochaetes		2.2	8.4	6.8			
Fibrobacteres	16.7						
Planctomycetes	4.4	2.9	0.6	3.4	0.8	1.1	
Chloroflexi	2.2		0.6		6.3	2.2	
Actinobacteria	5.6		0.6	0.7	1.6		
Verrucomicrobiae	1.1	0.7					
Aquificae					0.8		
Acidobacteria		0.7					
Unclassified	14.4	2.2	7.3	17.8	6.3	5.6	7.8

n.d, not determined.
*Data from ref. 2.

boundary, and bacterial abundance and ATP showed a peak indicating a layer with high cellular activity (Fig. 1 *B–D*). These results indicate that in the first 50 cm of interface 1, the high cell abundance is mainly due to a relatively low number of species. By contrast, in the lower part of interface 1, at salinities between 8.7% and 16%, there was a notable increase in bacterial diversity.

Screening of 16S rRNA libraries confirmed the stratification of the bacterial community (Tables 1 and 2). ∫-Libshuff analysis (16) demonstrated that the difference between RNA-based and DNA-based clone libraries is significant only between sequences with >90% similarity, suggesting that all of the bacterial lineages revealed by the clone libraries are active.

Upper Part of Interface 1 Is the most Active and Is Dominated by Sulfur-Cycling Microbes. ATP concentration, a general parameter indicating cellular activity, was up to 64 times higher in the upper part of interface 1 compared with the deep oxic seawater. In the first 50 cm of interface 1, ATP showed an average peak of 73.7 ± 20.2 ng L^{-1} compared with 22.6 ± 6.6 ng L^{-1} in Bannock DHAB (7), indicating higher biological activity in Urania DHAB (Fig. 1*D*). The increase in sulfate reduction rate (SRR) coincided with a sharp decrease of the redox potential in the first centimeters of the seawater–brine interface (Fig. 1). Between −40 and −100 mV, SRR exhibited a peak up to 20.6 μmol L^{-1} day^{-1} corresponding with maximum ATP concentrations and bacterial cell numbers, suggesting that sulfate reduction is a dominant process in the upper part of interface 1 (Fig. 1*E*). The SRR values found in Urania DHAB were 2–4 times higher than those in the Bannock DHAB (2, 7), which has a similar sulfate concentration, implying that sulfate reducing bacteria (SRB) contribute more to element cycling in Urania basin compared with Bannock basin. In brine 1, sulfide, that had previously been shown to be of biological origin (17),

Table 2. Percentage of clones in the different archaeal phylogenetic groups at different salinities within the deep oxic seawater (SW) above the Urania lake, the first chemocline (Interface 1) and the upper (Brine 1) and the lower (Brine 2) brines

	Clones, %					
Phylogenetic group	SW*	Interface 1			Brine 1	Brine 2
Salinity, %	n.d.	7	13.1	14.6	16.1	21.1
Euryarchaeota		18.9	26.4	100.0	100.0	100.0
MSBL1			21.1	81.8	57.1	85.7
Halobacteraceae			5.3	18.2	42.9	14.3
Thermoplasmatales		6.3				
Marine group II		6.3				
MSBL10		6.3				
Crenarchaeota	85.5	81.3	73.7			
Marine group I	63.3	62.5	73.7			
Unknown SW division	22.2					
Thermoprotei		12.5				
MSBL11		6.3				
Other, unclassified	15					

n.d., not determined.
*Data from ref. 2.

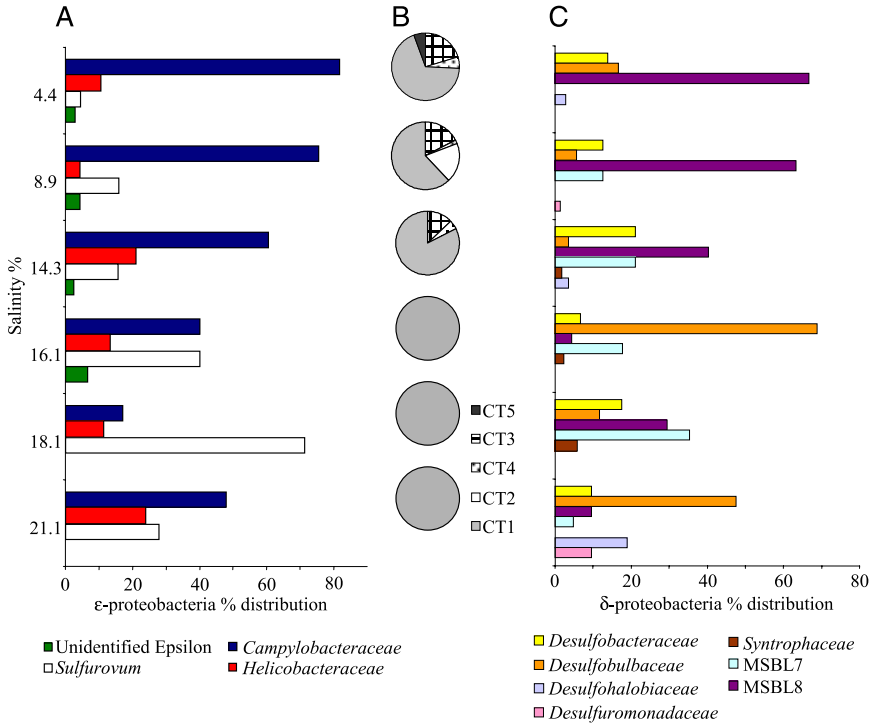

Fig. 2. δ- and ϵ-Proteobacteria are stratified along t he water column of Urania basin. (*A* and *C*) Relative distribution of sequences belonging to the ε- (*A*) and δ- (*C*) Proteobacteria. (*B*) Relative distribution of sequences at subfamily level, belonging to the *Campylobacteraceae*. CT, new candidate taxa.

reached a concentration of 16 mM (2). In contrast, L'Atalante brine had a higher concentration of sulfate and higher SRR but lower levels of sulfide (2), suggesting higher rates of sulfide consumption compared with Urania DHAB.

The overlying oxic seawater was dominated by α- and γ-Proteobacteria and Fibrobacteres (2), whereas Urania DHAB was dominated by δ- and ε-Proteobacteria (Table 1). Such organisms are typically found in environments with low oxygen levels and high concentrations of sulfur species (18–21). δ-Proteobacteria comprise one of the major groups of SRB (22), and constituted 22.2–30.8% of the 16S rRNA bacterial sequences along the whole depth profile of Urania DHAB (Table 1). The most represented families were *Desulfobacteraceae* and *Desulfobulbaceae*, which together made up between 18% and 76% of the total sequences within δ-Proteobacteria. *Desulfohalobiaceae* represented 19% of δ-Proteobacteria in brine 2, the most saline layer of the basin. A large fraction of the δ-Proteobacteria clones (14 to 76%) could not be affiliated to any known family. The MSBL8 group, related to *Desulfohalobiaceae*, was the dominant δ-Proteobacterial group in the upper interface 1 (Fig. 2*C*), concomitantly with the peak in SRR, suggesting a possible major role of the group in sulfate reduction. Sequences related to *Desulfohalobiaceae* and *Desulfobacteraceae* in the Urania interface have been detected in a previous phylogenetic analysis of dissimilatory sulfite reductase gene *dsrA* and proposed to be associated to oxygen-tolerant SRBs (23). In that study, alongside *Desulfohalobiaceae* and *Desulfobacteraceae*, the most abundant *dsrA* group included sequences related to the family *Peptococcaceae* (23), whose 16S rRNA signatures have not been detected in this and a previous study (2). However, because in that study a bulk sample of the whole interface was analyzed (23), it cannot be determined to which layer they were associated.

ε-Proteobacteria, constituting 11.7–47.8% of the bacterial clones, comprise many sulfide and sulfur oxidizers as well as manganese IV and nitrate reducers (18). Brines of Bannock and L'Atalante DHABs had lower sulfide concentrations (3 and 2.9 mM, respectively) than Urania (16 mM in brine 1) (2), and 16S rRNA gene libraries showed that ϵ-Proteobacteria were restricted to the brine of Bannock basin, or the medium-salinity layers of the seawater–brine interface of L'Atalante basin (2, 7,11). It seems therefore that the higher abundance and wider distribution of ε-Proteobacteria along the Urania DHAB chemocline is a consequence of its extremely high sulfide concentrations.

A sequence of sulfide oxidants can be hypothesized based on the standard Gibbs free energy for reactions (per mole of H_2S): O_2 ($\Delta G^{\circ\prime} = -967.9$ kJ), NO_3^- with the production of NH_3 and N_2 ($\Delta G^{\circ\prime} = -586.0$ 9 kJ), NO_3^- with the production of only NH_3 ($\Delta G^{\circ\prime} = -434.7$ 9 kJ (1) and MnO_2 ($\Delta G^{\circ\prime}: = -382.0$ kJ) (24). Indeed, a stratification of ε-Proteobacterial sequences related to bacteria capable of oxidation of reduced sulfur forms was observed along the Urania water column. Phylogenetic analysis of the ε-Proteobacteria (Fig. 2*A* and Fig. S3) showed that several clone sequences from Urania DHAB belong to new genera affiliated with the family *Campylobacteraceae*, related to *Arcobacter*, known to comprise species involved in sulfur oxidation/reduction and manganese reduction (18), that could explain Mn^{2+} concentrations found in the first 50 cm of interface 1. The most abundant taxonomic lineage within *Campylobacteraceae* belongs nevertheless to the new group CT1 not described previously and accounting for 46–58% of the ε-Proteobacteria, and constituting 100% of this family below interface 1 (Fig. 2*A*). In L'Atalante, seawater–brine interface sequences ascribed to this group were retrieved only in low salinity layers and were considered responsible for aerobic sulfide oxidation (11). Clones related to *Sulfurovum* and *Sulfurimonas*, both implicated in the redox cycle of sulfur, increased along with salinity and were particularly concentrated in the high-salinity layers constituting >70% of the ε-Proteobacteria in brine 2 (Fig. 2 and Fig. S3). This suggests that sulfur transformations could potentially take place along the whole depth profile. In other anoxic sulfidic systems like L'Atalante DHAB, the Cariaco basin, and the Black Sea, ε-Proteobacteria were found restricted to defined layers immediately across the oxic–anoxic boundary, putatively due to the lack of oxygen or other electron acceptors (8, 9, 11). For a phylogenetic tree of δ-Proteobacteria, see Fig. S4.

The archaeal clone libraries (Table 1 and Fig. S5) had low levels of taxonomic diversity. The only Archaea in the first 50 cm of interface 1 were Crenarchaeota, which consist of organisms having

sulfur-based metabolism, and hence could play a role in sulfur cycling in the upper interface.

Given the central role of sulfur cycling for energy generation in the majority of the representatives of the retrieved groups, the observed high rates of sulfate reduction, and the abundance of sulfide in this environment, it is reasonable to conclude that sulfur cycling is the main factor driving the functioning of this unique ecosystem, especially in the first 50 cm of interface 1.

Methanogenesis Greatly Exceeds Sulfate Reduction in the Most Saline Layers of the Basin. Dissolved methane increased from 7 to 2,761 μM in the first meter of interface 1 (Fig. 1*E*), suggesting either production only in the deep anoxic layers of the basin, and/or consumption at the oxic–anoxic boundary. In contrast to sulfate reduction, the methane production rate (MPR) was higher at lower redox potential. The MPR was minimal at redox potentials above −100 mV, and gradually increased down the chemocline to 169 μmol L^{-1} CH_4 day^{-1} in the brine 1 (Fig. 1*E*). This distribution may, in part, be explained by the high sensitivity of methanogens toward oxygen that restricts their presence to environments with low redox potential, whereas sulfate reducers are capable of sulfate reduction in a wider E_h range, including suboxic environments (25, 26). However, sulfate concentrations are high throughout the DHABs and sulfate reduction is more energetically favorable than methanogenesis, so why is MPR so much higher than SRR in the lower half of interface 1? The simple explanation of preferential inhibition of SRBs over methanogens due to high salinity or high sulfide concentrations are not strongly supported by evidence from the literature (27, 28). However, it is well known that, to cope with hypersaline conditions, microbes synthesize organic compatible solutes. Cell lysis will release such solutes into the environment, and their concentration will therefore increase in proportion with salinity. Glycine betaine, one of the most common compatible solutes, is readily fermented to methylamines, which serve as an energy source for many methanogens but not for SRBs (29, 30). This, however, does not explain the observations that there is neither a major change in relative abundance of Bacteria to Archaea (≈100:1), nor any decrease in the relative abundance of δ-Proteobacteria in brine 1. It is reasonable to assume that the microbes responsible for methanogenesis are Archaea (potentially the MSBL1 group) rather than Bacteria. So, based on the invariant ratios, it can be concluded that in the lower half of interface 1 and brine 1 the δ-Proteobacteria, known for their metabolic plasticity (31), are mainly performing functions other than sulfate reduction, possibly by using electron acceptors such as thiosulfate, sulfur, or dimethyl sulfoxide.

Methane production in the Urania water column is 10–30 times higher than in the water column of the Bannock basin, whereas the methane concentration in brine 1 (Fig. 1*E*) is >12 times higher (2, 7), indicating that halophilic methanogenesis is a major metabolic process in the carbon cycle in the deepest and most saline layers of the Urania basin. The prevalence of the euryarchaeal group MSBL1 in the most saline layers, reaching 85.7% of the clone libraries in brine 2 (Table 2), makes this group the prime candidate for methanogenesis in Urania and the other Mediterranean DHABs (2).

Chemoautotrophy Could Potentially Contribute to Interface Productivity. Autotrophic CO_2 fixation is an important function in deep-sea redoxclines, and has been extensively described in many ecosystems across oxic–anoxic boundaries (10). Chemoautotrophic activity and the detection of functional enzyme expressions have been documented for L'Atalante and Discovery DHABs (11, 32). Crenarchaeota, dominating the seawater and upper layers of the seawater–brine interface in Urania (Table 2) and L'Atalante DHABs have been considered responsible, at least partially, for autotrophic aerobic nitrification of ammonia (11). Few γ-Proteobacteria sequences belonging to the families *Piscirickettsiaceae* and *Ectothiorhodospiraceae*, aerobic sulfur oxidizing bacteria, have been retrieved in the first half of interface 1. The high productivity of the upper part of interface 1 of Urania DHAB is also supported by the abundant presence along all of the depths of ε-Proteobacteria (Table 1) that include key organisms responsible for chemolitotrophic nitrification in sulfidic environments. We cannot exclude that the new *Sulfurovum*-related species found in interface 1 could be responsible of chemosynthesis in the deepest layers of the basin.

Anaerobic ammonium oxidation (ANAMMOX) has been shown to be an important metabolic process along the oxic–anoxic interface in the Black Sea (12). Ammonia is one of the most abundant compounds along the Urania chemoclines and brines, and aerobic/microaerophilic nitrifying populations that could supply the above layers with nitrite diffusing from upwards, like Crenarchaeota or ε-Proteobacteria, were present in the first centimeters of interface 1. PCR using primers specifically designed for ANAMMOX bacteria revealed the presence of these bacteria in interface 1 in a large salinity interval (5.1% and 13.9%). Based on this finding, it is possible that ANAMMOX contributes to the productivity of Urania interface 1.

The presence of prokaryote populations responsible of primary productivity in the Urania basin was confirmed also by the isolation of 14 γ-Proteobacteria belonging to the species *Halothiobacillus hydrothermalis*. This species comprises halotolerant chemolithoautotrophs that obtain energy from reduced inorganic sulfur compounds (33).

Conclusions

The Urania DHAB is characterized by extreme chemistry and the interface between seawater and the anoxic hypersaline brine is a hot-spot of microbial activity. As in other DHABs, this can be explained by numerous redox combinations allowing many permutations of energy-generating reactions to occur. Urania brine has sulfide concentrations that have been shown elsewhere to inhibit microbial processes, but microbes in Urania basin have adapted to this exceptionally high sulfide concentration. Moreover, we provide evidence that sulfur cycling is a major driver in structuring the microbial communities, especially in the upper half of the chemocline. In addition to sulfide oxidation, other chemolithoautotrophic processes like manganese oxidation and ANNAMOX are implicated. Surprisingly, despite the presence of high concentrations of sulfate, rates of methanogenesis far exceed sulfate reduction at the base of the first chemocline. Known methanogens were not identified in clone libraries, but of several groups detected, the Euryarchaeota group MSBL1 emerges as the primary candidates for methane production. The chemoclines of Urania basin in the space of a few meters, without any physical barrier other than density, forced the evolution of different microbial communities exposed to geochemical conditions that change drastically with depth.

Methods

Sampling of Seawater–Brine Interface in Urania Basin. Sampling of the Urania basin was carried out from the research vessel Urania at location 35° 13.023′ N, 21° 30.682′ E (station BD16CT—for Interface I and II—9 June 2002), location 35° 13.715′ N, 21° 31.140′ E (station AB08MO for Interface 1—16 November 2003) and 35° 13.627′ N, 21° 31.247′ E (station AB17SCI for Interface I—19 November 2003). The halocline water fractions, sampled with the Modus–Scipack system as described in ref. 7, were analyzed for microbial activity, microbial abundance and diversity, and physicochemical properties.

Activity Measurements. Methane production and sulfate reduction rates were determined by measuring the production of methane and (^{35}S) sulfide production from radiolabeled sulfate [1–2 μCi (^{35}S) sulfate] as previously reported (7). ATP was measured on triplicate 10-mL samples filtered through 0.22-μm pore-size filters. ATP was extracted and measured directly on the filter with the luciferine–luciferase-based biomass test kit (Promicol), and relative luminescence

units were converted to ATP concentrations according to a standard ATP curve as previously described (7, 34).

DNA/RNA-Based Analysis and Direct Cell Count. Samples were filtered on to sterile 0.22-μm pore-size filters that were stored at -20 °C in 2 mL of sterile lysis buffer [EDTA 40 mM; Tris·HCl 50 mM (pH 5.8), sucrose 0.75 M]. DNA was extracted from each filter and quantified as previously described (7). Real-time PCR experiments for quantification of total Prokaryotes and Archaea were performed in triplicate by TaqMan assays according to methods described in ref. 35.

From each DNA fraction along the salinity gradient, ARISA was performed according to an established protocol (36). As a parameter for the structural diversity of a bacterial community a Shannon–Weaver index was calculated for representative ARISA profiles, and a similarity dendrogram was drawn by cluster analysis, as previously described (7). Construction and sequencing of 16S rRNA gene libraries from fractions with different salinities, were performed with a procedure previously described (2), using as forward primers B8F 5′-AGAGTTTGATCMTGGCTCAG-3′ for Bacteria and A2F 5′-TTCCGGTTGATCCYGCCGGA-3′ for Archaea and the universal reverse primer U1492R 5′-GGTTACCTTGTTACGACTT-3′ for both groups. A total of 925 clones (775 bacterial and 150 archaeal) have been sequenced. The ARB software was used to align sequences 350–500 bp long (37) and operational taxonomic unit (OTU) distribution at different salinities was calculated. Good coverage of the dominant OTU population was confirmed with rarefaction analysis of the clone libraries. The 16S rRNA gene sequences showing >97% homology were considered to belong to the same OTU. For RNA extraction and reverse transcription, the RNA/DNA Mini Isolation kit (Qiagen) and SuperScript II reverse transcriptase (Invitrogen) were used. Phylogenetic trees were constructed as previously described (2). $\int$-Libshuff analysis was done as previously described (16).

ANAMMOX signature 16S rRNA genes were amplified from the sample metagenome, according to ref. 38.

For total cell counts, a 3-mL aliquot of each sample was incubated immediately after its recovery with 4,6-diamidino-2-phenylindole (DAPI, final concentration 1 μg mL^{-1}) for 15 min in the dark, filtered through a black polycarbonate membrane (0.2-μm pore size, 25-mm diameter, Isopore; Millipore) and washed with 3 mL of PBS. Cell counts were determined in triplicate filters by counting stained cells on 20 randomly selected fields by using a Zeiss Axioplan epifluorescence microscope with excitation/emission filters of 365/420 nm.

Geochemical Analyses and Salinity-Related Measurements. Redox potential in each halocline fraction was measured onboard with a portable Eh-meter (39). Major elements in brines, including total S content, were analyzed by ICP-AES at Geosciences Utrecht after on-board acidification and dilution to 3.5‰ salinity; ammonia was analyzed by AutoAnalyzer at the Royal Netherlands Institute for Sea Research (Texel, The Netherlands); Mn at National Institute for Public Health and the Environment (Bilthoven, The Netherlands), by using high-resolution ICP-MS. Statistical analyses were performed with MATLAB 7.0.0 (The Mathworks).

Bacterial Isolation and Cultivation. *H. hydrothermalis* was isolated on CSBM medium and identified by sequencing the 16S rRNA gene.

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