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ORIGINAL ARTICLE

Primary producing prokaryotic communities of brine, interface and seawater above the halocline of deep anoxic lake L'Atalante, Eastern Mediterranean Sea

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Meso- and bathypelagic ecosystems represent the most common marine ecological niche on Earth and contain complex communities of microorganisms that are for the most part ecophysiologically poorly characterized. Gradients of physico-chemical factors (for example, depth-related gradients of light, temperature, salinity, nutrients and pressure) constitute major forces shaping ecosystems at activity 'hot spots' on the ocean floor, such as hydrothermal vents, cold seepages and mud volcanoes and hypersaline lakes, though the relationships between community composition, activities and environmental parameters remain largely elusive. We report here results of a detailed study of primary producing microbial communities in the deep Eastern Mediterranean Sea. The brine column of the deep anoxic hypersaline brine lake, L'Atalante, the overlying water column and the brine-seawater interface, were characterized physico- and geochemically, and microbiologically, in terms of their microbial community compositions, functional gene distributions and [^{14}C]bicarbonate assimilation activities. The depth distribution of genes encoding the crenarchaeal ammonia monooxygenase α subunit (*amo*A), and the bacterial ribulose-1,5-biphosphate carboxylase/oxygenase large subunit (RuBisCO), was found to coincide with two different types of chemoautotrophy. Meso- and bathypelagic microbial communities were enriched in ammonia-oxidizing *Crenarchaeota*, whereas the autotrophic community at the oxic/anoxic interface of L'Atalante lake was dominated by *Epsilonproteobacteria* and sulfur-oxidizing *Gammaproteobacteria*. These autotrophic microbes are thus the basis of the food webs populating these deep-sea ecosystems.
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Introduction

Marine prokaryotes are well recognized to play central roles in fixation of CO_2 into organic carbon (autotrophy) both at the ocean's surface and, as it has recently been demonstrated, in the aphotic bathypelagic and abyssal realms of the ocean (Herndl *et al.*, 2005; DeLong, 2006; Ingalls *et al.*, 2006). Absence of the sunlight restricts the occurrence of photosynthesis in deep-sea, but hydrogen sulfide, ammonia and some reduced inorganic chemicals can serve as electron donors needed for the CO_2 fixation in the dark. Indeed, chemolitoautotrophs are thought to be among the first organisms that appeared on early Earth (Russell and Hall, 1997; Campbell *et al.*, 2006; Wächtershäuser 1990, 2006, 2007).

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According to recent studies of deep-sea microbial ecosystems, the dark primary production (that is chemoautotrophy), occurs not only in specific 'hot spot' areas on the seafloor (for example, hydrothermal vents, cold seepages and mud volcanoes), but also takes place throughout the 'dark' water column. Since their discovery over a decade ago, a single group of marine *Crenarchaeota* is recognized to be a dominant fraction of marine bacterioplankton that accounts for up to 60% of the total prokaryotes in deep aphotic oceanic environments. Although the metabolism of these archaea is a subject of current debate, their successful isolation and subsequent genome analysis convincingly demonstrated their ability to grow by chemoautotrophic oxidation of ammonia (Francis *et al.*, 2005; Konneke *et al.*, 2005; Nicol and Schleper, 2006; Wuchter *et al.*, 2006; Hallam *et al.*, 2006a, b). Other studies based on microautoradiography also demonstrated the prevalence of marine crenarchaea in CO_2 fixation (Herndl *et al.*, 2005). Together with the new observations of autotrophic carbon assimilation by the archaeal communities in the deep ocean facilitated, these results reveal a major role of marine *Crenarchaeota* in both carbon and nitrogen cycling in marine ecosystems. Moreover, as the principal primary producers in the deep-sea, chemoautotrophic prokaryotes are likely to play a pivotal role in the functioning of such ecosystems, much more so than the input of sinking organic matter derived from photosynthetic producers in the photic zone (Sass *et al.*, 2001).

Deep-sea hypersaline anoxic lakes (DHALs) are fascinating but poorly studied, multiply extreme marine habitats. Four such lakes, Bannock, Discovery, l'Atalante and Urania are situated in the Mediterranean Ridge in the Eastern Mediterranean Sea, an accretionary complex subject to continental collision. They were formed 5000–40 000 years ago by dissolution of ancient subterranean salt deposits (Messinian evaporites) exposed to seawater by tectonic shifts and the downward flow of the dense brines into local abyssal depressions, followed by progressive development of anoxia in the brine lakes thereby formed (Fusi *et al.*, 1996; Wallmann *et al.*, 1997). Recent studies of DHALs revealed the existence of new microbial lineages flourishing within these harsh environments (Ferrer *et al.*, 2005; van der Wielen *et al.*, 2005; Daffonchio *et al.*, 2006; Hallsworth *et al.*, 2007; Yakimov *et al.*, 2007).

The aim of the present study was to characterize the chemoautotrophic processes occurring in such habitats and match them with the main microbial members of the prevailing communities. We therefore, created quantitative spatial profiles of planktonic crenarchaeal SSU rRNA and *amo*A-like genes, together with CO_2 fixation measurements, to obtain an initial assessment of *Crenarchaeota*-driven dark primary production in the bathypelagic zone of Mediterranean Sea. Remarkably, estimates of autotrophy and distribution of archaeal *amo*A copy numbers, normalized to concentration of DNA, correlated in all samples collected through the overlying water column. Since the halocline of all DHALs is enriched in hydrogen sulfide, occurrence of autotrophic sulfide-oxidizing bacteria was expected, so the distribution, diversity and phylogeny of ribulose-1,5-biphosphate carboxylase/oxygenase form I (RuBisCo, *cbb*L) was monitored. The highest abundance of metabolically active autotrophic sulfide-oxidizing bacterias, as detected by means of reverse transcription coupled with quantitative PCR of SSU rRNA and *cbb*L mRNAs, occurred within the upper, oxygenated level of the halocline coinciding with the maximum rate of dark CO_2 fixation.

Materials and methods

Sampling site and sample collection

The deep hypersaline anoxic lake l'Atalante is located at lat 35°18.27′N and 21°23.46′E on the South-East Mediterranean Sea at a water depth of 3500 m. The seawater: l'Atalante brine interface and the l'Atalante brine were sampled during two cruises BIODEEP-3 (November 2003) and MEDBIO06 (October 2006). The interface was captured and fractionated as described previously (Daffonchio *et al.*, 2006; Hallsworth *et al.*, 2007). Briefly, 10-litre Niskin bottles housed on a rosette with conductivity–temperature-depth sensors were closed when a large increase in conductivity, indicating that the interface had been entered, was observed. This was confirmed on board by measuring the refractive index of the top and bottom of brine in the Niskin bottles. Two litre fractions of the captured interface were subsampled and either preserved in sealed bottles for determination of chemolithoautotrophic activity or immediately filtered through 0.2 μm Nucleopore filters (Millipore, Billerica, MA, USA) for nucleic acid analysis. Additionally, the water column overlying the l'Atalante evaporite lake was sampled at water depths of 50, 500, 1000, 3400 and 3495 m water depths. For the total nucleic acids extraction, 20 l samples of seawater from each depth were filtered through a 47 mm diameter, 0.2 μm pore-size Nuclepore filters (Millipore). Collected material was resuspended in 100 μl of TE buffer (pH 8.0) containing lysozyme (5 mg ml^{-1}), lysed by addition of 300 μl of lysis buffer QRL1 (Qiagen, Hilden, Germany) and stored at −20 °C until processing.

Geochemical analyses

Fluid chemical parameters were determined immediately after sample recovery. The pHs of unfiltered seawater and oxic/anoxic interface samples were measured at 25 °C in unfiltered sample aliquots with an Ag/AgCl reference electrode (Mettler-Toledo, Novate Milanese, Italy). Sulfide concentrations were measured photometrically by the methylene blue

method (Cline, 1969). Redox potentials (Eh) of unfiltered sample aliquots were measured immediately according to the procedure described by Daffonchio *et al*. (2006) and Hallsworth *et al*. (2007). Major elements including nitrate and ammonia were analyzed by Stayer 1C isocratic chromatography system for routine ion determination (Portlab, Sulmona, Italy).

Direct cell count and fluorescent in situ *hybridization (FISH)*

To determine the abundance of bacteria and archaea in collected samples, three oligonucleotide probes were used: EUB338I (Amann *et al.*, 1990), ARCH915 (Amann *et al.*, 1992) and Cren512 (Jürgens *et al.*, 2000). Filters were fixed on board with formaldehyde (final concentration 4% v/v) for 4 h, washed twice with 1X PBS (phosphate-buffered saline), fixed in 1 ml ethanol : 1X PBS (1:1) and stored at 4 °C until further treatment. FISH analysis with cyanine dye CY3-labeled oligonucleotide probes (final concentration, 50 ng μl^{-1}) and DAPI (4′,6-diamidino-2-phenylindole) staining were conducted according to Glockner *et al*. (1999). Filter sections on slides were embedded in Citiflour AF1 antifadent (Plano, Wetzar, Germany), covered with a coverslip and inspected using an Axiophot epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with filters 02 (DAPI), 10 (FLUOS, DTAF) and 20 (Cy3), a mercury-arc lamp and an AxioCam digital camera. For each hybridization approach and sample, at least 750 DAPI stained cells in 30 randomly chosen counting squares were manually counted. The number of FISH-stained cells was calculated by counting probe-specific positive signals relative to DAPI counts. Counting signals were always corrected by subtracting signals obtained with the negative control probe NON338 (Wallner *et al.*, 1993).

Primary production measurements

Primary production was calculated through uptake of [^{14}C]bicarbonate (100 μCi, 2 mCi ml^{-1}, Amersham, Buckinghamshire, UK), according to the protocol of Herndl *et al*. (2005), in 45-ml vials samples in duplicate with a formaldehyde-fixed blank. Samples were incubated for 9 days in the dark at *in situ* temperatures (13 °C), after which incubation was terminated by the addition of formaldehyde to a final concentration of 2% (v/v), and samples were filtered through 0.1-μm polycarbonate filters (Millipore). Filters were washed three times with 10 ml of ultra-filtered (0.1-μm) seawater and then exposed to concentrated HCl vapor for 12 h to eliminate any residue of radioactive bicarbonate. Filters were then placed in scintillation vials and stored in the dark at −20 °C until counted. The values obtained in disintegrations per minute were corrected, using the disintegrations per minute of the abiotic control and converted into rates of production of organic carbon, according to Herndl *et al*. (2005).

Nucleic acid extraction and synthesis of cDNA by reverse transcription reaction

Total RNA and genomic DNA were extracted from membrane filters prepared as described above (see section Sampling site and sample collection of Materials and methods) using Qiagen RNA/DNA Mini Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. Total RNA was precipitated with *iso*-propanol, washed with 70% ethanol and after air-drying, re-suspended in 50 μl of diethylpyrocarbonate-treated sterile water and subsequently purified using a 'DNA-free' kit (Ambion, Austin, TX, USA). The quality of the RNA samples was assessed by agarose gel electrophoresis and concentrations were determined by spectrophotometry (Biophotometer, Eppendorf, Hamburg, Germany). Subsequently, 50–100 ng of RNA, heat denatured at 70 °C for 10 min, were mixed with random hexameric primers, the dNTP mixture and RNase inhibitor and reverse transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 60 min at 50 °C. The reaction was stopped by heating at 80 °C for 5 min, and the resulting cDNA was used as a template for further PCR amplification. Possible DNA contamination of ribosomal RNA templates was routinely monitored by PCR amplification of RNA aliquots that were not reverse transcribed. No contaminating DNA was detected in any of these reactions.

PCR amplification, cloning and sequencing of complementary 16S ribosomal DNA

Archaeal and bacterial complementary 16S ribosomal DNA (crDNA) was PCR-amplified using the corresponding primer sets consisting of Arc20_F and 1390R (Mincer *et al*., 2007), and 27F and 1492R (Lane, 1991), respectively. The PCR mix contained 10–50 ng of crDNA, 1X Qiagen reaction buffer, 1X solution Q (Qiagen), 1 pM of each primer, 200 μM dNTPs (Gibco, Carlsbad, CA, USA) and 2.5 U of Qiagen *Taq* polymerase. An initial denaturation step (95 °C for 3 min) was followed by 30 cycles each consisting of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C, with a final extension of 10 min at 72 °C. PCR products were purified with QIAQuick PCR purification columns (Qiagen) and subcloned into the pGEM T-Easy Vector II according to the manufacturer's instructions (Promega, Madison, WI, USA). Inserts were subsequently PCR-amplified from lyzed colonies with the M13F and M13R vector primers. Sequencing was performed with an ABI PRISM 3100-*Avant* Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) using the ABI PRISM BigDye Terminator v 3.1 Cycle Sequencing Kit (PE Applied Biosystems).

PCR amplification, cloning and sequencing of cbbL and amoA

Crenarchaeal ammonia mono-oxygenase subunit A (*amo*A) genes were detected using primer set

ArcamoAF (5′-STAATGGTCTGGCTTAGACG-3′) and ArcamoAR (5′-GCGGCCATCCATCTGTATGT-3′) (Francis *et al.*, 2005). Bacterial *amo*A PCR detection was carried out using primer set AmoA1-F and AmoA2-R, described previously by Rotthauwe *et al.* (1997). The DNA and cDNA samples extracted or derived from 3400 m seawater column samples, oxic/anoxic interface and brine, were additionally investigated for the presence of the *cbb*L gene, encoding the RuBisCO, using primers cbbL1F (5′-GACTTCACCAAAGACGACGA-3′) and cbbL1R (5′-TCGAACTTGATTTCTTTCCA-3′) (Elsaied *et al.*, 2007). The PCR amplification was carried out in a 2700 Gene Amp PCR System (Applied Biosystem), with cycling parameters according to the above report. PCR products were visualized on 1% agarose gels and corresponding bands were cut out and purified using QIAQuick gel extraction kit (Qiagen). Purified PCR products were cloned and subsequently sequenced as described above.

Sequencing and phylogenetic analysis

Sequence analysis was performed as described previously (Hallsworth *et al.*, 2007; Yakimov *et al.*, 2007). Briefly, all sequences were edited with MacVector Software version 7.2.2 (Accelrys, San Diego, CA, USA) and compared with DNA or amino-acid sequences in the public domain through the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). Before alignment, the sequences were checked for chimeras by means of the CHECK_CHIMERA program available from Ribosomal Database Project (Cole *et al.*, 2003). Resulting 16S rRNA gene alignments were manually verified against known secondary structure regions. Phylogenetic trees were constructed using the Neighbor Joining method and Jukes-Cantor distance matrix; 1000 bootstrap resamplings were performed to estimate the reproducibility of the tree. Random order input of sequences, single jumbling and the global rearrangement option were used. A multiple sequence alignment and the phylogeny of AmoA and CbbL protein homologs were constructed using MacVector 7.2.2 software (Accelrys) with BLOSUM matrix, open and extend gap penalties of 10.0 and 0.05, respectively. The alignment file was then analyzed with the same software for calculation of the distance matrix. Neighbor Joining algorithm and Poisson correction of distances were used to construct the phylogenetic tree; 1000 bootstrap resamplings were performed to estimate the reproducibility of the tree.

Quantitative PCR analyses of environmental DNA templates and estimation of amoA gene copies

The TaqMan gene expression assay was designed for quantitative analysis of crenarchaeal *amoA*-like gene distribution. All Q-PCR experiments were run using an ABI 7500 Fast Real-Time PCR System thermocycler. Specific primers and a TaqMan probe for crenarchaeal *amoA*-like gene were designed using Primer Express software v.2.0 (Applied Biosystems). The primers were AmoAF379 (5′-CA CTCGCTGATATTCTTTGG-3′) and AmoAR495 (5′-GG TCTTGGRT ACTTGAATGC-3′), while the TaqMan probe was AmoTaq404 (5′-TTTTRGTYGGWATGT CAATGCCRCT-3′). 5′-6-FAM and 3′-TAMRA labeled TaqMan probe was obtained from PE Applied Biosystems. Both DNA and cDNA templates originating from all compartments sampled were tested in triplicates along with a 'No Template Control'. The mixtures for Q-PCRs were as follows: 0.8 μM final concentration of each primer, 0.2 μM final concentration of TaqMan probe, 2 ng of template, 12.5 μl of 2X TaqMan Universal PCR Master Mix (PE Applied Biosystems) and water to make a final volume of 25 μl. Reaction conditions were: AmpErase UNG Activation step at 50 °C for 2 min, initial denaturation for 10 min at 95 °C, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Q-PCR amplification was analyzed with automatic setting of the baseline and threshold values, using the relative standard curve method. The pGEM T-Easy Vector II harboring the 598-bp long fragment of crenarchaeal *amoA*-like gene (AM747269) was quantified using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA), and used as a standard in a dilution series ranging from 1.0×10^7 to 10 copies of *amo*A. The cycle-threshold values (CT) indicate the quantity of the target gene in each sample. Standard curves for the *amo*A gene quantification were generated by serial dilution ranging from 10^7 until 10 copies of plasmid containing *amoA* cDNA to quantify the mRNA concentrations.

Nucleotide accession numbers

Sequence data have been submitted in the DDBJ/EMBL/GenBank databases under accession numbers DQ453160-DQ453256 and DQ453461-DQ453476 (16S rRNA gene), AM747269 and AM747270 (*amo*A), AM747271-AM747277 (*cbb*L).

Results and discussion

Geochemical and microbiological characterization of the sampling site

The brine lake of the L'Atalante Basin, some 3500 m below the surface of the Eastern Mediterranean Sea, ca. 200 km off the western coast of Crete (Figure 1), is the smallest of four deep hypersaline anoxic lakes (DHAL) that have been extensively studied over recent years (Ferrer *et al.*, 2005; van der Wielen *et al.*, 2005; Daffonchio *et al.*, 2006; van der Wielen, 2006; Hallsworth *et al.*, 2007; van der Wielen and Heijs, 2007; Yakimov *et al.*, 2007). The lake is filled by brine originating from Messinian evaporites dissolved in seawater to almost saturation level ($365\ g\ l^{-1}$). The water column overlaying the

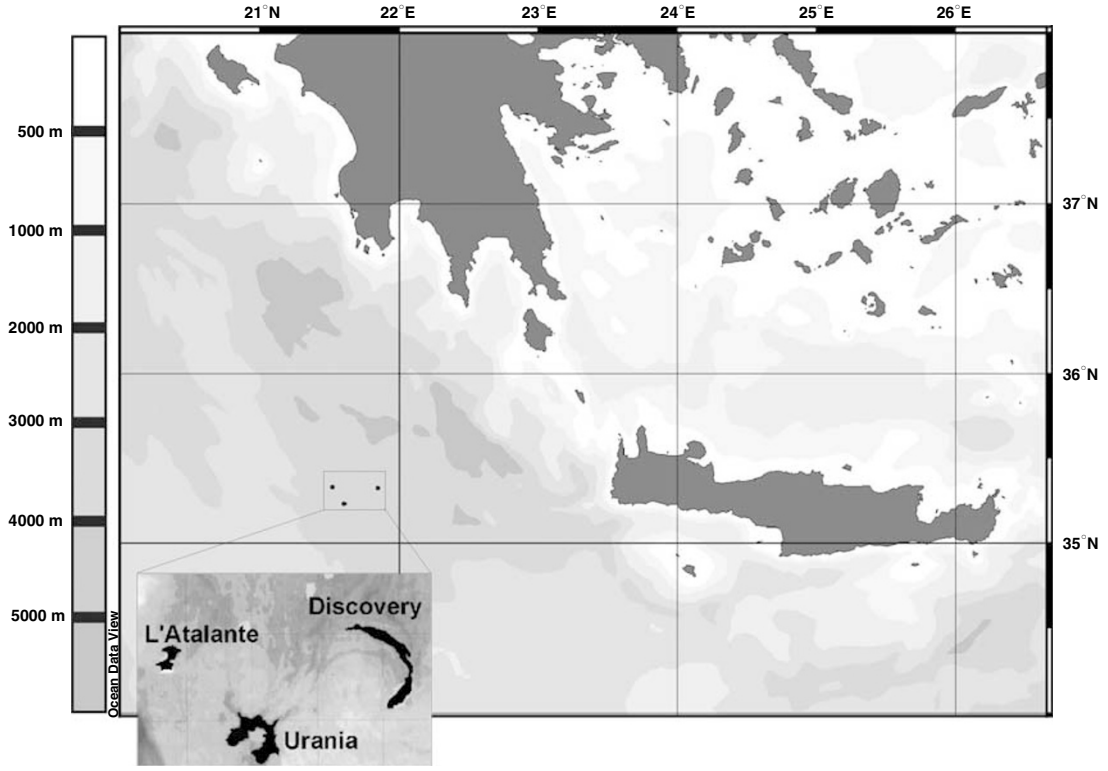

Figure 1 Location of deep anoxic hypersaline lakes in the Eastern Mediterranean Sea. The map was constructed with Ocean data View software (http://awi-bremerhaven.de/GEO/ODV).

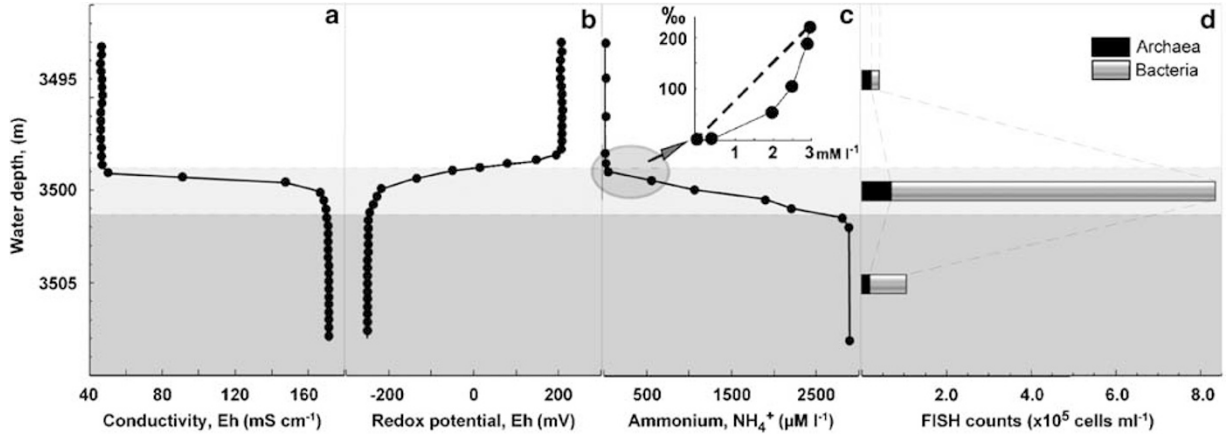

Figure 2 Depth profiles of geochemical and microbiological markers along the seawater/halocline/l'Atalante brine lake: conductivity (**a**); redox potential (**b**); ammonium concentration (**c**) and prokaryotic cell counts performed using FISH (**d**). Small insert in C indicates the depletion of NH_4^+ profiling (mM l^{-1}) towards the salinity increase (%).

L'Atalante Basin lake, halocline and brine were sampled in November 2003 and October 2006 during BIODEEP-3 and MedBio cruises on the RV 'Urania', respectively. The previously developed sampling strategy was successfully applied to sample the oxic/anoxic interface (Daffonchio *et al.*, 2006; Hallsworth *et al.*, 2007). As shown in Figure 2a, the L'Atalante Basin brine lake, like all Mediterranean DHALs, has a sharp, ~1.5 meter-thick halocline, exhibiting a steep gradient of salinity ranging in concentration from 39‰ at its upper face to 365‰ at its lower face. The redox potential rapidly decreased to negative values within the initial part of the halocline (Figure 2b), indicating a total depletion of oxygen and strong anoxia in the underlying compartments of the brine lake. A progressive increase in the ammonia from 5.5 to almost 3000 µM was measured along the halocline (Figure 2c). Ammonia, accumulating in the brine as a final product of anoxic organic matter

mineralization, seems to be a characteristic feature of all Mediterranean DHALs studied (van der Wielen *et al.*, 2005; Daffonchio *et al.*, 2006). The brine of the Bannock Basin lake, contains 3450 μM of dissolved ammonia (Daffonchio *et al.*, 2006), a concentration that is 1–5 orders of magnitude greater than that found in the open ocean and coastal waters (<0.03–100 μM; Konneke *et al.*, 2005). Whereas the increase in Na^+ concentration follows a predictable pattern across the haloclines of deep-sea brine lakes (Daffonchio *et al.*, 2006), the ammonia concentrations are non-linear, presumably reflecting its biologically-mediated depletion in the 'sub-oxic' zone (inset in Figure 2c). In typical marine environments, such a vast pool of ammonia would fuel at least three different microbial metabolic processes: utilization as a source of nitrogen, and exploitation as an electron donor and energy source, either in aerobic or anaerobic oxidation activities. Taking into account the anoxia in the halocline deeper than 30–50 cm from the surface, aerobic oxidation of ammonia in L'Atalante brine lake may occur only in the superficial layer. The anaerobic oxidation of ammonia (the anammox process) in the brine would seem unlikely, as so far no halophilic planctomycete-like bacteria have been found that perform anaerobic ammonia oxidation (Jetten *et al.*, 2003; Schmid *et al.*, 2007).

Total prokaryote numbers were significantly higher in the halocline layer ($8.33 \pm 0.11 \times 10^5$ cells ml^{-1}) than in the overlying deep seawater ($3.57 \pm 0.08 \times 10^4$ cells ml^{-1}) or underlying brine ($1.07 \pm 0.15 \times 10^5$ cells ml^{-1}) (Figure 2d). Comparison of archaeal and bacterial abundance revealed that the members of these domains of life were present at almost equivalent levels in oxygenated deep seawater overlaying the hypersaline brine (3495 m), but *Bacteria* were predominant in samples from the interface (91.2%) and brine lake (86%). This finding is in good agreement with those reported previously for similar environments of the deep-sea (Henneke and De Lange, 1990; Herndl *et al.*, 2005; Daffonchio *et al.*, 2006).

Diversity of prokaryotic SSU rRNAs

To survey the distribution of actively metabolizing *Bacteria* and *Archaea*, their 16S rRNA sequences were amplified from the pool of total RNA by reverse transcription-PCR, cloned and sequenced. Phylogenetic analysis revealed a pronounced stratification of prokaryotes along the halocline.

Members of the domain *Archaea* in communities of both adjacent seawater and the upper level of halocline (up to 80‰ of salinity) were dominated by Marine Group I *Crenarchaeota* (DeLong, 1998), whereas the lower compartments were inhabited by different groups of halophilic *Euryarchaeota* (Figure 3a). Remarkably, members of the *Haloarchaea* were found only in a narrow window of the halocline at 130‰ salinity. Proliferation of these aerobic heterotrophic halophiles in upper and deeper zones is probably prevented by low salinity and the anoxia, respectively. In contrast, the distribution of the ANME-1 group of strictly anaerobic methane-oxidizers is shaped by their low tolerance of oxygen toxicity and elevated salinity. Organisms affiliated with the Mediterranean Sea Brine Lake 1 (MSBL-1) Candidate Division exhibited a distribution similar to that of ANME-1, but also a broader halotolerance. Members of this division have only thus far been found in anoxic, hypersaline environments, like Mediterranean DHALs, deep-sea mud volcanoes and endoevaporitic microbial mats (Sørensen *et al.*, 2005; van der Wielen *et al.*, 2005; Daffonchio *et al.*, 2006; Yakimov *et al.*, 2007), which

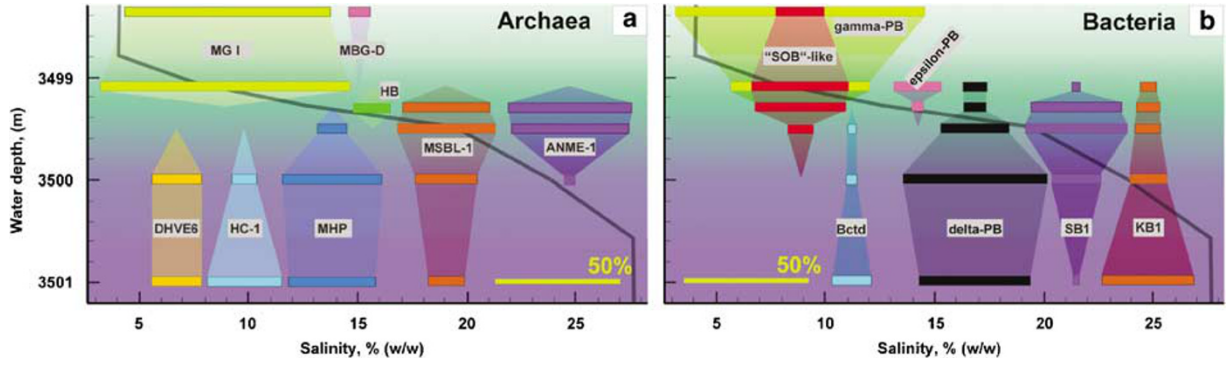

Figure 3 Overview on prokaryotic diversity and relative abundance of phylogenetic groups recovered from A*rchaea* (**a**) and *Bacteria* (**b**) SSU rRNA clone libraries of the seawater : l'Atalante brine interface. The salinity gradient is plotted behind of the bars representing the phylogenetic groups. The breadth of the bar indicates the percentage of clones related to the indicated phylogenetic group (to be applied only to the same depth—at a time). one which were recovered from clone library of the each of six depths studied. Scale bar represent 50% of clones in clone library. Abbreviation for A*rchaea* used: ANME-1, Anaerobic Methane Oxidizers group 1; DHVE6, Deep-sea Hydrothermal Vent *Euryarchaeota* Group 6; HB, *Halobacteriaceae*; HC-1, *Euryarchaeota* Halophilic Cluster 1; MBG-D, *Euryarchaeota* Marine Benthic Group D; MG-I, *Crenarchaeota* Marine Group I; MHP, *Methanohalophilus*; MSBL-1, *Euryarchaeota* Mediterranean Sea Brine Lake Group 1. Abbreviation for *Eubacteria* used: Bctd, *Bacteroidetes*; KB1, Kebrit Deep candidate division 1; PB, *Proteobacteria*; SB1, Shaban Deep candidate division 1; SOB, sulfur-oxidizing bacteria.

indicates an anaerobic lifestyle. The deeper regions of the halocline and the L'Atalante brine itself, were inhabited by three additional major groups of halophilic anaerobic euryarchaea, found in hypersaline anoxic ecosystems, deep-sea habitats or associated with sulfide-rich hydrothermal vents and gas hydrates (Takai and Horikoshi, 1999; Vetriani *et al.*, 1999; Lanoil *et al.*, 2001; Sørensen *et al.*, 2004; Lloyd *et al.*, 2006). The high relative abundance of members of *Methanohalophilus* indicated that methylotrophic methanogenesis is one of the prevalent metabolic processes of this environment. Unfortunately, neither of the other two euryarchaeal clusters yet have cultivated representatives, which speculate on their physiological roles.

Metabolically-active members of the domain *Bacteria* in communities of bathypelagic seawater overlaying the L'Atalante brine lake were represented exclusively by members of the class *Gammaproteobacteria* (Figure 3b). In all 20% of these riboclones were related to the cluster of thioautotrophic endosymbionts and sulfur-oxidizing bacteria of the genus *Thiomicrospira*, which became more dominant in the upper halocline and infiltrated into the 130–200‰-salinity layer, in which they represented 100% of all *Gammaproteobacteria*-like clones. Their penetration into deeper layers seems to be prevented by both the strong anoxia and elevated salinity. Similarly, the distribution of *Epsilonproteobacteria* was probably limited by salinity, but also possibly by oxygen toxicity in the upper zone of the halocline, given their known microaerophilic/anaerobic metabolisms (Campbell *et al.*, 2006). The class of *Deltaproteobacteria* appeared to be the most prominent bacterial group in the L'Atalante ecosystem since sequences corresponding to this group accounted for 57 and 46% of all clones retrieved from the deepest level of the halocline and the hypersaline brine, respectively. A salinity of 130–200‰ seems to be optimal for members of the Shaban Deep 1 (SB1) Candidate Division, whereas representatives of the Kebrit Deep 1 (KB1) lineage, also found exclusively in hypersaline anoxic environments (Eder *et al.*, 2002; Mounè *et al.*, 2003), were localized in more saline compartments. Since no cultured organisms belonging to these phylogenetic groups are known so far, their physiology remains enigmatic.

Chemoautotrophy in the L'Atalante Lake halocline and overlying water column

The relatively high concentration of metabolically active microbes in the halocline is indicative of a community well adapted to the prevailing environmental conditions. The halocline acts as a physical barrier to particulate material, sinking through the seawater column and its upper horizon collects organic carbon, nutrients and particulate materials with reactive surfaces that can support microbial growth. The halocline also receives from the underlying brine reduced compounds, like methane, ammonia and sulfide, which diffuse upwards towards the halocline/oxic seawater interface, where they may be oxidized by chemoautotrophic prokaryotes.

To determine whether chemoautrophic metabolism occurs in the L'Atalante Basin lake halocline and overlying water column, the uptake of dissolved inorganic carbon by un-amended samples of halocline and seawater collected at different depths (50, 500, 1000 and 3400 m) spiked with 2.5 μCi ml^{-1} [^{14}C]bicarbonate was measured in batch cultures over a 9-day incubation period at *in situ* temperature in the dark. Since marine chemoautotrophic crenarchaeota have very small sizes (Konneke *et al.*, 2005), samples collected from the 3400 m depth and from the halocline were additionally filtered through 0.45-μm cellulose nitrate filters (Millipore) to physically discriminate between the free-living and particle-associated cells. Values of dark primary production calculated from the measurements of incorporated bicarbonate for each depth level showed a continuous increasing trend with depth (Figure 4a), culminating in a maximum value (6.45 ± 0.26 mgC m^{-3} per day) for the sample from the halocline coinciding with the highest prokaryotic biomass ($8.33 \pm 0.11 \times 10^5$ cells ml^{-1}). No labeled bicarbonate incorporation was detected in samples from lower depths in the halocline. Interestingly, filtration had no effect on CO_2 fixation in bathypelagic seawater, but removed more than 95% of autotrophic capacity from halocline samples (Figure 4a, inset). This implies that autotrophs in seawater are mostly small and free-living, whereas those thriving in the halocline are larger and/or

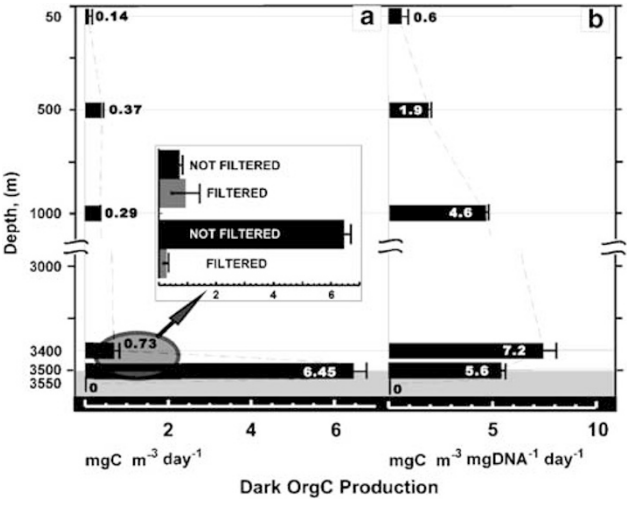

Figure 4 Prokaryotic primary production (**a**) and relative turnover rate (production biomass^{-1}) (**b**) detected at different depth horizons of water column and in halocline of L'Atalante brine lake. Small insert in (**a**) indicates the values of primary production detected in untreated (black bar) and 0.47 μm-prefiltered (gray bar) samples. All error bars indicate one standard error from a mean of two replicate measurements.

integral components of particulates. Autotrophic activity values, normalized to cell density and expressed as mgC m^{-3} per day per mg DNA^{-1}, clearly indicated that autotrophic organisms are abundant in the seawater microbial community of the 3400 m depth (Figure 4b).

Distribution and abundance of archaeal amoA-like genes through the water column

Until recently, only members of the domain *Bacteria* were thought to be capable of autotrophic ammonia oxidation. Several new lines of evidence, however, have suggested a role for non-thermophilic planktonic crenarchaea in a first step of nitrification (that is transformation of NH_3 to NO_2^-) in the ocean (Venter *et al.*, 2004; Konneke *et al.*, 2005; Wuchter *et al.*, 2006; Hallam *et al.*, 2006a, b). The energy gained from ammonia oxidation allows these tiny marine organisms ($0.17–0.22 \times 0.5–0.9\,\mu m$) to use dissolved bicarbonate as a principal carbon source (Herndl *et al.*, 2005; Konneke *et al.*, 2005; DeLong, 2006; Ingalls *et al.*, 2006). This, combined with the fact, that marine crenarchaeota represent a dominant fraction of marine microplankton in meso- and bathypelagic ocean, indicates that they may represent a significant but hitherto little-appreciated sink for inorganic carbon in the largest oceanic ecosystem (Herndl *et al.*, 2005).

We, therefore analyzed the distribution and abundance of ammonia-oxidizing prokaryotes in the brine, within the halocline and in overlying seawater of l'Atalante basin, using probes specific for *amo*A-like genes. With primers specific for *Gamma-* and *Betaproteobacteria* (Rotthauwe *et al.*, 1997; Horz *et al.*, 2000; Mincer *et al.*, 2007), no amplification of bacterial *amo*A-like genes was obtained with samples from depths greater than 500 m. In contrast, archaeal *amo*A-like genes were amplified from meso- and bathypelagic depths of the water column, but not from the surface waters. Cloning and sequencing of the amplicons revealed a monophyletic group of sequences (AM747269 and AM747270), very similar (97% identity of deduced protein) to the putative ammonia mono-oxygenase subunit A of deep-sea ammonia-oxidizing crenarchaeota CBK-G3N136 and ES_HI_2 (Francis *et al.* 2005). The high degree of conservation among all retrieved sequences allowed us to design a TaqMan probe for quantitative PCR analysis of *amo*A-like gene depth distribution across the seawater/brine interface. The pool of *amo*A-like genes examined by this approach showed their highest abundance in the 500 m-depth sample and in sample from the depth closest to the surface of the L'Atalante halocline (Figure 5a). Interestingly, the number of archaeal cells, $21\,420 \pm 400$ cells ml^{-1}, determined by FISH counting in the sample from the depth of 3495 m was essentially the same as the number of *amo*A-like gene copies, $20\,575 \pm 140$ cells ml^{-1}. This one-to-one relationship between archaeal cells and

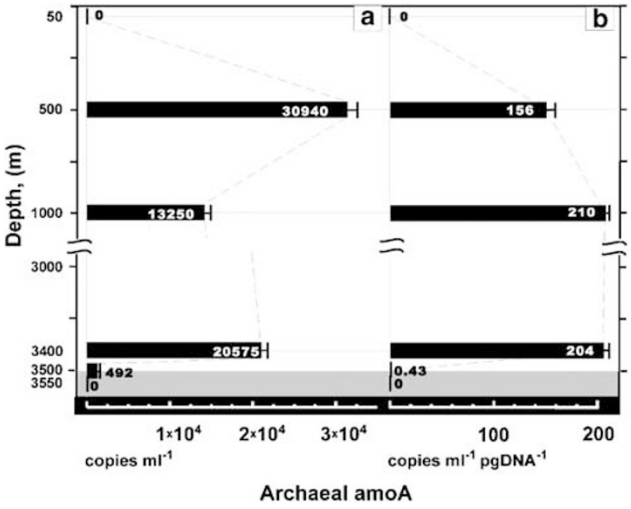

Figure 5 Q-PCR monitored distribution of crenarchaeal *amo*A-like gene copy number at different depth horizons of water column and in halocline of L'Atalante brine lake given in absolute (gene count ml^{-1}) (**a**) and relative (gene count per ml biomass^{-1}) (**b**) units. All error bars indicate one s.e. from a mean of three replicate reactions.

*amo*A gene copies suggests an absolute dominance of the deep-sea planktonic ammonia-oxidizing *Crenarchaea* in the bathypelagic realm of Mediterranean Sea.

Subsequent normalization of the *amo*A-like gene copies to the amount of total DNA, recovered from each sample, produced a depth profile of *amo*A distribution, that was remarkably similar to the CO_2 fixation profiles (Figures 4b and 5b). Although the archaeal *amo*A-like gene copy numbers in seawater collected at the depth of 1000 and 3400 m depths amounted to one half and two-thirds, respectively, of that detected at 500 m, the extracted DNA at these depths contained 1.5 times more *amo*A gene copies (210 and 204 copies pgDNA^{-1} ml^{-1}), indicating an increase in crenarchaeal *amo*A with the depth.

The remarkable coincidence of the depth distribution profiles of crenarchaeal *amo*A-like genes and autotrophic activity in the water column provides further evidence that marine *Crenarchaea* are important contributors to the first step in oceanic nitrification (transformation of NH_3 to NO_2^-), that they couple this process with CO_2 fixation, and thus they play a pivotal role in two major biogeochemical cycles in marine systems.

Distribution and abundance of autotrophic bacteria in the halocline of L'Atalante Lake

In spite of high rates of [^{14}C]bicarbonate incorporation in the L'Atalante halocline, the concentration of archaeal *amo*A-like genes was extremely low, $<4\%$ of the levels measured in the overlying meso- and bathypelagic layers of seawater (Figures 5a and b), which indicates that this niche harbors quite different types of autotrophic chemolitotrophs. As

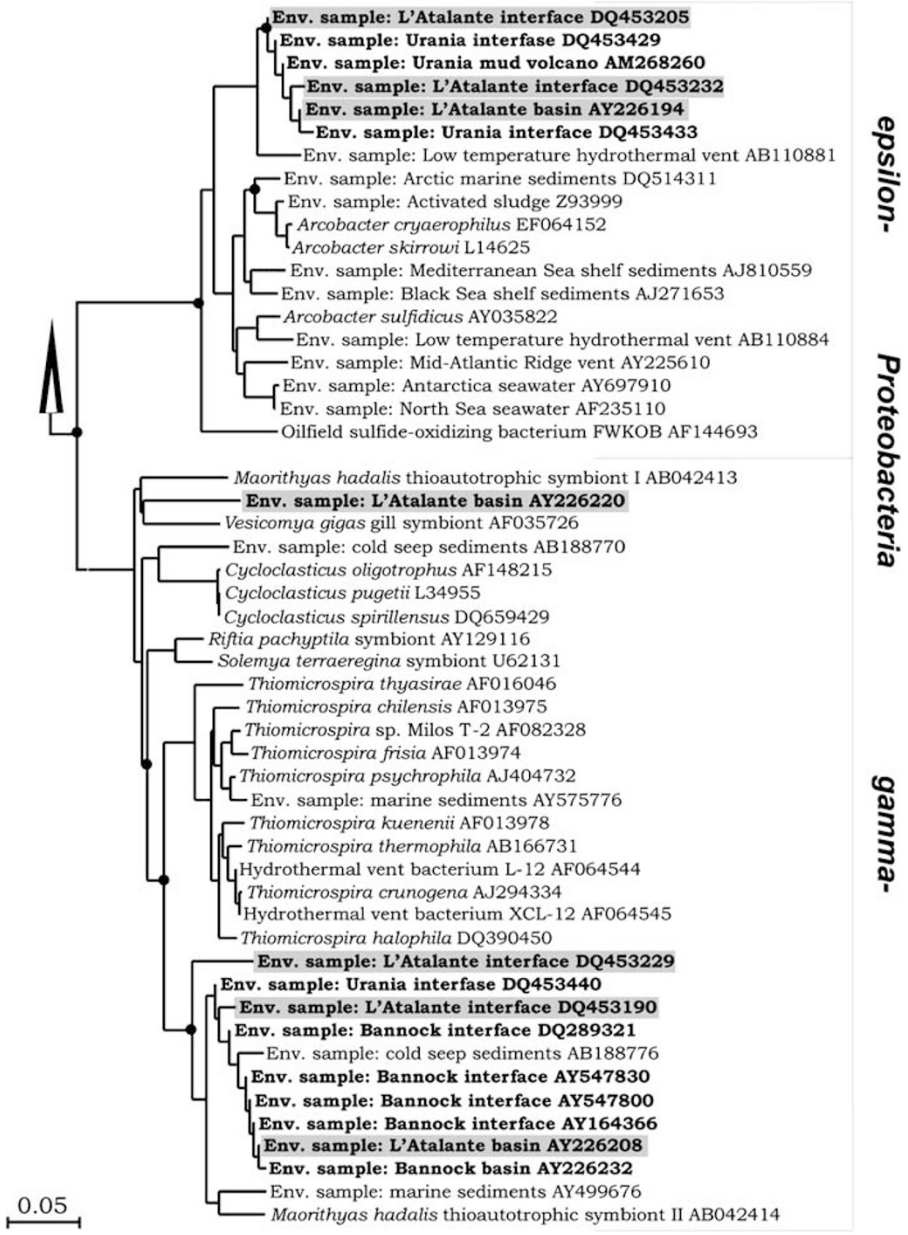

Figure 6 Phylogenetic tree of 16S rRNA sequences of putative chemolitotrophic *Epsilon-* and *Gammaproteobacteria* phylotypes recovered in the l'Atalante oxic/anoxic interface. The tree was constructed using sequences of comparable region of the 16S rRNA gene sequences available in public databases. The tree is rooted with *Thermotoga maritima* 16S rDNA sequence (AJ401017). Neighbor-joining analysis using 1000 bootstrap replicates was used to infer tree topology and the nodes with the percentage of bootstrap re-sampling above 75% is indicated by filled cycles. All clones retrieved from deep-sea hypersaline anoxic lakes are listed in bold. Additionally, L'Atalante clones are evidenced as gray-shaded. The scale bar represents 5% sequence divergence.

discussed above, the 16S rRNA-based phylogenetic survey revealed the presence in the halocline of *Gamma-* and *Epsilonproteobacteria* potentially involved in chemolitoautotrophic processes (Figure 3b). The majority of L'Atalante *Epsilonproteobacteria* formed a tight cluster distantly related to the uncultured proteobacterium SERP3279-31 (Figure 6), recovered from a sulfide-rich low-temperature deep hydrothermal vent fluid in the Southern East Pacific Rise (Higashi *et al.*, 2004). Remarkably, riboclones related to the L'Atalante halocline sequence were recovered only from the Urania Basins, another Mediterranean DHAL filled with a brine of similar composition (van der Wielen *et al.*, 2005), suggesting the presence of an endemic monophyletic group of *Epsilonproteobacteria*, highly adapted to this particular environment. Since no organisms related to this group have so far been cultivated, assumption about their metabolic features are purely speculative. The narrow window of their distribution within oxic/anoxic interface, and oriented towards deeper layers, suggests that they

are microaerophilic or anaerobic, like all current representatives of deep-sea hydrothermal *Epsilonproteobacteria* (Campbell *et al.*, 2006). The distant relationship of this DHAL-specific group to the nitrate-reducing, sulfide-oxidizing autotrophic *Arcobacter* sp. FWKO B isolated from an oil field brine having a similar salinity (Gevertz *et al.*, 2000), suggests the possibility of their ability to carry out these biogeochemical processes. In contrast, *Gammaproteobacteria*-related riboclones were unambiguously affiliated with thioautotrophic endosymbionts and sulfur-oxidizing *Thiomicrospira* spp. (Fujiwara *et al.*, 2001; Fang *et al.*, 2006). This physiological group is characterized by the ability to gain energy from the oxidation of sulfide and other reduced sulfur products, which drives fixation of CO_2 to organic carbon by means of the enzymes of the Calvin-Bassham-Benson (CBB) cycle, similar to those used by oxygenic phototrophs (Wirsen *et al.*, 2002).

The *cbb*L gene codes for the large subunit of RuBisCO and its expression is considered to be an unambiguous indicator of active CO_2 fixation. *cbb*L gene mRNA levels in the halocline and brine of L'Atalante lake were therefore determined. No RuBisCo expression could be detected in any compartment other than the halocline, from which a strong positive signal was obtained by RT-PCR amplification of total RNA. As shown in Figure 7, phylogenetic analyses of the clone sequences

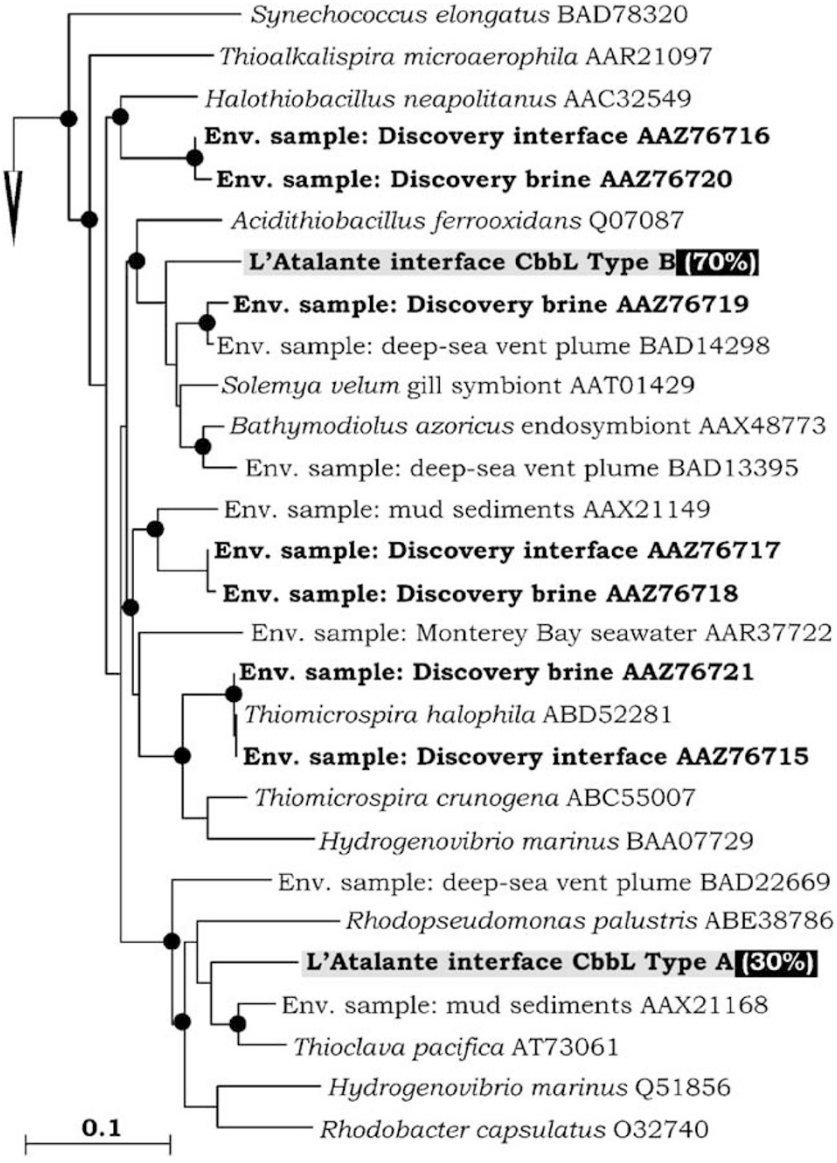

Figure 7 Phylogenetic relationships of two CbbL phylotypes of *Gammaproteobacteria* as determined by Neighbor-Joining analysis and Poisson correction of distances. The percentage of bootstrap re-sampling above 75% is indicated at the corresponding nodes by filled cycles. Sequences obtained from deep-sea hypersaline anoxic lakes are listed in bold. Additionally, L'Atalante clones are evidenced as gray-shaded. The scale bar represents 10% amino acid sequence divergence.

revealed the presence of two distinct groups with a predominance of sequences corresponding to the CbbL type A subunit, a protein similar to the large RuBisCo subunit of *Thioclava pacifica*, a marine facultatively autotrophic, sulfur-oxidizing bacterium isolated from a near-shore sulfidic hydrothermal area (Sorokin *et al.*, 2005). Sequences corresponding to the L'Atalante CbbL type B subunit formed a deep branch within the cluster of RuBisCo enzymes found in chemoautotrophic symbionts of various hydrothermal vent mussels (Schwedock *et al.*, 2004). Recently, the *cbb*L genes similar to those encoding the L'Atalante type B subunit were retrieved from the Discovery Basin, another Mediterranean DHAL filled with 5 M of $MgCl_2$ (van der Wielen, 2006), and from a hydrothermal vent plume collected from the Mariana Trench (Elsaied *et al.*, 2007). These results, in conjunction with the high number of halocline riboclones assigned to thioautotrophic symbionts, support the view that *Gammaproteobacteria* may be responsible for the high CO_2 fixation activity detected in the L'Atalante halocline.

Conclusions

We report here a description of the autotrophic component of deep-sea microbial communities inhabiting the L'Atalante hypersaline brine lake, and its overlying chemocline and seawater column, obtained through analysis of prevailing physical and geochemical parameters and characterization of physiological and molecular-taxonomical diversity of the microbial flora. It was shown that representatives of *Crenarchaeota* Marine Group I are the predominant members of the meso- and bathypelagic microbial communities of Mediterranean Sea, and most likely play a pivotal role in dark deep-sea autotrophic carbon assimilation. The correlation of the depth-profile of CO_2 fixation activity with the distribution of crenarchaeal *amo*A-like gene copies suggests that ammonium oxidation is the main energy-generation process sustaining crenarchaeal autotrophy. In contrast, the high abundance of reduced sulfur compounds probably represents the predominant energy source for chemolitotrophic bacteria thriving in the upper, oxic/anoxic interface of L'Atalante brine lake. However, other chemical compounds such as hydrogen and methane, also present in this environment, may also be of importance. Bacteria, whose metabolism is probably sulfur oxidation-based, included thioautotrophic *Gammaproteobacteria* and some affiliates of *Epsilonproteobacteria*. The former were identified in the halocline on the basis of genes encoding RuBisCO, the key enzyme of the CBB cycle and it seemingly dominated the bacterial community. The role of the DHAL-endemic group of *Epsilonproteobacteria* in primary production remains unknown. However, inferring from their environmental characteristics, a contribution in chemolitoautotrophy is anticipated. Further efforts to understand in detail the chemoautotrophic community thriving in the oxic/anoxic interface of l'Atalante, involving the activity measurements, culture isolation/enrichment strategies and environmental genomics are in progress.

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