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RESEARCH ARTICLE

Active bacteria and archaea cells fixing bicarbonate in the dark along the water column of a stratified eutrophic lagoon

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Abstract

We studied the carbon dioxide fixation activity in a stratified hypereutrophic karstic lagoon using a combination of fingerprinting techniques targeting bacterial and archaeal 16S rRNA genes, functional gene cloning [the acetyl-CoA carboxylase (*accC*)], and isotopic labelling (^{14}C-bicarbonate) coupled to single-cell analyses [microautoradiography combined with catalyzed reported deposition-FISH (MAR-CARD-FISH)]. The microbial planktonic community was dominated by bacteria with maximal abundances of archaea just below the oxic/anoxic transition zone (7% of total cells). *In situ* incubations with radiolabelled bicarbonate showed maximal photoassimilation activity in the oxic epilimnion, whereas dark CO_2 fixation was consistently observed throughout the water column, with a maximum at the oxic/anoxic interface (8.6 mg C m^{-3} h^{-1}). The contributions of light and dark carbon fixation activities in the whole water column were 69% and 31% of the total C incorporated, respectively. MAR-CARD-FISH incubations corroborated these results and revealed that the highest fraction of bacterial and archaeal cells actively uptaking bicarbonate in the light was found at the surface. The bacterial community was mainly composed of green sulfur bacteria (*Chlorobi*) and members of the *Betaproteobacteria* and the *Bacteroidetes*. The archaeal assemblage was composed of phylotypes of the *Miscellaneous Crenarchaeotic Group* and a few methanogens. Clone libraries of the *accC* gene showed an absolute dominance of bacterial carboxylases. Our results suggest that the dark carbon fixation activity measured was mainly related to CO_2 incorporation by heterotrophs rather than to the activity of true chemoautotrophs.

Introduction

Inorganic carbon photoassimilation has traditionally been considered the dominant fixation process in planktonic environments. However, high dark CO_2 fixation rates measured in several stratified lakes with sharp oxic/anoxic interfaces have called this assumption into question (Culver & Brunskill, 1969; Jorgensen *et al.*, 1979; Cloern *et al.*, 1983; Pedrós-Alió & Guerrero, 1991; Shively *et al.*, 1998; Camacho *et al.*, 2001; Hadas *et al.*, 2001; Casamayor *et al.*, 2008; Casamayor, 2010). In some of these habitats, the values of dark fixation rates integrated for the entire water column were equivalent to those calculated for light-driven processes, indicating that dark assimilation could not be easily disregarded. Besides, the complexity of the microbial assemblages thriving in environments with oxic/anoxic interfaces and the availability of different energy sources and electron donors favor the presence of a large diversity of microorganisms involved in chemoautotrophic activities (Casamayor, 2010).

In the last decade, several studies have provided detailed analyses of dark CO_2 assimilation by distinct microbial guilds in stratified lakes (Camacho *et al.*, 2001; García-Cantizano *et al.*, 2005; Casamayor *et al.*, 2008; Casamayor, 2010), but none have focused on the potential contribution of planktonic archaea in the process. This is of special

interest considering the general distribution and abundance of archaea in aquatic ecosystems (e.g. DeLong, 1992, 1998; DeLong & Pace, 2001; Robertson *et al.*, 2005; Schleper *et al.*, 2005; Chaban *et al.*, 2006; Auguet *et al.*, 2010) and the particular high richness of archaeal taxa in anoxic waters of stratified lakes (Llirós *et al.*, 2008, 2010). To date, archaeal carbon fixation has mainly been studied in pure cultures of extremophiles, most of which have been in relation to sulfur metabolism (Kletzin *et al.*, 2004; Berg *et al.*, 2010) and methanogens (Jones *et al.*, 1987; Simpson & Whitman, 1993). Very recently, the incorporation of inorganic carbon by ammonia-oxidizing archaea (AOA) has been confirmed both in the laboratory (Könneke *et al.*, 2005; Martens-Habbena *et al.*, 2009) and *in situ* (Wuchter *et al.*, 2003; Herndl *et al.*, 2005; Ingalls *et al.*, 2006; Varela *et al.*, 2008). Besides, isotopic analyses of membrane lipids from marine AOA have provided additional evidence of their ability to incorporate inorganic carbon into their cell structures (Schouten *et al.*, 2000, 2008; Sinninghe Damste *et al.*, 2002). Moreover, the discovery of a new biosynthetic route for the assimilation of CO_2 by autotrophic crenarchaeota – the 3-hydroxypropionate/4-hydroxybutyrate cycle – has provided a metabolic framework to explain how these carboxylation reactions take place (Berg *et al.*, 2007, 2010; Walker *et al.*, 2010). In this regard, the identification of acetyl-CoA/propionyl CoA carboxylase (*accC*) as a key enzyme in this pathway allowed the design of new molecular markers to trace the potential autotrophic activity of mesophilic crenarchaeota in environmental samples (Auguet *et al.*, 2008; Yakimov *et al.*, 2009). Thus, planktonic archaea may play a relevant role linking the sulfur, nitrogen, and carbon cycling in aquatic environments. The lack of cultivated representatives for most of the ubiquitous archaeal lineages in aquatic and terrestrial environments (Könneke *et al.*, 2005) hinders the proper interpretation of activity measurements under an ecophysiological and biogeochemical framework.

In the present work, we described the structure of the microbial planktonic community in an ammonium- and sulfide-rich stratified karstic lagoon, and attempted to ascertain whether or not planktonic archaea were able to incorporate inorganic carbon in the dark and how they contributed to the overall dark fixation processes along the water column. An experimental approach combining 16S rRNA gene fingerprinting [denaturing gradient gel electrophoresis (DGGE)], isotopic labelling (^{14}C) coupled to single-cell analyses [microautoradiography combined with catalyzed reported deposition-FISH (MAR-CARD-FISH)], and cloning the acetyl-CoA carboxylase gene was applied to samples collected at depths selected along the vertical physico-chemical gradient to represent the fully oxic epilimnion, the oxic/anoxic interface, and the sulfide-rich, anoxic hypolimnion.

Materials and methods

Study site and sample collection

Sampling was carried out in the Coromina lagoon, (42°8′N, 2°45′E) located in the karstic lacustrine system of Banyoles in the Northeast of Spain, on May 2007. The lagoon is round-shaped and protected against wind due to its sharp shore slope ($> 50°$) and surrounding vegetation. Similar to other water bodies of the area, the lagoon develops a thermal stratification during late spring-summer and it has been classified as holomictic (Borrego & Garcia-Gil, 1994). The lagoon receives high external inputs of organic matter from adjacent crops and a cattle farm that heavily affect its trophic status (the maximal reported values of ammonia and total phosphorous at the hypolimnion are of 60 and 3.5 mg L^{-1}, respectively; Borrego & Garcia-Gil, 1994). Blooms of photosynthetic microbial populations composed both by green algae (e.g. *Chlamydomonas* sp. and *Chlorella* sp.) and by anoxygenic phototrophic sulfur bacteria (both purple- and green-colored species) have consistently been reported (Borrego & Garcia-Gil, 1994; Gich *et al.*, 2001).

Depth profiles for water temperature, conductivity, pH, redox potential (E_H), and oxygen concentration were determined *in situ* using a multiparametric probe YSI-556MPS (Yellow Spring Instruments, OH). Water samples for biological and chemical analyses were selected along the vertical physico-chemical gradient to represent the fully oxic epilimnion, the oxic/anoxic interface, and the sulfide-rich, anoxic hypolimnion. Samples were then collected from different depths in 1-L sterile glass bottles using a weighted double cone device designed to minimize disruption of the vertical water stratification (Jorgensen *et al.*, 1979). The double cone is connected to a battery-powered pump by a 10-m calibrated plastic hose. This system allows the cone to be placed at the selected depth and water to be pumped to the surface, where it is collected. On the boat, water samples were kept on ice and protected from light in a portable icebox until further analysis within 24 h. For sulfide analysis, 10 mL of water was collected in sterile screw-capped glass tubes and fixed *in situ* by adding zinc acetate (0.1 M final concentration) under alkaline conditions (NaOH, 0.1 M final concentration).

Chemical and pigment analyses

The sulfide concentration was measured using the leucomethylene blue method (Brock *et al.*, 1971). Dissolved organic carbon (DOC) and humic substance concentrations were estimated from optical absorbance at 250 and 365 nm of 0.2-μm-filtrated water samples as described previously (Jezberova *et al.*, 2010). The ratio $A_{250\,nm}/A_{365\,nm}$ was used as a proxy for DOC quality because this ratio is related to the

proportion of small molecules in the DOC pool (De Haan, 1972).

For pigment analyses, 500 mL of water samples collected at selected depths were passed through 0.45 mm pore-size membrane filters (47 mm filter diameter) previously covered with a thin layer of 2.5% $MgCO_3$ (Guerrero *et al.*, 1985). Cells retained on the filters were scrapped using a sterile spatula and transferred to light-preserved, screw-capped glass tubes containing the extraction solvent (acetone : methanol 7 : 2 v/v, HPLC grade, Scharlau, Germany). Pigments were extracted after a mild sonication in the dark at 4 °C for 30 s (B.Braun-Labsonic 2000, B.Braun, Germany) and stored at −30 °C for 24 h. HPLC analyses were performed as described previously (Borrego & Garcia-Gil, 1995).

Biological uptake of inorganic carbon

The bulk uptake of inorganic carbon was measured in 70-mL plastic flasks fully filled to minimize aeration in hypolimnetic samples. Samples were spiked with radiolabelled bicarbonate ($NaH^{14}CO_3$; specific activity 4 μCi mL^{-1}; DHI, Denmark) at a final concentration of 0.15–2.0 μCi mL^{-1}. The incubation lasted 4 h (noon period) under *in situ* light conditions. The incubation set included two clear (light) and two dark incubation flasks and one additional formaldehyde-killed flask as a control. After incubation, water samples were immediately fixed with formaldehyde (final concentration 3.7%) to stop microbial activity (Camacho & 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 radioactivity was measured in a Beckton-Dickinson LS6000 scintillation counter (Beckman). 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 chemosynthetic incorporation was calculated by subtracting d.p.m. measured in the killed control from d.p.m. measured in 'dark' flasks (Pedrós-Alió *et al.*, 1993). Almost identical results were obtained from duplicate subsamples and the results are presented as mean values.

CARD-FISH

The abundance of different prokaryotic groups was analyzed by CARD-FISH (Pernthaler *et al.*, 2002) using horseradish peroxidase-labelled oligonucleotide probes targeting members of the Domain *Bacteria* and *Archaea* (Table 1). Because biases of the archaeal probe ARC915 unspecifically hybridizing with some members of the *Bacteroidetes* – formerly known as *Cytophaga–Flavobacteria–Bacteroides* cluster – have been reported by several authors (Battin *et al.*, 2001; Klammer *et al.*, 2002; Amann & Fuchs, 2008), the abundance of members of the *Bacteroidetes* was measured in parallel samples using the specific probe CF319a (Manz *et al.*, 1996)

Table 1. Oligonucleotides sequences for the PCR primers and probes used in this study

Primer/probe name	Target	Sequence (5′–3′)	Source
16S rRNA gene primers			
341f	*Bacteria*	CCTACgggAggCAgCAg	Muyzer & Ramsing (1995)
907r	*Bacteria*	CCgTCAATTCCTTTgAgTTT	Muyzer & Ramsing (1995)
Arch21F	*Archaea*	TTCCggTTgATCCYgCCggA	DeLong (1992)
Arch958R	*Archaea*	YCCggCgTTgAMTCCAATT	DeLong (1992)
ARC344f	*Archaea*	ACggggYgCAgCAggCgCgA	Casamayor *et al.* (2001)
ARC337f	*Crenarchaeota**	ATgggCACTgAgACAAgg	Llirós *et al.* (2008)
ARC915r	*Archaea*	gTgCTCCCCCgCCAATTCCT	Raskin *et al.* (1994)
accC gene primers			
ACAC254f	*accC*	gCTgATgCTATACATCCWggWTAYgg	Auguet *et al.* (2008)
ACAC720r	*accC*	gCTggAgATggAgCYTCYTCWATA	Auguet *et al.* (2008)
AccAF573	*accC*	gTTYgTYACDggDCCYgAYG	Yakimov *et al.* (2009)
AccAR279	*accC*	TgATRTRRTCCATRCAHTCRTA	Yakimov *et al.* (2009)
Probes			
EUB338-I†	Most *Bacteria*	gCTgCCTCCCgTAggAgT	Amann *et al.* (1990)
EUB338-II†	*Planctomycetales*	gCAgCCACCCgTAggTgT	Daims *et al.* (1999)
EUB338-III†	*Verrucomicrobia*	gCAgCCACCCgTAggTgT	Daims *et al.* (1999)
ARC915	*Archaea*	gTgCTCCCCCgCCAATTCCT	Raskin *et al.* (1994)
CF319a	*Bacteroidetes*	TggTCCgTgTCTCAgTAC	Manz *et al.* (1996)

*In nested PCR, this primer is biased towards lacustrine *Crenarchaeota*, but a few euryarchaeotal phylotypes can also be unspecifically amplified (Llirós *et al.*, 2008).

†EUB338 mix = UB338-I, -II and -III.

to detect double hybridizations and correct false positives in archaeal counts. Hybridizations were performed overnight at 35 °C and by adding 55% formamide to hybridization buffers. Filter sections were counter-stained with 4′-6-diamidino-2-phenylindole (DAPI) (1 μg mL^{-1}). Between 250 and 600 DAPI-stained cells were counted in 10 randomly selected microscopic fields using an Axioskop epifluorescence microscope (Zeiss, Germany).

MAR-CARD-FISH

MAR-CARD-FISH was carried out as described previously (Alonso & Pernthaler, 2005). Briefly, between 0.1 and 2.0 mL water subsamples from $NaH^{14}CO_3$ uptake incubations were passed through 0.22 μm pore-size, 25 mm diameter white polycarbonate filters (Millipore, Germany) at a low vacuum pressure. Filters were then hybridized following the CARD-FISH protocol described above and glued on glass slides using epoxy adhesive (UHU plus, UHU GmbH, Germany). For autoradiography, slides were embedded in 46 °C tempered photographic emulsion (KODAK NTB-2) containing 0.1% low-gelling-point agarose in a darkroom. The slides were then placed on black boxes containing a drying agent and incubated at 4 °C until development. The optimal exposure time was determined for each sample and resulted in 14 and 24 days (data not shown). Exposed slides were developed and fixed following the manufacturer's specifications, i.e. a 3-min immersion in photographic commercial developer (KODAK D19; 1 : 1 dilution with Milli-Q water), 30-s rinsing in Milli-Q water, 3 min of emulsion fixation (KODAK T_{max}; 1 : 4 dilution with Milli-Q water), and two final 30-s rinsing steps with distilled and tap water. Afterwards, the slides were dried in a desiccator overnight, counter-stained 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. Cells showing positive hybridization by the bacterial and archaeal probes and surrounded by silver grains are named from now on as EUB+M+ and ARC+M+, respectively.

DNA extraction, PCR, and DGGE fingerprinting

DNA extraction was carried out from water samples as described previously (Llirós *et al.*, 2008). Extraction of DNA from the sediment was carried out on 10 g of fresh sample using the PowerMax™ Soil DNA Extraction Kit (MoBio Laboratories Inc.) according to the manufacturer's instructions. Amplifications of bacterial and archaeal 16S rRNA gene fragments were carried out using a universal primer combinations for Bacteria and Archaea as described by Casamayor *et al.* (2000) (Table 1). To increase the resolution for the archaeal community, nested-PCR reactions using the primer combination ARC337F-ARC915R (Llirós *et al.*, 2008) were run on amplicons obtained with the universal primer pair Arch21F-Arch958R (DeLong, 1992). Nested products of the expected size (~578 bp) were obtained from all samples analyzed. DGGE for Bacteria and Archaea were run as described previously (Casamayor *et al.*, 2000; Llirós *et al.*, 2008). A DGGE ladder composed by a mixture of known SSU rRNA gene fragments was loaded in all gels to allow intergel comparison of band migration. After the run, gels were stained for 30 min with 1 × SYBR Gold nucleic acid stain (Molecular Probes Inc.) in 1 × TAE buffer, rinsed, and visualized under UV radiation using a GelPrinter system (TDI, Spain). Individual DNA bands were excised using a sterile scalpel and rehydrated overnight in 50 μL of Tris-HCl 10 mM buffer (pH = 7.4). DNA was eluted after incubation at 65 °C for 3 h and reamplified using the corresponding primer pairs (without GC clamp) and sizing down the number of PCR cycles up to 20. Reamplified products were further purified and sequenced on both strands by an external company (Macrogen Inc., Seoul, Korea).

Phylogenetic analysis

Bacterial and archaeal 16S rRNA gene sequences obtained from excised DGGE bands were analyzed for the presence of chimeras using BELLEROPHON (Huber *et al.*, 2004). Consensus sequences were obtained after the alignment of forward and reverse strands using BIOEDIT (Hall, 1999) and then aligned in MOTHUR (http://www.mothur.org; Schloss *et al.*, 2009) using the SILVA bacterial and archaeal reference alignments provided by the MOTHUR project. Neighbor-joining (NJ) (Saitou & Nei, 1987) distance matrices were calculated by MOTHUR using the Jukes–Cantor (JC) correction and used to assign sequences to operational taxonomic units (OTUs) defined at the 97% cutoff using the furthest-neighbor algorithm. Representative sequences for each OTU were identified using the implemented tool in MOTHUR.

Bacterial and archaeal phylogenetic trees were constructed after importing MOTHUR alignments into the ARB software package (Ludwig *et al.*, 2004) loaded with the SILVA 16S rRNA gene-ARB-compatible database (SSURef-102, February 2010) and checked manually for errors. Bacterial and archaeal backbone trees were built with reference sequences of at least 900 bp in length using the NJ algorithm and JC-corrected distances. The aligned bacterial and archaeal sequences were then added to the corresponding trees using the parsimony 'quick add marked tool', thereby maintaining the overall tree topology. Bootstrap support (1000 replicates) was calculated in PHYLIP (Felsenstein, 2007) using JC evolutionary distances and the NJ method. The archaeal cluster names and grouping used in this work were based on widely used cluster definitions for *Euryarchaeota* and *Crenarchaeota* (Takai *et al.*, 2001;

Inagaki *et al.*, 2003) and recently reviewed by Teske & Sorensen (2008).

Clone libraries of acetyl-CoA carboxylase (*accC*) genes

Genes for the biotin carboxylase subunit (*accC*) of the acetyl-CoA carboxylase enzyme (ACCase) were amplified using two primer combinations: ACAC-254F/-720R (Auguet *et al.*, 2008) and AccA-F573/-R279 (Yakimov *et al.*, 2009) (Table 1). Although originally designed to selectively target archaeal *accC* genes, the application of the primer pair ACAC-254F/-720R to environmental samples resulted in unspecific amplification of bacterial *accC* gene and genes coding for related enzymes (propionyl and pyruvate carboxylases and carbamoyl-phosphate synthases) (Auguet *et al.*, 2008; Alonso-Saez *et al.*, 2010). In turn, the primer pair AccA-F573/-R279 seems to be highly specific for autotrophic AOA (Yakimov *et al.*, 2009) and was used to increase the resolution on the detection of these microorganisms if present.

DNA extracts from the oxic epilimnion (a mixture of equal volumes of extracts from 0 and 1 m depths), the oxic/anoxic interphase (2 m depth), the anoxic hypolimnion (4 m depth), and a sediment sample were used as templates for PCR reactions targeting the *accC* gene as described previously (Auguet *et al.*, 2008; Yakimov *et al.*, 2009). PCR products (50 μL) were purified using the QIAGEN PCR purification kit and cloned using the TOPO-TA cloning kit as described previously (Eiler & Bertilsson, 2004). For each sample, 50 clones were picked and checked for the presence of the correct insert, purified, and sequenced on both strands by an external company (Macrogen Inc.). The resulting sequences were checked for chimeras using BELLEROPHON (Huber *et al.*, 2004) and aligned and translated to the predicted protein sequences using BIOEDIT (Hall, 1999). Functional gene annotation of the final set of sequences (155) was carried out using BLAST2GO software (http://www.blast2go.org; Conesa *et al.*, 2005).

Nucleotide sequence accession numbers

Archaeal and bacterial 16S rRNA gene sequences were deposited in GenBank under accession numbers HQ890472–HQ890486 and HQ890487–HQ890502, respectively. Carboxylase clone sequences were deposited under accession numbers HQ901203–HQ901357.

Results

Physico-chemical profiles

The lagoon was thermally stratified showing three water compartments defined by the physico-chemical vertical gradient: an oxic epilimnion ranging from the surface to 2 m depth, an oxic/anoxic transition zone (metalimnion) from 2 to 2.25 m depth, and an anoxic hypolimnion from this latter depth to the bottom (Fig. 1a). Oxygen concentrations decreased from *c.* 17 mg L^{-1} at the surface to extinction at 2.25 m depth, with a noticeable oxygen peak at 1 m depth (17.2 mg L^{-1}). Sulfide increased with depth, reaching values of 381 μM at the sediment surface (4.75 m), with the maximum values (*c.* 450 μM) at 4 m. The slight increase in conductivity with depth was due to the accumulation of reduced compounds that created a mild biogenic meromixis (Borrego & Garcia-Gil, 1994). $A_{250\,nm}$ indicated a high concentration of dissolved humic material ($A_{250\,nm}$ of 0.49 and 0.23 at 1.25 m and 4 m depth, respectively). The $A_{250\,nm}/A_{365\,nm}$ ratio at 1.25 and 4 m depth (11.3 and 6.2, respectively) indicated a DOC pool rich in labile molecules (De Haan, 1972).

Vertical distribution of photosynthetic microbial populations

Different planktonic populations of oxygenic and anoxygenic photosynthetic microorganisms were identified along the water column, showing a distinct distribution along the vertical gradient according to their physiological requirements (Fig. 1b). High chlorophyll *a* (Chl *a*) concentrations were measured in the oxic epilimnion, reaching maximal values (175 μg L^{-1}) at the upper layer of the oxic/anoxic transition (2 m depth). Microscopical observations revealed a conspicuous abundance of *Cryptomonas* spp. (mainly *Cryptomonas phaseolus*). This species has consistently been found in other stratified, sulfide-rich lakes forming dense populations at the oxic/anoxic interface (Gasol *et al.*, 1993; Camacho & Vicente, 1998; García-Cantizano *et al.*, 2005). The comparison of the vertical profiles of Chl *a* and dissolved oxygen concentrations indicated a high photosynthetic activity in epilimnetic waters. Anoxic conditions and the accumulation of sulfide below 2.25 m depth favored the growth of anoxygenic green and purple sulfur bacterial populations. In this regard, the presence of green-colored three-dimensional nets composed by cells with ternary fission resembling those of *Chlorobium clathratiforme* (formerly *Pelodictyon clathratiforme*) were easily distinguished on the microscope. This branching species of green sulfur bacteria (GSB) contains bacteriochlorophyll (BChl) *c* and *d* (Overmann, 2006) and it has repeatedly been found as the dominant species of GSB in the lagoon (Borrego & Garcia-Gil, 1994; Gich *et al.*, 2001). In this study, *Chl. clathratiforme* constituted a dense population as indicated by the high concentrations of their signature pigments (970 μg L^{-1} of BChl *c*+*d*, Fig. 1b) measured at the metalimnion and the intensity of bands assigned to phylum *Chlorobi* in bacterial 16S rRNA gene fingerprints (bands B13 and B10 in

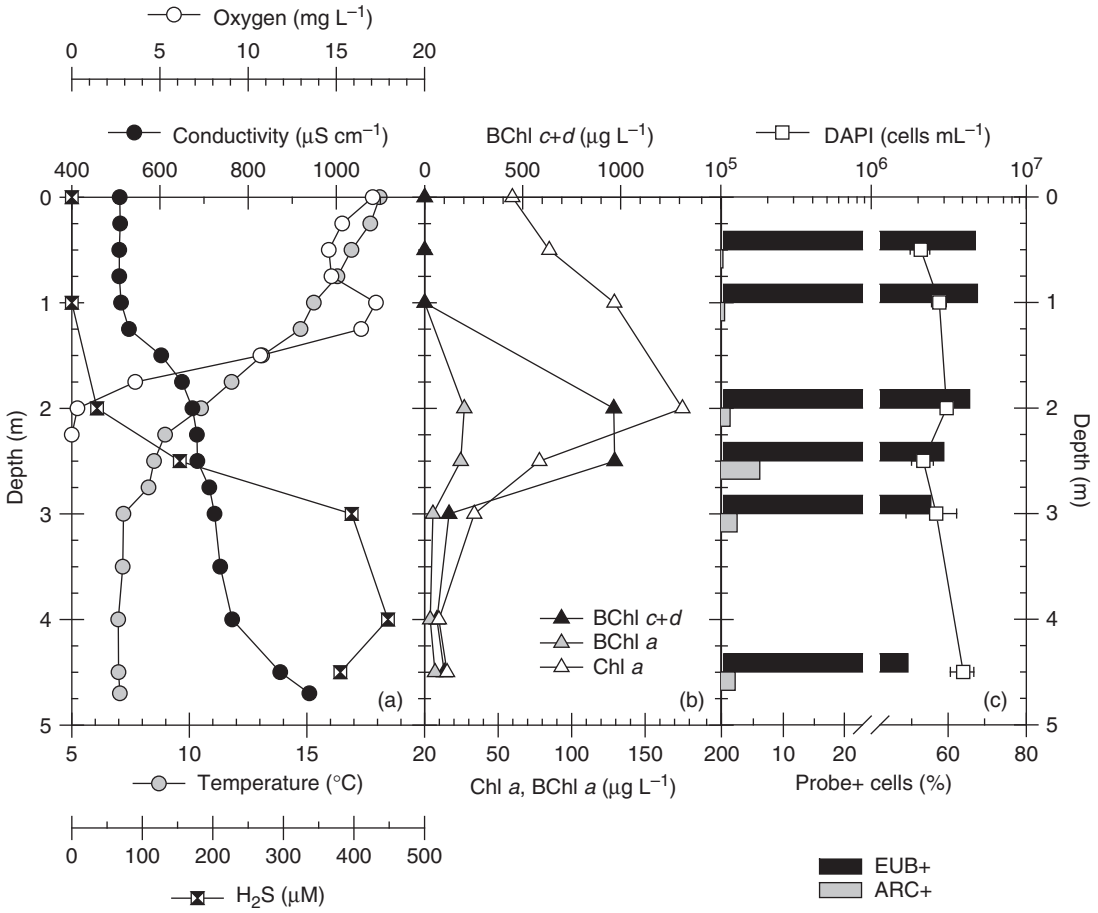

Fig. 1. Depth profiles of main physico-chemical variables (a), photosynthetic pigments (b), and total cells (DAPI staining) and the relative abundances of bacterial and archaeal cells targeted by specific CARD-FISH probes (c) in Coromina lagoon on the day of sampling.

Supporting Information, Fig. S1). In turn, purple sulfur bacteria were rarely observed on the microscope, agreeing both with the low BChl *a* concentrations measured (27 µg L^{-1}, Fig. 1b) and with previous studies (Gich *et al.*, 2001).

Abundance of planktonic prokaryotes

The total abundances (DAPI-stained cells) ranged from $2.1 \pm 0.3 \times 10^6$ to $4.0 \pm 0.7 \times 10^6$ cells mL^{-1}, but no consistent differences were observed along the vertical profile (Fig. 1c). CARD-FISH counts indicated the dominance of bacteria over archaea (averaged relative contributions of 60.7% and 2.3% of DAPI cell counts, respectively) and a different vertical distribution pattern for both groups. While Bacteria had a homogeneous distribution along the water column with a slight decrease in concentration with depth, archaeal cells were practically absent from the oxygenic epilimnion and showed maximal abundances just below the oxic/anoxic transition (7% of the total DAPI counts at 2.5 m depth, Fig. 1c). To correct potential biases on archaeal counts due to unspecific hybridization of probe ARC915 to cells of *Bacteroidetes* (Battin *et al.*, 2001; Klammer *et al.*, 2002; Amann & Fuchs, 2008), additional hybridization tests using probes ARC915 and CF319a were carried out in parallel samples. No correlations were observed for the estimated abundance of Archaea and *Bacteroidetes* under the hybridization conditions used (55% formamide). Further tests using double hybridizations did not detect cross-interference by both probes ARC915 and CF319a. Accordingly, ARC915 counts were considered valid archaeal cell estimations. However, a high unspecific hybridization of probe ARC915 with CF319a-positive cells was observed after reducing hybridization stringency (formamide concentration $< 55\%$, data not shown), indicating a strong dependence of probe ARC915 specificity on formamide concentration.

Phylogenetic identification of the dominant microbial populations

A total of 16 sequences of bacterial 16S rRNA gene were retrieved from DGGE fingerprints (Fig. S1). These sequences were grouped into 13 OTUs (defined at 97% cutoff,

Table S1) that affiliated to *Alphaproteobacteria* (OTU-5), *Betaproteobacteria* (OTU-3, -4, -8, -9, and -13), *Bacteroidetes* (OTU-2 and -12), *Chlorobi* (OTU-7 and -10), high G+C Gram-positive bacteria (i.e. *Actinobacteria*; OTU-11), and low G+C Gram-positive bacteria (i.e. *Firmicutes*; OTU-6). The remaining three sequences were recovered from the oxic epilimnion and grouped into a single OTU affiliated with plastids from diatoms (OTU-1 in Fig. 2 and bands b1, b4, and b5 in Fig. S1).

Fingerprinting of archaeal 16S rRNA gene fragments using the general primer pair ARC344f-ARC915r only retrieved four sequences from water samples collected at the anoxic hypolimnion. All these sequences were assigned to OTUs affiliated to methanogenic euryarchaeota from the *Methanosaeta* cluster (OTU-1 and OTU-2, Fig. 3 and Table S2) and *Methanobacteriales* (A5, OTU-3). Considering the low archaeal richness detected using this general primer pair, a second primer combination (ARC337f-ARC915r) was applied to detect archaeal phylotypes potentially overlooked by the primer pair ARC344F-ARC915R. When used in nested reactions, the primer pair ARC337f-ARC915r is biased towards lacustrine crenarchaeota and it considerably improves the recovery of crenarchaeota phylotypes as described previously for the neighboring Lake Vilar (Llirós *et al.*, 2008). Using this primer pair, 11 additional sequences were recovered (Fig. S1). These sequences grouped into six archaeal OTUs (97% cutoff, Table S2) affiliated to crenarchaeal lineages such as the *Soil Group 1.1b* (OTU-6) and the *Miscellaneous Crenarchaeotic Group* (MCG, OTU-8 and -9) and to the euryarchaeal *Miscellaneous Euryarchaeotic Group* (MEG, OTU-4 and -5) and *Thermoplasmatales* (OTU-7) (Fig. 3 and Table S2).

Carbon dioxide incorporation by the planktonic prokaryotic community

Samples from each water compartment (oxic epilimnion, oxic/anoxic interface, and anoxic hypolimnion) were collected to study the vertical distribution of bulk inorganic carbon incorporation under light and dark incubation conditions. Incubations carried out in the epilimnion showed the maximal contribution of photosynthetic

Fig. 2. NJ phylogenetic tree generated by the ARB software package showing the affiliation of the bacterial 16S rRNA gene sequences retrieved from the lagoon (in bold) and grouped into OTUs (only the representative sequence for each OTU is shown; see Table S1 for details). Bootstrap support values > 50% (1000 replicates) are shown. The scale bar indicates 10% estimated sequence divergence. The OTU number corresponds to the identification number shown in Table S1. Sequences named following the code used in DGGE fingerprints (Fig. S1).

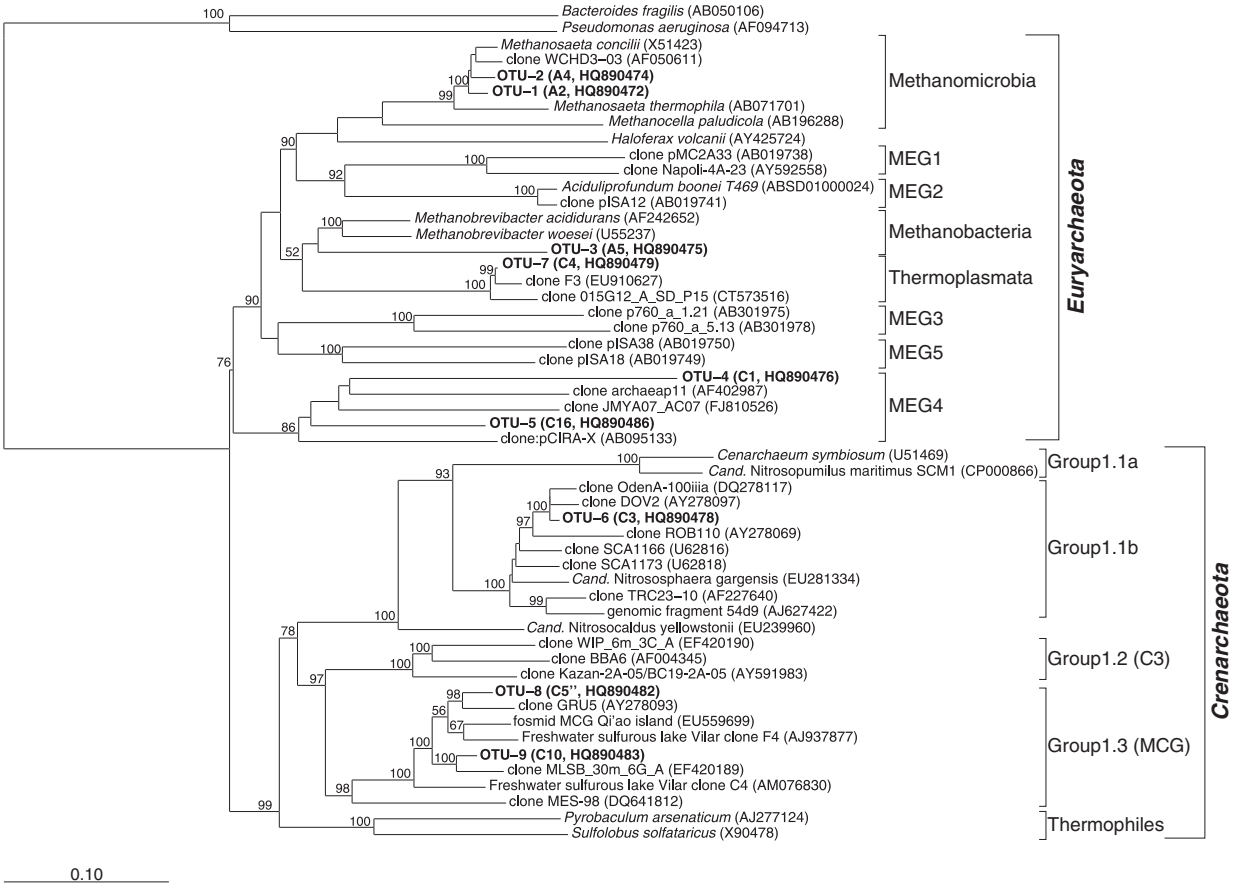

Fig. 3. Same as Fig. 2, but for archaeal 16S rRNA gene sequences. OTUs ID, OTUs members, and their representative sequences were obtained with MOTHUR (see Materials and methods) and compiled in Table S2. MEG, *Miscellaneous Euryarchaeotic Group* (Takai *et al*., 2001); MCG, *Miscellaneous Crenarchaeotic Group* (Inagaki *et al*., 2003).

Table 2. Bulk carbon incorporation by the planktonic microbial community under light and dark conditions and % labelled cells in MAR-CARD-FISH analyses

Depth (m)	Bulk C incorporation (mg C m^{-3} h^{-1})		^{14}C-labelled cells (%)*			
			Bacteria		Archaea	
	Light	Dark	Light	Dark	Light	Dark
0.5	61.0	4.4	13.8	7.5	11.9	2.4
1.0	40.8	3.8	8.9	6.6	0.0	8.3
2.0	0.0	8.6	0.0	12.6	0.0	4.2
2.5	–	3.6	–	4.4	–	1.1
3.0	–	3.8	–	4.4	–	1.2
4.5	–	6.1	–	4.2	–	3.7

*Probe+MAR+cells in relation to probe+cells for each treatment.

processes (94% and 91.5% of the total C incorporation at 0 and 1 m depth, respectively) (Table 2). Below 1 m depth, carbon incorporation measured in 'clear' flasks was negligible because of the strong shading caused by the algal population thriving above (integrated concentration of Chl *a* between 0 and 1 m depth of 1021 mg m^{-2}) (Fig. 1). In turn, bulk C fixation in the dark was measured along the vertical profile with a consistent maximum (8.6 mg C m^{-3} h^{-1}) at the oxic/anoxic interface (2 m depth, Table 2). When these values were integrated for the entire water column, light and dark carbon fixation rates were 45.8 and 20.6 mg C m^{-2} h^{-1}, respectively, showing that up to 31% of the total C incorporated by the planktonic community was carried out by chemosynthetic metabolisms.

These results were substantiated by MAR-CARD-FISH experiments in light and dark incubations. The relative contribution of cells uptaking bicarbonate (i.e. Probe+ MAR+) in relation to the total cells hybridized (i.e. Probe+cells) was generally higher for Bacteria than for Archaea at all depths measured and under both conditions assayed (Table 2). The highest fraction of bacterial cells uptaking bicarbonate was found at 0.5 m in light incubations and at the oxic/anoxic interface in the dark. In the hypolimnion, the relative contribution of active Bacteria uptaking bicarbonate remained fairly constant (4% of the total

EUB+hybridized cells). For Archaea, the highest percentages of cells uptaking bicarbonate were observed again at 0.5 m in light incubations (~12% of the total ARC915+ hybridized cells), and both at the epilimnion (with a clear maximum located at 1 m depth) and at the bottom of the lagoon, in the dark.

Cloning of the biotin acetyl-CoA carboxylase gene

Amplification of *accC* genes from water samples only yielded positive results with the primer set described by Auguet *et al.* (2008), whereas the AccA-F573/-R279 primer combination yielded no amplicons (data not shown) even after careful DNA purification and optimization of the original PCR conditions (Yakimov *et al.*, 2009). Clone libraries were constructed for the oxic epilimnion (0–1 m depth), the metalimnion (2–2.25 m depth), the hypolimnion (4 m depth), and the sediment of the lagoon. Overall, 133 biotin acetyl-CoA carboxylase sequences were obtained (Table 3). Annotation and sequence comparison against databases showed only bacterial sequences, which affiliated to a broad range of species, the *Betaproteobacteria* (63%) and the *Bacteroidetes* (8%) being the most frequent taxa in the top-hit BLAST species distribution (Table S3). Particularly, 46 and 30 *accC* clones showed high sequence identity (> 95%) with gene sequences from *Polynucleobacter necessarius* ssp. *asymbioticus* QLW-P1DMWA-1 and *Ralstonia pickettii* 12J, respectively. The clone library constructed from the sediment sample recovered 17 sequences (Table 3), with identities ranging from 74% to 83.4% to pyruvate carboxylases from *Dehalococcoides ethenogenes* (15 sequences, Table S3), *Candidatus* 'Methanoregula boonei' (one sequence), and *Croceibacter atlanticus* (one sequence). Five sequences retrieved from different libraries (Table 3) showed a high identity to carbamoyl-phosphate synthases from the *Betaproteobacteria* (*Polaromonas* sp. JS666 and *Leptothrix chlolodnii* SP-6) and the *Chloroflexi* (*Oscillochloris trichoides*) (Table S3).

Discussion

The assimilation of inorganic carbon in the dark has traditionally been considered negligible in photic zones of both marine and freshwater environments due to the overwhelming dominance of photosynthetic processes. However, in environments characterized by the presence of oxic/anoxic interfaces, chemoautotrophic assimilation of CO_2 by planktonic microorganisms has been shown to be as important as photosynthesis (Cloern *et al.*, 1983; Camacho *et al.*, 2001; García-Cantizano *et al.*, 2005). The relevance of dark carbon fixation within the C cycle in such environments progressively gained significance as new data accumulated in the literature (Camacho *et al.*, 2001; Hadas *et al.*, 2001; García-Cantizano *et al.*, 2005; Casamayor *et al.*, 2008; Grote *et al.*, 2008; Glaubitz *et al.*, 2009, 2010; Casamayor, 2010; Jost *et al.*, 2010). Most of these studies also identified the different bacterial taxa potentially involved in these processes such as the purple sulfur bacteria (García-Cantizano *et al.*, 2005; Casamayor *et al.*, 2008), the *Epsilonproteobacteria* [e.g. *Sulfurimonas* (Grote *et al.*, 2007, 2008)], and the aerobic sulfur-oxidizing bacteria [e.g. *Thiobacilli* (Casamayor, 2010)]. However, the capability of lacustrine archaea other than ammonia oxidizers to incorporate CO_2 in the dark has not been assessed in aquatic environments with well-defined oxic/anoxic interfaces. To date, archaeal chemoautotrophy has been found in (hyper)thermophiles (Berg *et al.*, 2010), methanogens (Jones *et al.*, 1987; Simpson & Whitman, 1993), and ammonia-oxidizing crenarchaeota (Könneke *et al.*, 2005; Hallam *et al.*, 2006; Martens-Habbena *et al.*, 2009; Walker *et al.*, 2010), although the high diversity of archaea in the most diverse habitats (Schleper *et al.*, 2005) provides the possibility of a wider occurrence of autotrophy over the entire archaeal domain.

Coromina is a small lagoon of the Banyoles Karstic System characterized by the high concentrations of ammonium and sulfide in the anoxic water compartment and the prevalence of photosynthetic primary producers that are either eukaryotic (green and brown algae) or prokaryotic

Table 3. Distribution of gene sequences in the four *accC* clone libraries constructed from water and sediment samples of the lagoon (see text for details)

Clone library	Clones with correct insert (%)*	Unspecific sequences (%)	Valid sequences (%)	Acetyl-CoA carboxylase† (%)	Pyruvate carboxylase† (%)	Carbamoyl-phosphate synthase† (%)
Epilimnion (0–1 m)	44 (88)	7 (16)	37 (84)	36 (97)	0	1 (3)
Metalimnion (2–2.5 m)	46 (92)	7 (15)	39 (85)	36 (92)	0	3 (8)
Hypolimnion (4 m)	48 (96)	10 (21)	38 (79)	38 (100)	0	0
Sediment	49 (98)	8 (16)	41 (84)	23 (56)	17 (42)	1 (2)
Total	187 (93.5)	32 (17)	155 (83)	133 (86)	17 (11)	5 (3)

*A total of 50 clones were picked for each library (200 screened clones in total).

†Sequence description was obtained by automatic annotation of nucleotide sequences using BLAST2GO software.

The relative contribution (in %) of each sequence cluster with respect to the number of correct sequences for that group is also indicated.

Table 4. Values of dark carbon fixation rates integrated for the entire water column and their relative contribution to total assimilated C in different lakes with an oxygen/sulfide interface

System	Dark C fixation (mg C m^{-2} h^{-1})	Contribution (%)	Source
Coromina	20.6	31	This study
Vilar	18.9	32	Casamayor *et al.* (2008)
Cisó	14.5	52	García-Cantizano *et al.* (2005)
Big Soda Lake	17	30	Cloern *et al.* (1983)
Kinneret	13	16	Hadas *et al.* (2001)

(green anoxygenic photosynthetic bacteria) (Borrego & Garcia-Gil, 1994; Gich *et al.*, 2001). In fact, the data presented here agree with this picture because bicarbonate photoassimilation accounted for up to 69% of the total carbon incorporated by the planktonic community in the entire water column. However, when integrated values of light and dark C fixation rates were calculated on a daily basis assuming: (1) an average value of 10 h of solar radiation and (2) that both processes operate at constant rates, the bulk dark C fixation (494 mg C m^{-2} day^{-1}) exceeds photosynthetic assimilation (459 mg C m^{-2} day^{-1}). These data support the significance of dark C fixation processes mediated by planktonic microorganisms in stratified freshwater systems and agree with those calculated in other karstic lakes with oxic/anoxic interfaces (Table 4).

These results were corroborated by MAR-CARD-FISH experiments. Archaeal abundance (up to 7% of the total DAPI counts) was within the range reported for other freshwater environments (Casamayor & Borrego, 2009). The maximum archaeal counts were found at the upper layers of the hypolimnion, although both the bulk dark C incorporation rates and the abundance of active archaeal cells in MAR-CARD-FISH incubations showed their maximal values at the oxic/anoxic interface and above (Table 2). Of particular interest was the relatively high fraction of archaeal cells (~12%) actively uptaking bicarbonate under light conditions at the surface. Stimulation of CO_2 fixation in the light has been reported in proteorhodopsin-containing marine *Flavobacteria* (Gomez-Consarnau *et al.*, 2007; Gonzalez *et al.*, 2008), but it has never been reported for archaea. Current data are not sufficient to properly explain this observation and further work is needed to resolve the extent of such stimulation of CO_2 uptake by light in archaea, if any. Besides, we cannot rule out a mixotrophic or even a heterotrophic metabolism on labelled organic compounds excreted by phototrophs, especially considering that several authors have reported the ability of some marine archaeal groups to incorporate organic matter (Ouverney & Fuhrman, 2000; Pérez *et al.*, 2003; Kirchman *et al.*, 2007).

At the oxic/anoxic interface and in the hypolimnion, all the archaeal phylotypes recovered affiliated to crenarchaeota of the *Miscellaneous Crenarchaeotic Group* (MCG, OTU-8, and -9) (Fig. 3) and to methanogenic euryarchaeotal lineages [either to acetoclastic *Methanosaeta* (OTU-1 and -2) or to hydrogenotrophic representatives close to *Methanobrevibacter woesei* (OTU-3)]. A high richness of MCG phylotypes is a distinctive feature of anoxic water layers in stratified, sulfide-rich lakes (Llirós *et al.*, 2008, 2010), although the use of a PCR primer combination biased towards lacustrine crenarchaeota may overestimate their actual relevance in the ecosystem (for details on primer design and specificity, see Llirós *et al.*, 2008). In clear contrast to the wealth of information available for ammonia-oxidizing crenarchaeota, very little is known about the metabolic capabilities and ecological role of the MCG archaea. This phylogenetically diverse group seems to be especially abundant and active in anoxic, nutrient-rich habitats such as deep subsurface sediments (Parkes *et al.*, 2005; Biddle *et al.*, 2006) and they have been classified as heterotrophic anaerobes (Teske & Sorensen, 2008). This putative metabolism agrees with the physico-chemical conditions prevalent in the lagoon.

The co-occurrence of methanogenic archaea with sulfate-reducing bacteria is not unusual and has been reported for other freshwater environments (Lovley & Klug, 1983; Sinke *et al.*, 1992; Holmer & Storkholm, 2001), although methanogenesis is usually low in comparison with sulfate respiration when sulfate is not limiting (Holmer & Storkholm, 2001; Kallistova *et al.*, 2006). Although no data on methane concentration are available for the studied lagoon, the vertical profile of sulfide concentration (Fig. 1) and the high amount of sulfate present in underground waters of the Banyoles karstic system [~10 mM, (Guerrero *et al.*, 1985)] suggest an active sulfate reduction both in the planktonic compartment and in the sediment. On the other hand, no evidences on the presence of archaea potentially involved in anaerobic oxidation of methane, either free-living or associated with sulfate-reducing bacteria [ANME groups (Hinrichs & Boetius, 2002)], were found in the molecular fingerprints, although the detection limit of the DGGE may explain that low abundant (< 1%) microbial populations remained undetected (Casamayor *et al.*, 2000). The detection of archaeal cells actively uptaking bicarbonate in the anoxic waters (Table 2) may be related either by the activity of autotrophic methanogens (Simpson & Whitman, 1993) or by the heterotrophic incorporation of CO_2 by archaea as reported for bacteria.

The bacterial planktonic community was mainly composed by phylotypes affiliated to *Betaproteobacteria*, *Bacteroidetes*, and *Chlorobi* (Fig. 2). Minor components of the community were ascribed to *Alphaproteobacteria*, high and low G+C Gram-positive bacteria, and algal plastids. With the exception of those phylotypes affiliated to either algae or GSB recovered from the oxic epilimnion and the

metalimnion, respectively, the remaining OTUs showed close similarities to heterotrophic bacteria capable of growth on complex organic matter (Table S1). Besides, the observation that all analyzed *accC* clones were most closely related to carboxylases from *Betaproteobacteria* and the *Bacteroidetes* points to a large relevance of heterotrophs at the meta- and hipolimnion. Particularly interesting is the large number of *accC* clones showing high sequence identity (> 95%) to *accC* genes from both *P. necessarius* ssp. *asymbioticus* QLW-P1DMWA-1 (PnecC) and *R. pickettii* 12J (Table S3). The latter is a usual inhabitant of polluted soils and aquatic environments having the capacity to degrade a wide range of complex organic substances (e.g. tricloroethylene and aromatic hydrocarbons) (Ryan *et al.*, 2007). In turn, PnecC is an obligately free-living *Polynucleobacter* strain that is ubiquitous in freshwater habitats characterized by high concentrations of humic substances (Hahn *et al.*, 2009; Jezberova *et al.*, 2010). The measured values for proxies of humic substances suggest a high content of these organic compounds in the lagoon, which may derive from the high external inputs of organic matter into the system (Borrego & Garcia-Gil, 1994). The capacity of *P. necessarius* to grow on photodegradation products of humic substances (Buck *et al.*, 2009; Hahn *et al.*, 2009; Watanabe *et al.*, 2009) might then confer some competitive advantage over other bacterial species as suggested previously to explain their ubiquity in freshwater lakes (Jezberova *et al.*, 2010).

Overall, the results from MAR-CARD-FISH incubations, 16S rRNA gene fingerprinting, and *accC* clone libraries suggest that the dark CO_2 assimilation activity measured was mainly carried out by heterotrophs rather than by true chemoautotrophs. Heterotrophic CO_2 assimilation is a well-known process in chemoorganotrophic microorganisms either to equilibrate their C metabolism via anaplerotic reactions or to fuel carboxylation reactions for the synthesis of different cellular constituents, for example fatty acids, nucleotides, and amino acids (Dijkhuizen & Harder, 1984, 1985; Roslev *et al.*, 2004). The occurrence of these peripheral metabolic pathways was demonstrated in environmental samples and pure cultures of heterotrophic bacteria (Roslev *et al.*, 2004) and it has been proposed to explain the high rates of dark carbon fixation both in oxic/anoxic interfaces (Casamayor *et al.*, 2008) and in laboratory-controlled batch enrichments of Arctic seawater and dominated by heterotrophs (Alonso-Saez *et al.*, 2010). Although no evidences of heterotrophic CO_2 fixation by archaea have been reported so far, it would be reasonable to assume that heterotrophic archaea also need to compensate their carbon metabolism using different anaplerotic pathways. In this regard, the potential capacity of mesophilic archaea other than ammonia-oxidizers to fix inorganic carbon is an interesting issue that should be addressed properly. Considering the high microbial diversity and activity found in oxic/anoxic interfaces of stratified lakes, these microenvironments constitute optimal systems for the investigation of such processes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DGGE fingerprints for 16S rRNA bacterial (left) and archaeal (middle and right gels) 16S rRNA gene fragments recovered in the water column of the lagoon.

Table S1. Identification number for the assigned bacterial OTUs, phylogenetic affiliation, representative sequence for each OTU, OTU members, and % similarity of the OTU representative sequence with the first BLAST and the closest cultured representative found in the BLAST search result list.

Table S2. Identification number for the assigned archaeal OTUs, phylogenetic affiliation, representative sequence for each OTU, OTU members, and % similarity of the representative sequence with the first BLAST and the closest cultured representative found in BLAST search result list.

Table S3. Top-hit BLAST species, phylogenetic affiliation, sequence description, enzyme codes, average identity values, and number of clones for each BLAST hit obtained in accC clone libraries constructed from different depths of the water column and the sediment (see text for details).

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