Diversity of freshwater Epsilonproteobacteria and dark inorganic carbon fixation in the sulphidic redoxcline of a meromictic karstic lake

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One sentence summary: A freshwater member of the genus Arcobacter fix inorganic carbon in the dark in the sulphidic redoxcline of a meromictic karstic lake.

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ABSTRACT

Sulphidic redoxclines are a suitable niche for the growth and activity of different chemo- and photolithotrophic sulphide-oxidizing microbial groups such as the Epsilonproteobacteria and the green sulfur bacteria (GSB). We have investigated the diversity, abundance and contribution to inorganic carbon uptake of Epsilonproteobacteria in a meromictic basin of Lake Banyoles. CARD-FISH counts revealed that Epsilonproteobacteria were prevalent at the redoxcline in winter (maximum abundance of 2 × 10⁶ cells mL⁻¹, ≈60% of total cells) but they were nearly absent in summer, when GSB bloomed. This seasonal trend was supported by 16S rRNA gene pyrotag datasets, which revealed that the epsilonproteobacterial community was mainly composed of a member of the genus Arcobacter. In situ incubations using NaH¹⁴CO₃ and MAR-CARD-FISH observations showed that this population assimilated CO₂ in the dark, likely being mainly responsible for the autotrophic activity at the redoxcline in winter. Clone libraries targeting the aclB gene provided additional evidence of the potential capacity of these epsilonproteobacteria to fix carbon via rTCA cycle. Our data reinforce the key role of Epsilonproteobacteria in linking carbon and sulphur cycles, extend their influence to freshwater karstic lakes and raise questions about the actual contribution of chemolithotrophy at their redoxcline and euxinic water compartments.

Keywords: Arcobacter; dark inorganic carbon fixation; Epsilonproteobacteria; euxinic waters; Green Sulfur Bacteria; karstic lakes; sulphidic redoxclines
INTRODUCTION

Sulfidic redoxlines are well-defined transition zones in the water column of both marine and freshwater environments characterized by opposite gradients of oxygen and sulphide (Grote et al. 2007; Lavik et al. 2008; Camacho 2009). Sulfide produced in anoxic water layers and sediments by sulphate-reducing bacteria diffuses upwards creating a gradient of decreasing concentration that provides optimal niches for the growth and activity of different groups of sulphur-oxidizing microorganisms, both chemolithoautotrophic (either obligate or facultatively) and photolithoautotrophic (Camacho et al. 2001; Camacho 2009; Ghosh and Dam 2009; Overmann 2008). These microbial populations actively link C and S cycles and constitute an efficient biological barrier for sulphide diffusion to upper water layers thus preventing its toxic effects on the aquatic biota (Lavik et al. 2008; Walsh et al. 2009). Members of the class Epsilonproteobacteria represent the major fraction of chemolithoautotrophic bacteria in sulfidic redoxlines of anoxic marine basins (Madrid et al. 2001; Lin et al. 2006; Grote et al. 2007, 2008), coastal sediments (Wirsig et al. 2002) and hydrothermal vents where they thrive either as free-living populations (Campbell and Craig Cary 2004; Takai et al. 2005) or as symbionts of deep-sea polychaetes (Campbell, Stein and Craig Cary 2003). In all these habitats, Epsilonproteobacteria have been recognized as important players in the biogeochemical cycling of C and S, encompassing diverse sulphur-based metabolisms ranging from strict autotrophy to mixotrophy and heterotrophy (Campbell et al. 2006). In this regard, several studies highlighted the key role of members of this class in linking carbon, sulphur and nitrogen cycles through autotrophic denitrification (Hannig et al. 2007; Bruckner et al. 2013).

In freshwater systems, the distribution of epsilonproteobacterial sulphide oxidizers has only been reported in terrestrial caves and springs, where they largely contribute to the development of sulphide-oxidizing streambed biofilms (Elshahed et al. 2003; Porter and Engel 2008; Porter et al. 2009; Rossmasler et al. 2012). Remarkably, very little information is available for the distribution, abundance and activity of Epsilonproteobacteria in stratified lakes (Ridere-Petit et al. 2011) especially considering that these waterbodies are usually characterized by sharp $O_2/H_2S$ interphases that provide an optimal niche for the growth and activity of epsilonproteobacterial sulphide oxidizers. In most freshwater environments, however, sulphidic redoxlines are located at photic depths (5–30 m depending on the system) that favour the bloom of anoxygenic phototrophs that efficiently compete for available electron donors (i.e. sulphur-reduced compounds) (Borrego, Bañeras and García-Gil 1999; Camacho, Vicente and Miracle 2000; Camacho et al. 2005; Camacho 2009). In turn, the occurrence of purple and green sulphur bacteria in anoxic marine basins where epsilonproteobacteria have conspicuously been found is not common (Lin et al. 2006; Grote et al. 2008, 2007) though documented (Repeta et al. 1989; Manske et al. 2005; Marschall et al. 2010).

Previous studies carried out in different lakes and lagoons of the Banyoles Karstic System reported a high contribution of dark processes to overall inorganic carbon fixation at the oxic–anoxic interface (i.e. the redoxcline) and the euxinic monimolimnion (García-Cantizano et al. 2005; Casamayor, García-Cantizano and Pedrós-Alió 2008; Casamayor 2010). Similar results were obtained in other stratified lakes with sulphidic redoxlines (Cloern, Cole and Oremland 1983; Camacho et al. 2001; Hadas, Pinkas and Erez 2001; Casamayor et al. 2011) pointing out the ecological significance of chemolithothrophic processes even in the presence of oxygenic and anoxygenic autotrophs.

The aim of this work was to investigate whether or not Epsilonproteobacteria constitute stable populations at the redoxcline depth of a meromictic basin in Lake Banyoles where green sulphur bacteria (GSB) seasonally bloom, providing data on their diversity, activity and contribution to the (dark) inorganic carbon fixation in the system. According to the physical-chemical conditions of the habitat and the physiological requirements of chemolithothrophic sulphide oxidizers under investigation, we hypothesized that the latter would be prevalent in winter, when GSB are rare.

MATERIALS AND METHODS

Study site, sample collection and chemical analyses

Lake Banyoles (Catalonia, NE of Spain, 42° 07′ N, 2° 45′ E) is a karstic lake composed of six basins with different morphometric and limnological features (Moreno-Amich and García-Berthou 1989). Water enters the lake by subterranean bottom springs creating strong chemical gradients along the water column due to high concentration of dissolved sulphate (∼13 mM, Guerrero et al. 1985). This sulphate-rich water together with anoxic conditions at deep layers favoured by the chemical and thermal stratification allows an active sulphate reduction both in the anoxic water layers and in the sediment. The current study was carried out in basin C-III, a meromictic, circular, regular-shaped basin of 32 m depth located in the northern lobe of the lake (Fig. S1, Supporting Information).

Sampling was carried out in winter 2011 (25th January), spring and summer 2012 (27 March and 7 July, respectively), and winter and summer 2013 (15 January and 16 July, respectively). Depth profiles of temperature, conductivity, pH, redox potential $(E_{HYS})$ and oxygen concentration were determined in situ using a multiparametric probe OTT-Hydrolab M55 (Hatch Hydromet, Loveland, CO, USA). Photosynthetically active radiation (PAR) profiles were performed with a submersible PAR sensor (LI-250 Light Meter; LI-Cor Inc.). Water samples for biological and chemical analyses were collected at selected depths according to the physicochemical gradient with special focus on the oxic–anoxic interface (19–22 m depth) where samples were collected at 25–50 cm intervals using a weighted double cone connected to a battery-driven pump that allowed a laminar water sampling and a minimal disruption of microstratification (Jorgensen, Kuenen and Cohen 1979; García-Gil and Camacho 2001). On boat, water samples were kept on ice and protected from light in a portable icebox until further analysis within less than 24 h. For sulfide analysis, 10 mL of water was collected in sterile screw-capped glass tubes, alkalinized by adding NaOH (0.1 M final concentration) and sulphide immediately fixed by adding zinc acetate (0.1 M final concentration). Sulfide was later analysed by the leucemethylene-blue method (Tripler and Schlegel 1964). Samples for the determination of ammonia were collected in sterile 50 mL Falcon tubes after filtration through 0.22-μm pore-size Millipore membrane filters and acidified with HCl. For nitrate, nitrite and sulphate determinations, 10 mL subsamples were filtered through 0.22-μm pore-size Millipore membrane filters and kept frozen at −30°C until analysis. Sulfate, ammonia, nitrate and nitrite were measured by ionic chromatography (DIONEX, Model IC5000) using IonPac® AS18 anion-exchange column (4 × 250 mm) with the AG Guard column (4 × 50 mm), using 30 mM MSA for the production of the mobile phase for cations and 22–40 mM KOH for anions. For both (anions and cations), the injection volume was 25 μL with an eluent flow rate of 1 mL min$^{-1}$. For
dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) determinations, water subsamples of 50 mL were filtered through Millex-HA, MF 0.45-μm pore-size, 25-mm-diameter Nylon filters and kept at 4°C until measurement. DOC and DIC were oxidized through combustion and analysed in a Total Organic Carbon Analyzer (TOC-V CSH, Shimadzu). Total phosphorous (TP) was measured according to UNE-EN ISO 6878.

CARD-FISH

The abundance of members of the class Epsilonproteobacteria and the phylum Chlorobi was analysed by Catalyzed-Reported Deposition Fluorescence In Situ Hybridization (CARD-FISH) (Pernthaler et al. 2002) using specific probes EPSY914 (Loy et al. 2007) and GSB-532 (Tuschak, Glaser and Overmann 1999), respectively. Filter sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg mL⁻¹). Between 250 and 600 DAPI-stained cells were counted in 20 randomly selected microscopic fields using an Axioskop epifluorescence microscope (Zeiss, Germany).

DNA extraction

Water samples for DNA extraction were filtered through 0.22-μm pore-size, 47-mm-diameter polycarbonate filters (ISOPORE, Millipore, MA) and kept frozen at −80°C until processing. Total DNA was extracted from filters using a combination of enzymatic cell lysis with lysozyme and proteinase K followed by a modified CTAB extraction protocol as previously described (Lliró, Casamayor and Borrego 2008). Dry DNA pellets were finally rehydrated in 50 μL of 10 mM Tris-HCl buffer (pH 7.4). DNA concentration was determined using QUBIT® 2.0 fluorometer (Invitrogen, Molecular probes Inc., Oslo, Norway). Purified DNA extracts were stored at −80°C until use.

Sequence generation and processing

Pyrosequencing of total extracted genomic DNA from selected depths and seasons was performed at Research and Testing Laboratory facilities (RTL) (TX, USA) using Roche 454 FLX-Titanium technology (Dowd et al. 2008). Amplification reactions were performed using primers 28F/519R targeting the V1/V3 region of the bacterial 16S rRNA gene complemented with 454-adapters and sample-specific barcodes. Sequence dataset was pre-processed in RTL facilities to reduce pyrosequencing noise. After this initial denoising, sequences were demultiplexed according to sample barcodes, quality-filtered, chimera checked and clustered into OTUs (97% cutoff) using MOTHUR (Schloss et al. 2009). MOTHUR was also used for the alignment and taxonomic classification of the representative sequences of each OTU using the SILVA reference alignment and taxonomy database, respectively, available at MOTHUR website (http://www.mothur.org). α-Diversity indicators of richness (observed richness and Chao1) and diversity (Shannon) were calculated in MOTHUR after normalization of the number of sequences in each sample by randomly selecting a subset of 1600 sequences from each sample to minimize bias due to different sampling efforts.

Cloning

PCR amplification of the bacterial 16S rRNA gene and the beta subunit of the ATP citrate lyase (aclB) were carried out using specific primers Bac27F/Univ1492R (Suzuki and Giovannoni 1996; Engberg et al. 2000) and aclB892F/aclB1204R (Campbell, Stein and Craig Cary 2003), respectively. The bacterial 16S rRNA clone library was constructed using PCR amplicons obtained from a sample collected at the O₂/H₂S interface in winter 2011 (25 January 2011, 21.75 m). The aclB gene amplicons were obtained from samples where maximal dark fixation rates were measured during both winter (15 January 2013) and summer (16 July 2013) in situ incubations. To improve cloning efficiencies, purified PCR products were polyadenylated by adding 26 μL of the clean PCR product to a 4.5 μL of a PCR mix [0.98X PCR buffer (Invitrogen, Paisley, UK), 0.16 mM of MgCl₂ (QIAGEN, Valencia, USA), 0.26 mM of dATP (PROMEGA, Madison, USA) and Taq polymerase (QIAGEN, Valencia, USA)]. Polyadenylation reaction mixtures were incubated for 10 min at 72°C in the thermal cycler and products were used for cloning without further processing. Cloning was performed using TOPO TA Cloning® Kit for sequencing (Invitrogen, Paisley, United Kingdom) following manufacturer instructions. A total of 192 and 250 clones were randomly picked for bacteria 16S rRNA library and aclB gene libraries, respectively. All clones were checked for the presence of the correct insert, purified and sequenced using the M13F primer by an external company (Macrogen Inc.). Alignment of bacterial 16S rRNA gene sequences was carried out in MOTHUR (Schloss et al. 2009) using the Silva reference alignment available at MOTHUR website. MOTHUR was also used for OTU delineation (97% cutoff) and to obtain representative sequences for each OTU. aclB sequences were aligned and translated to the predicted protein sequences using MEGA v6 (Tamura et al. 2013). Sequences yielding incorrect protein sequences or non-specific products were discarded for further analysis. Correct aclB nucleotide sequences (193) were annotated using BLAST2GO software (http://www.blast2go.com/b2ghome; Conesa et al. 2005). The distance matrix of the final set of aclB amino acid sequences was computed in MEGA (JTT algorithm and pairwise deletion as computational parameters) and then loaded into MOTHUR to delineate OTUs (97% cutoff) and to obtain representative sequence for each OTU. Phylogenetic trees were generated in MEGA v6 (Tamura et al. 2013) using maximum likelihood (for bacterial 16S rRNA genes) and neighbor joining (for aclB amino acid sequences, Saitou and Nei 1987) based on the JTT matrix-based model (Jones, Taylor and Thornton 1992) and bootstrapping (1000 replicates).

Biological uptake of inorganic carbon

The bulk uptake of inorganic carbon was measured by in situ incubations carried out in winter (15 January 2013) and summer (16 July 2013) as previously described (Camacho and Vicente 1998). For each sample, a 70-ml plastic flask fully filled to minimize aeration was used. Samples were spiked with radiolabelled bicarbonate (NaH¹⁴CO₃; specific activity 4 μCi, DHI, Denmark) to a final concentration of 0.07 μCi mL⁻¹. The in situ incubation lasted 4 h (noon period) under at-depth light conditions. The incubation set included two clear (light) and two dark incubation flasks and one additional formaldehyde-killed flask as a control at every sampled depth. After incubation, water samples were immediately fixed with formaldehyde (final concentration 3.7%) to stop microbial activity (Camacho and Vicente 1998), and cells were collected on white 0.22-μm pore-size nitrocellulose filters (25-mm filter diameter) at a low vacuum pressure. Filters were exposed overnight to HCl fumes to release precipitated bicarbonate. Scintillation cocktail (4 mL; Optiphase Hisafe 2) was added and then radioactivity was measured in a Becton-Dickinson LS6000 scintillation counter. Alkalinity and pH were determined for each sample to estimate the total inorganic carbon content (Margalef 1982). Photosynthetic carbon
incorporation was calculated by subtracting the disintegrations per minute (d.p.m.) measured in the ‘dark’ flasks from that measured in the ‘clear’ flasks, whereas chemolithotrophic carbon incorporation was calculated by subtracting d.p.m. measured in killed controls from d.p.m. measured in ‘dark’ flasks (Pedrós-Alió, García-Cantizano and Calderón-Paz 1993). Almost identical results were obtained from duplicate subsamples and the results are presented as mean values.

Estimations of areal primary production (µg C m⁻² h⁻¹) for each sampling date were obtained using Simpson’s rule for numerical integration (Fee 1969; Auer and Canale 1986) of the measurements of inorganic carbon assimilation (oxygenic photosynthesis, anoxygenic photosynthesis and chemolithotrophy) obtained at discrete depths within the water column. Results were weighted considering the hypsography of basin C-III, determined according to available bathymetric data (Moreno-Amich and García-Berthou 1989; Casamitjana et al. 2006).

Microautoradiography-CARD-FISH

MAR-CARD-FISH was carried out as previously described (Llirós et al. 2011). Briefly, water samples were filtered [0.22-µm pore-size, 25-mm diameter white polycarbonate filters (Millipore, Germany)] and were subjected to CARD-FISH as described above. Afterwards, filters were processed for autoradiography to reveal the incorporated radiolabelled bicarbonate into active cells according to Llirós et al. (2011). Finally, the slides were dried in a desiccator overnight, counterstained with DAPI (1 µg mL⁻¹ final concentration) and examined under an Axioskop epifluorescence microscope (Zeiss, Germany). Active cells were distinguished by the presence of silver grains surrounding the cell.

Statistical analyses

Spearman rank correlation was calculated on log-transformed data of Epsilonproteobacteria and GSB abundance obtained from CARD-FISH counts for winter and summer redoxcline samples. Statistical analysis was carried out in SPSS software package (v21, SPSS Chicago, IL, USA)

GenBank submission and accession numbers

Pyrosequencing data from this study have been deposited in the NCBI database via the Biosample Submission Portal (http://www.ncbi.nlm.nih.gov/biosample/) under accession number PRNA282702. 16S rRNA and aclB gene sequences obtained in the corresponding clone libraries were deposited in GenBank under accession numbers KR537292–KR537427 and KR559042–KR559234, respectively.

RESULTS

Physicochemical characterization of the water column

Basin C-III is a crenogenic, meromictic basin in Lake Banyoles that maintains a permanent chemical stratification of the water column. In winter, the chemocline was located at 22 m depth coinciding with a step decrease in oxygen concentration (Fig. 1A). In late spring and summer, the water column became thermally stratified with the thermocline spanning from 7 to 12 m depth. Summer stratification caused an upward movement of the chemocline that displaced the oxic–anoxic interface to shallower depths (18.5–19.5 m depth) (Fig. 1B). This interface showed a large variability for most variables measured evidencing its dynamic state in comparison to the more stable conditions at the upper and lower water compartments (Fig. S2, Supporting information). Oxygen peaks of up to 14.3 mg L⁻¹ were measured at the mixolimnion (8–10 m depth) in spring and summer as a result of oxygenic photosynthesis occurring at these depths. Measured pH values decreased from 7.8 units at the mixolimnion to nearly neutrality at the chemocline and reaching values close to 6.7 at the monimolimnion (Fig. S2, Supporting Information). Redox potential dropped at the chemocline depth coinciding with oxygen extinction and sulphide diffusion from anoxic bottom waters, generating a sharp redoxcline (Fig. 1). Sulfide concentrations measured in the monimolimnion were higher in winter (average 511.99 ± 311.72 µM) than in summer (average 89.37 ± 135.50 µM). In turn, sulphate concentrations in this euxinic bottom water layer were similar in both winter and summer periods (average of 12.16 ± 0.80 and 13.00 ± 0.05 mM, in winter and summer, respectively). Ammonia concentrations were always higher in the anoxic monimolimnion (average of 98.07 ± 16.83 and 54.79 ± 35.05 µM in winter and summer, respectively) and diminished in upper water layers. In turn, nitrate was always below the detection limit in the monimolimnetic waters but showed high variability at the oxic–anoxic interface and the mixolimnion in winter (Fig. S2, Supporting Information). Nitrite was always below detection limit in all samples analysed from all water layers and sampling dates (data not shown). Average light intensities reaching the oxic–anoxic interface were always lower in winter (average of 0.14% of surface incident light) than in summer (average of 0.31%) (data not shown).

Phylogenetic composition of the planktonic bacterial assemblage at the redoxcline

The phylogenetic structure of the bacterial assemblage at the redoxcline zone was assessed by 16S rRNA gene pyrotag sequencing of samples collected at selected depths and seasons (21.75 m on 25 January 2011; 22.5 m on 27 March 2012; 19.75 and 21 m on 12 July 2012; 22 m on 15 January 2013; and 20.25 m on 16 July 2013) (Fig. 2). Sequences affiliated to class Epsilonproteobacteria were predominant in pyrotag libraries from the two winter samples analysed (relative abundances of 42 and 81%, in winter 2011 and 2013, respectively). In clear contrast, samples collected at the redoxcline and at the upper monimolimnion during summer season showed a clear dominance of sequences affiliated to phylum Chlorobi (90 and 61% in summer 2012 and 2013, respectively), which also constituted an important fraction of the community (45% of total sequences) at 22.5 m depth in spring 2012. On this date, most sequences (52% of total) affiliated to Deltaproteobacteria (genus Desulfomonomile, 63.6% of total deltaproteobacterial sequences). Other bacterial taxa typical from freshwater planktonic environments such as the Actinobacteria, the Alphaproteobacteria and the Cyanobacteria (mainly sequences affiliated to Synechococcus) showed high variability in their relative abundances across the six sampling depths analysed, reaching their maximal contributions at the upper layers of the redoxcline in summer 2012 (19.75 m, Fig. 2). Richness and diversity indices calculated from pyrotag libraries showed little variation between winter and summer samples (Shannon index of 1.48 ± 0.44 and 1.96 ± 0.53 for winter and summer, respectively) (Table S1, Supporting Information).

A detailed analysis of the epsilonproteobacterial assemblage revealed that 96.8% (winter 2011) and 90.7% (winter 2013) of assigned sequences affiliated to genus Arcobacter (Fig. 2). These sequences grouped into two OTUs (97% cutoff) that clustered together and were closely related to Arcobacter butzleri (Fig. 3). To better resolve the taxonomic assignment of the dominant
Figure 1. Physicochemical profiles of the water column of basin C-III in (A) winter 2011 (25 January) and (B) summer 2012 (12 July). Right panels show an enlarged view of the physicochemical gradient at the sulphidic redoxcline (shaded in grey).
member of the epsilonproteobacterial community occurring in basin C-III, longer 16S rRNA gene sequences were obtained after cloning a winter sample from the oxic–anoxic interface (21.75 m, 25 January 2011). Sequences affiliated to Epsilonproteobacteria were dominant in the clone library (104 out of 135 clone sequences, 77%). Besides, most of these sequences (99 out of 104; 95%) grouped into a single OTU closely related to Candidatus Arcobacter sulfidicus, an autotrophic, sulphide-oxidizer marine Epsilonproteobacterium (Wirsen et al. 2002) (Fig. S3, Supporting Information). Of the remaining five clones, three of them grouped into unique OTUs also showing high-sequence identity to Candidatus Arcobacter sulfidicus whereas two of them (OTUs 15 and 17) clustered with Sulfuricurvum kuijense DSM 16994. These results agreed with the low representativeness of Epsilonproteobacteria other than Arcobacter in pyrotag libraries (e.g. Sulfurimonas (OTU-13 and -15) and Sulfuricurvum (OTU-11 and -14)) (Fig. 3).

**Abundance of target bacterial groups in the planktonic assemblage**

Samples for the quantification of Epsilonproteobacteria and GSB were collected at different depths at the oxic–anoxic interface (redoxcline) in different seasons (winter, spring and summer) and years (2011, 2012 and 2013) and analysed by CARD-FISH using specific probes for both groups. Total cell counts were always maximal at the redoxcline, coinciding with the maximal abundance of Epsilonproteobacteria in winter (22.5 m in January 2011 and January 2103) and GSB in summer (20.75 m in July 2012 and 20.25 in July 2013) (Fig. 2), suggesting that both groups contributed the most to the total biomass of the prokaryotic planktonic community at the redoxcline depth. Higher concentrations of Epsilonproteobacteria were always measured at the upper redoxcline depth where oxygen and sulphide cooccurred whereas abundance of GSB was maximal at upper monimolimnetic water layers. According to CARD-FISH data, Epsilonproteobacteria were prevalent in winter samples whereas the abundance of GSB was higher in summer (Fig. 2). This general trend of seasonal distribution was also confirmed after analysis of pyrotag libraries from selected depths (Fig. 2). Nevertheless, large discrepancies were observed between the relative abundance of Epsilonproteobacteria in spring and summer samples calculated from CARD-FISH counts and pyrotag datasets, even after correction of the latter figures by the average number of 16S rRNA operons in genomes of Epsilonproteobacteria (4.8 copies/genome, 2002).
Figure 3. Maximum likelihood phylogenetic tree showing the affiliation of OTUs identified in pyrotag 16S rRNA gene libraries from basin C-III at different dates. OTUs were delineated at 97% cutoff. The number of sequences for each OTU is indicated between brackets. Node numbers refer to bootstrap support (1000 replicates).

Klappenbach et al. 2001). Particularly, whereas the relative abundance of Epsilonproteobacteria ranged between 9 and 78% of total cells in spring and summer samples according to CARD-FISH counts, no sequences assigned to this class were identified in corresponding pyrotag datasets (Fig. 2). A specificity check of probe EPSY914 using the TestProbe tool available at the SILVA database (http://www.arb-silva.de/search/testprobe/) confirmed non-specificities of this probe against members of the phylum Chlorobi (Chlorobium limicola and Chl. phaeobacteroides) and the class Deltaproteobacteria (Desulfovomonile tiedjei) with two mismatches (Table S2, Supporting Information). Both phyla were prevalent in the water column of basin C-III in summer and spring, respectively (Fig. 2). The strong correlation observed between CARD-FISH counts of GSB and Epsilonproteobacteria in summer samples (Spearman rho = 0.860, p < 0.001) provided additional support to the potential interference of Chlorobi cells on the quantification of Epsilonproteobacteria by CARD-FISH using probe EPSY914 (Fig. S4, Supporting Information). In turn, this interference was minimal for winter samples (Spearman rho = 0.483, p = 0.187) when GSB were rare and both methodologies showed a high relative abundance of Epsilonproteobacteria (Fig. 2). The low representativeness of GSB in pelagic redoxclines of marine basins might probably explain why probe EPSY914 yielded accurate estimations of epsilonproteobacterial abundance in these habitats (Grote et al. 2008).

Inorganic carbon fixation by the planktonic assemblage

In situ incubations were carried out to calculate the rates of inorganic carbon fixation in two different periods, covering both the already demonstrated winter dominance of chemolithoautotrophic sulphide oxidizers (i.e. Epsilonproteobacteria) and the summer prevalence of anoxygenic photoautotrophs (i.e. GSB). The highest inorganic carbon fixation rates were measured in winter at the redoxcline depth (≈22 m) coinciding with the maximum abundance of Epsilonproteobacteria and mainly due to chemolithoautotrophic dark carbon fixation (Fig. 4A). In winter, these rates were almost 6-fold higher than maximum rates of
Figure 4. $^{14}$C fixation rates by oxygenic and anoxygenic phototrophs and by chemolithotrophic microorganisms at different water compartments (mixolimnion, redoxcline, monimolimnion) of basin C-III calculated from (A) winter and (B) summer in situ incubation data. Values are the average of measurements made on sample duplicates (± standard error of the mean). Note the differences in the scale of X-axis.

Oxygenic photosynthesis measured in the upper mixolimnion, and accounted for most of the inorganic carbon fixed at the redoxcline depth (82.5% of total fixed C) and the euxinic monimolimnion (77.3% of total fixed C) (Fig. 5A). Even though these high rates at the redoxcline were measured in a very narrow water layer, dark fixation occurred throughout the water column, and then its contribution to the carbon fixation in the whole basin, estimated after considering the lake hypsography, was relatively high (29.2%). Oxygenic photosynthesis that occurred almost exclusively in the mixolimnion accounted for most inorganic carbon fixation (69.8% of the total carbon fixed in the whole basin), whereas anoxygenic photosynthesis at the redoxcline and the monimolimnion represented less than 1% of the inorganic carbon fixed in the basin. When in situ
incubations were performed in summer, coinciding with the minimum abundance of Epsilonproteobacteria and the dominance of GSB, very low rates of both dark carbon fixation and anoxygenic photosynthesis were measured (≈ 0.2 mg C L⁻¹ h⁻¹) in comparison to the rates measured for oxygenic photoassimilation (ranging from 1 to 3.5 mg C L⁻¹ h⁻¹) (Fig. 4B). Accordingly, the relative contribution of both anoxygenic photosynthesis (0.3%) and dark fixation (4.8%) to overall inorganic carbon fixation in the whole basin was much lower in summer than in winter.

To elucidate whether or not planktonic Epsilonproteobacteria were responsible for the high dark inorganic carbon uptake rates measured at the redoxcline in winter, MAR-CARD-FISH analyses were carried out in the samples where the maximum dark carbon fixation rate was measured (22 m depth, 16 January 2013). Deposition of silver granules around cells hybridized with probe EPSY914 was clearly visible in MAR-CAR-FISH images (Fig. S5, Supporting Information). Remarkably, the proportion of cells identified as Epsilonproteobacteria actively uptaking bicarbonate was similar (37.5% of total epsilonproteobacterial cells on average) in both dark and light incubations (Table 1). Being aware of the potential overestimation of Epsilonproteobacteria due to non-specific binding of probe EPSY914 (see above), we analysed a cDNA-based pyrotag library from the same sample. This dataset revealed the prevalence of epsilonproteobacteria-assigned reads (88.2% of total sequences, Table 1) and provided additional evidence that epsilonproteobacterial cells were active members of the planktonic assemblage at this depth.

Assuming that chemolithoautotrophic Epsilonproteobacteria fix CO₂ via the reductive tricarboxylic acid cycle (rTCA) (Hügler et al. 2005), further pieces of evidence of the potential capacity of planktonic Epsilonproteobacteria in basin C-III to assimilate CO₂ were provided after cloning the aclB gene in samples collected at selected depths and dates using specific primers (Campbell, Stein and Craig Cary 2003). The gene aclB encodes for the beta subunit of ATP citrate lyase, a key enzyme in the rTCA (Antranikian, Herzberg and Gottschalk 1982; Campbell, Stein and Craig Cary 2003; Hügler et al. 2005). A total of 193 valid aclB clones were finally sequenced, aligned and translated to protein sequences (Table S3, Supporting Information). These environmental sequences clustered into five OTUs (97% cutoff) with high-sequence identity at amino acid level to reference aclB sequences from other Epsilonproteobacteria (Table S3, Supporting Information). Remarkably, representative sequences of the most populated OTU (OTU-1, 157 sequences recovered from both winter and summer libraries) were phylogenetically more related to aclB sequences from Thiouolum spp. than to aclB from

Table 1. Abundance of Bacteria and Epsilonproteobacteria (EPS), contribution of EPS to total Bacteria, percentage of active EPS uptaking CO₂ and percentage of cDNA-based pyrotag reads assigned to EPS in the planktonic sample where maximum inorganic CO₂ fixation rate was measured (22 m, 16 January 2013).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Abundance¹ (cells mL⁻¹)</th>
<th>EPS² (%)</th>
<th>¹⁴CO₂-positive EPS³ (%)</th>
<th>EPS-assigned reads (%)⁴</th>
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<tr>
<td>Light</td>
<td>Bacteria</td>
<td>3.4 × 10⁶</td>
<td>85.9</td>
<td>35.2</td>
<td>88.2</td>
</tr>
<tr>
<td></td>
<td>Epsilonproteobacteria</td>
<td>2.9 × 10⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>Bacteria</td>
<td>5.6 × 10⁶</td>
<td>77.8</td>
<td>39.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epsilonproteobacteria</td>
<td>4.3 × 10⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Positive hybridization with either EUB I-II-III or EPSY914 probe, respectively.
² Relative abundance of Epsilonproteobacteria to total Bacteria calculated from CARD-FISH counts.
³ Percentage of Epsilonproteobacteria (positive hybridization with probe EPSY914) that showed deposition of silver granules around the cell periphery with respect to total EPS cell counts.
⁴ Percentage of pyrotags assigned to class Epsilonproteobacteria in the cDNA pyrotag library obtained from the same sample (22 m, 16 January 2013). Data were corrected according to the average number of rRNA operons in genomes of Epsilonproteobacteria (4.8, Klappenbach et al. 2001).
**DISCUSSION**

Sulfidic redoxclines in both marine and freshwater habitats are suitable niches for the growth and activity of chemo- and photolithothrophic sulphur-oxidizing microorganisms, which actively contribute to sulphur and carbon biogeochemical cycles. Several studies have reported the outstanding role of chemolithotrophic, sulphide-oxidizing *Epsilonproteobacteria* in marine pelagic redoxlines 
(Madrid et al. 2001; Lin et al. 2006; Grote et al. 2007, 2008), but less information is available about their presence and contribution to carbon fixation in stratified freshwater lakes. In the current work, we have investigated the diversity, abundance and activity of *Epsilonproteobacteria* in a meromictic basin of Lake Banyoles characterized by a sulphidic redoxcline located at photic depths, a shallow location rarely occurring in marine anoxic basins. Our work provides pieces of evidence about the seasonal dominance of a chemolithotrophic member of the genus *Arcobacter* and raises questions about the actual contribution of chemolithotrophy at the redoxcline and euxinic bottom waters of stratified lakes, which would be of interest according to the expected increase of hypoxic and anoxic zones in aquatic systems due to global change (Diaz and Rosenberg 2008; Wright, Konwar and Hallam 2012).

**Diversity and abundance of freshwater *Epsilonproteobacteria* in basin C-III**

The vertical distribution of *Epsilonproteobacteria* at the redoxcline of basin C-III is similar to that reported for pelagic redoxlines in anoxic marine basins (Lin et al. 2006; Grote et al. 2007, 2008; Glaubitz et al. 2010). Analogously, *Epsilonproteobacteria* were nearby absent in upper oxygenated water layers and reached maximal abundances (ranging from 48 to 71% of total cell counts in winter) at the redoxcline depth. Despite these similarities, the maximum abundance of *Epsilonproteobacteria* in basin C-III was an order of magnitude higher (1.75 and 2.5 × 10^6 cells mL^-1 in samples from winter 2011 and 2012, respectively) compared to maximal abundances obtained by Grote and co-workers in the Baltic Sea (~2.5 and 2 × 10^5 cells mL^-1) (Grote et al. 2007, 2008).

Besides, *Epsilonproteobacteria* were mainly prevalent during winter, when GSB were scarce due to unfavourable light conditions caused by the deeper location of the O2/H2S interface (22 and 19 m in winter and summer, respectively) and the lower irradiance reaching these depths in winter. In late spring and summer, the increase in light intensities reaching sulphide-rich water layers stimulated, however, anoxygenic sulphur phototrophs (Borrego et al. 1997; Borrego, Bañeras and García-Gil 1999).

In anoxic marine basins of the Central Baltic Sea, epsilonproteobacterial communities are dominated by a single population of *Sulfurimonas* subgroup GD17 (Grote et al. 2007, 2008), later described as *Sulfurimonas* gotlandica (Grote et al. 2012; Labrenz et al. 2013). Studies carried out in the Black Sea and Cariaco Basin reported more diverse communities composed of phyotypes related to *Sulfurimonas* spp., *Arcobacter nitrofigilis*, *Thiomicrospira denitrificans* and even free-living relatives of deep-sea polychaetes symbionts (Madrid et al. 2001; Lin et al. 2006; Grote et al. 2008; Glaubitz et al. 2010). Remarkably, in these pelagic redoxlines chemolithoautotrophic planktonic assemblages also comprise gammaproteobacterial sulphur oxidizers of the SUP05 clade (Glaubitz et al. 2009, 2010, 2013). In freshwater lakes, Biderre-Petit et al. (2011) identified *Sulfurimonas* and *Sulfuricurvum* as main sulphide-oxidizing prokaryotes in the low-sulphate Lake Pavín through phylogenetic analysis of 16S rRNA and aprA gene markers. Moreover, conspicuous populations of *Sulfuritalea hydrogenivorans* (*Betaproteobacteria*) have recently been identified as the major planktonic sulphur-oxidizing prokaryote in Lake Mizugaki, a meromictic Japanese lake where epsilonproteobacteria were absent (Kojima et al. 2014). In contrast, the chemolithoautotrophic planktonic assemblage in sulphate-rich, meromictic basin C-III is composed of *Epsilonproteobacteria* sulphur oxidizers whereas no sequences affiliated to gammaproteobacterial SUP05 clade were identified in any of the pyrotag libraries analysed. Besides, the epsilonproteobacterial community was dominated by a phyotype closely related to *Candidatus Arcobacter sulfidicus*, an autotrophic, marine sulphide-oxidizing *Epsilonproteobacteria* (Würsinger et al. 2002; Sievert et al. 2007), whereas other epsilonproteobacterial representatives prevalent in marine habitats such as *Sulfurimonas* spp. and *Sulfuricurvum* spp. were rarely found. Members of the genus *Arcobacter* have been identified in environments such as salt marsh and marine sediments (McClung, Patriquin and Davis 1983; Vandamme et al. 1991; Llobet-Brossa, Rosselló-Mora and Amann 1998), marine bacterioplankton (Eilers et al. 2000; Madrid et al. 2001; Fera et al. 2004), deep-sea vents and associated fauna (Naganuma et al. 1997) and activated sludge (Snaidr et al. 1997) but to our knowledge this is the first report of a non-marine, sulphide-oxidizing member of this genus. In this regard, and considering that hypolimnetic waters of Lake Banyoles are slightly brackish (conductivity ~ 2000–2500 μS cm^-1), the identified *Arcobacter* could not be strictly considered a true freshwater bacterium. Unfortunately, lack of cultured isolates precluded any characterization of its salt requirements for growth. Besides, and according to the in situ fixation measurements and MAR-CARD-FISH observations, this Arcobacter representative would be capable of chemolithoautotrophic growth, probably gaining energy from the oxidation of reduced sulphur compounds similarly to its marine counterpart (Würsinger et al. 2002). No pieces of evidence of filamentous sulphur formation (Würsinger et al. 2002; Sievert et al. 2007) or autotrophic denitrification or chemooorganotrophic growth that are characteristic of other epsilonproteobacterial sulphide oxidizers (Grote et al. 2012; Bruckner et al. 2013; Glaubitz et al. 2014) could be obtained. Notwithstanding this, the identification of a large proportion of denitrification genes associated to order Campylobacterales (*Epsilonproteobacteria*) after metagenomic analyses of samples collected at oxic–anoxic interphases and euxinic water layers of basin C-III (Llorens-Márés et al. 2015) suggest that planktonic *Epsilonproteobacteria* might be able to couple sulphide oxidation to autotrophic denitrification in this habitat.

**Dark carbon fixation by freshwater *Epsilonproteobacteria* in basin C-III**

The cooccurrence of hydrogen sulphide and potential electron acceptors such as oxygen and nitrate at the redoxcline depth offers a suitable niche for the growth and activity of chemolithoautotrophic sulphide oxidizers. Results of the current study provide direct evidence that *Epsilonproteobacteria* contributed the most on the chemolithotrophic carbon uptake at the redoxcline depth of basin C-III. Identification of *Epsilonproteobacteria* as being responsible for dark inorganic carbon fixation rates measured in marine pelagic redoxlines is well documented for the Cariaco Basin (Taylor et al. 2001), the Black Sea (Jørgensen et al. 1991; Sorokin et al. 1995; Glaubitz et al. 2010; Grote et al. 2008), the Mariager Fjord (Zopfi et al. 2001) and the Central
Baltic Sea (Labrenz et al. 2005; Grote et al. 2008; Jost et al. 2008; Bruckner et al. 2013). Previous studies carried out in different lakes and lagoons of the Banyoles Karstic System measured high dark carbon fixation rates at the oxic–anoxic interface and euxinic bottom waters, raising questions about the identity of microbial populations involved in such processes, either photosynthetic bacteria (García-Cantizano et al. 2005; Casamayor, García-Cantizano and Pedrós-Alió 2008) or planktonic thiobacilli (Casamayor 2010). Although centred in basin C-III, results from the current work point to an active contribution of Epsilonproteobacteria to chemolithoautotrophic activity in these systems. Results from MAR-CARD-FISH and phylogenetic analyses of cDNA-based libraries from samples where maximum dark carbon fixation rates were measured clearly identify Epsilonproteobacteria as active members of the planktonic community in uptaking CO₂. Besides, the average number of active epsilonproteobacterial cells (37% of total Epsilonproteobacteria at 22 m depth in winter) was similar to that reported for marine redoxclines in the Baltic and Black seas (Grote et al. 2008). Metagenomic analyses of samples collected at the oxic–anoxic interface and the euxinic monimolimnion of basin C-III identified genes related to carbon fixation via rTCA and sulphide oxidation that affiliated to class Epsilonproteobacteria providing further support to the contribution of this group to C and S cycles (Llorens-Marés et al. 2015).

The phylogenetic analysis of aclB genes retrieved in clone libraries from redoxcline samples clearly indicated that the epsilonproteobacterial community has the potential to carry out the rTCA cycle, the main carbon fixation pathway used by autotrophic Epsilonproteobacteria (Wirsen et al. 2002; Campbell, Stein and Craig Cary 2003; Campbell and Craig Cary 2004; Hügler et al. 2005; Takai et al. 2005). Interestingly, aclB clone sequences from basin C-III were distantly related to aclB from Candidatus Arcobacter sulfidicus but showed close phylogenetic relatedness to aclB from Thio vulum spp. The lack of congruent phylogenies between 16S rRNA and protein-coding genes is a well-known consequence of horizontal gene transfer (HGT) among prokaryotes (Klein et al. 2001; Boucher et al. 2003; Zverlov et al. 2005; Hügler et al. 2007) and might explain the discrepancies found in our study. Particularly, Moussard et al. (2006) also reported similar discrepancies between 16S rRNA and aclB phylogenies in epsilonproteobacterial assemblages dominated by Arcobacter-related phylotypes in hydrothermal vents of the East Pacific Rise. In this regard, further research is needed to identify whether or not HGT is the ultimate cause of the lack of congruency between 16S rRNA and aclB phylogenies or the result of biased amplification of aclB sequences due to the primers used.

### Dark carbon fixation at ecosystem level

Though most of the water column of the whole Lake Banyoles is well oxygenated and dark carbon fixation rates are low, the high rates of this activity occurring at the redoxcline depth and anoxic water layers deserve further attention. Calculations made from data obtained in winter incubations in basin C-III, when highest inorganic dark carbon fixation rates were measured at depths coinciding with the maximum abundance of Epsilonproteobacteria, showed that almost a third (29.2%) of total carbon fixation corresponded to dark fixation, even though these higher rates occurred in a sharp water layer. Comparatively, anoxygenic photosynthesis, which originally could be seen as the main productive process other than oxygenic photosynthesis, accounted only for ≈1% in winter and even less in summer (0.3%), when dark fixation represented a lower, but still important, fraction of total fixed carbon (4.8%). These relative contributions do not only highlight the relative importance of dark CO₂ fixation at the ecosystem level but also pose the question of whether chemolithotrophic metabolisms are a complementary source of organic carbon other than oxygenic/anoxygenic photosynthesis and settled particulate organic matter from upper layers for sustaining anoxic food webs in stratified lakes.

The ecological significance of dark inorganic carbon fixation processes for the carbon balance and the food web of the whole lake are yet to be properly determined. The Epsilonproteobacteria responsible for most of the dark carbon fixation in winter in basin C-III are small cells that could be preyed by specific organisms thriving within the oxic–anoxic interface such as rotifers, ciliates and heterotrophic flagellates (Camacho 2006; Saccá et al. 2009). Regardless of this impact, the energy transfer (as food source) to upper water layers might be limited according to the reduced migration capabilities of potential predators. Thus, the fate of most of the dark primary production at the redoxcline depth would be: (i) to sustain the microbial food web within the redoxcline, and (ii) to provide organic matter to euxinic bottom waters and sediments to fuel anaerobic metabolisms (e.g. anaerobic respiration and fermentation). Yet, the possibility of a certain energy transfer, though small, to upper trophic levels could not be ruled out if some of these bacterial consumers could be preyed by small crustaceans with a higher migratory capacity and these, in turn, could be consumed by fish.

Comparison of the relative contribution of chemolithoautotrophy to the total carbon uptake in different lacustrine and marine ecosystems is misleading due to the lack of data on dark carbon fixation that take into account the system hypsography. Only few reports have evaluated the importance of this process in lakes at the whole ecosystem level (e.g. Camacho et al. 2001; Casamayor, García-Cantizano and Pedrós-Alió 2008; Casamayor et al. 2011). In most of these cases, the contribution of dark carbon fixation process is from low to medium in percentage of the inorganic carbon fixed within the whole lake although actual dark carbon fixation rates at the redoxcline can be extraordinarily high and could far multiply those of oxygenic photosynthesis in the epilimnion (Camacho and Vicente 1998; Wetzel 2001). Compared to marine or coastal environments, actual rates of dark carbon fixation reported for stratified lakes are far higher (Table 2). Overall, the major relative contribution of dark fixation to total inorganic carbon fixation in stratified lakes has been found in small, doline type, sulphide-rich lakes, like lakes Arcas, Cisó and Vilarr (Table 2), where anoxygenic photosynthesis or chemolithotrophic metabolisms can even dominate over oxygenic photosynthesis. High relative contribution of dark CO₂ fixation processes has also been reported for bigger lakes with euxinic bottom waters, such as Lake Cadagno (Camacho et al. 2001) or basin C-III in Lake Banyoles (this work), where abundant and active populations of chemolithoautotrophic sulphide oxidizers accumulate taking advantage of the simultaneous presence of both oxidized and reduced compounds at the oxic–anoxic transition zone (i.e. the redoxcline). Besides, the measurement of high rates of dark inorganic carbon fixation in sulphide-poor environments such as Lake La Cruz and Lake El Tobar (Casamayor et al. 2011) poses the question of whether this activity is fuelled by the trace amounts of sulphide available or if it is carried out by microorganisms not involved in the sulphur cycle such as ferro lithotrophic nitrate reducers (Walter et al. 2014). Though not totally well known, the processes involved in dark inorganic carbon fixation at redoxclines of stratified lakes are, with studies like ours, well documented and explained, although the relatively high rates of dark
Table 2. Maximum dark carbon fixation rates measured at the O₂/H₂S interface (i.e. redoxcline) of different marine and freshwater environments.

<table>
<thead>
<tr>
<th>System</th>
<th>Depth (m)</th>
<th>Max. depth (m)</th>
<th>Maximum dark CO₂ fixation rate (µg CO₂ L⁻¹ h⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine basins</td>
<td></td>
<td></td>
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<tr>
<td>Black Sea</td>
<td>115</td>
<td>2,212</td>
<td>0.3</td>
<td>Grote et al. (2008)</td>
</tr>
<tr>
<td>Baltic Sea</td>
<td>142</td>
<td>459</td>
<td>1.1</td>
<td>Grote et al. (2008)</td>
</tr>
<tr>
<td>Cariaco Basin</td>
<td>350</td>
<td>1,400</td>
<td>1.3</td>
<td>Taylor et al. (2001)</td>
</tr>
<tr>
<td>Mariager Fjord</td>
<td>13.5</td>
<td>30</td>
<td>43.2</td>
<td>Zopfi et al. (2001)</td>
</tr>
<tr>
<td>Karstic lakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisó</td>
<td>0-0.2</td>
<td>6.5</td>
<td>12.4</td>
<td>Casamayor et al. (2008)</td>
</tr>
<tr>
<td>Vilar</td>
<td>5.5</td>
<td>9</td>
<td>22.8</td>
<td>Casamayor et al. (2008)</td>
</tr>
<tr>
<td>Estanya</td>
<td>12</td>
<td>22</td>
<td>17.6</td>
<td>Casamayor et al. (2008)</td>
</tr>
<tr>
<td>Arcas</td>
<td>8.3</td>
<td>14.2</td>
<td>41.2</td>
<td>Camacho and Vicente (1998)</td>
</tr>
<tr>
<td>Cadagno</td>
<td>11.4</td>
<td>21</td>
<td>94.1</td>
<td>Camacho et al. (2001)</td>
</tr>
<tr>
<td>Lagunillo del Tejo</td>
<td>5.5</td>
<td>6</td>
<td>1.8</td>
<td>Casamayor et al. (2011)</td>
</tr>
<tr>
<td>La Cruz</td>
<td>19.5</td>
<td>20</td>
<td>23</td>
<td>Casamayor et al. (2011)</td>
</tr>
<tr>
<td>El Tobar</td>
<td>13</td>
<td>18</td>
<td>18.1</td>
<td>Casamayor et al. (2011)</td>
</tr>
<tr>
<td>Banyoles Basin C-III</td>
<td>22</td>
<td>32</td>
<td>4.9</td>
<td>This study</td>
</tr>
<tr>
<td>Coastal lagoon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>La Massona</td>
<td>5.5</td>
<td>10.5</td>
<td>0.6</td>
<td>Casamayor et al. (2008)</td>
</tr>
</tbody>
</table>

inorganic carbon fixation measured in deep anoxic layers—with very low redox potentials and paucity of oxidized substances—deserve further investigation.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSEC online.

**ACKNOWLEDGEMENTS**

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**Conflict of interest.** None declared.

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Evidence for autotrophic CO$_2$ fixation among planktonic microbial guilds


