Universitat de Girona

DIVERSITY, DYNAMICS AND ACTIVITY OF EPSILONPROTEOBACTERIA IN A STRATIFIED KARSTIC LAKE.
IMPLICATIONS IN CARGBON AND SULFUR CYCLES

Imma Noguerola Solà

DOCTORAL THESIS

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Doctorate program in Water Science and Technology

Thesis supervisor	**PhD candidate**
Dr. Carles Borrego Moré	**Imma Noguerola Solà**

This thesis is submitted in fulfilment of the requirements to obtain the doctoral degree from the Universitat de Girona

Hereby, Dr. Carles Borrego Moré, of the Universitat de Girona,

CERTIFY:

That this doctoral thesis entitled "**Diversity, dynamics and activity of Epsilonproteobacteria in a stratified karstic lake. Implications in carbon and sulfur cycles**", that Imma Noguerola Solà has submitted to obtain the doctoral degree from the Universitat de Girona has been completed under my supervision, and meets the requirements to opt for the *International Doctor* mention.

In witness whereof and for such purposes as may arise, the following certification is signed:

Thesis supervisor
Dr. Carles Borrego Moré

Girona, 2016

Agraïments
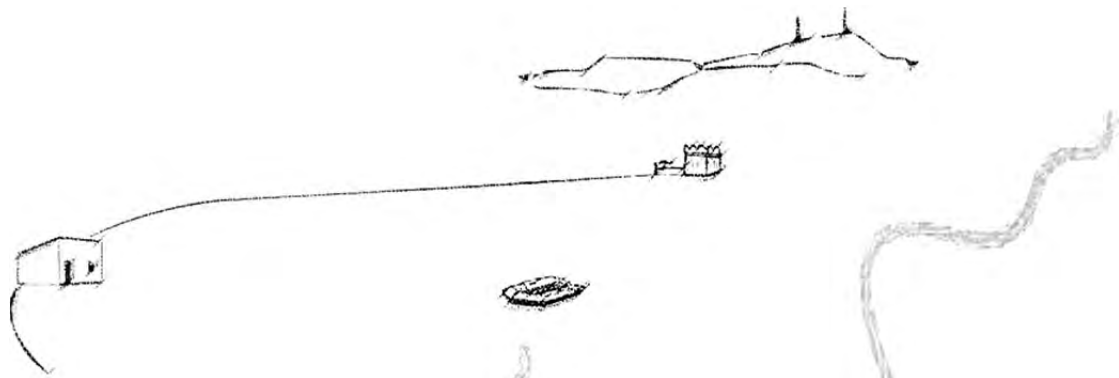
Es tractava de triangular els punts correctes: alinear-nos amb les antenes de Rocacorba i la pesquera del Castellet, i triangular-nos amb la pesquera petita de l'extrem mig de la cubeta. I aquí, situats sobre el punt més profund de la cubeta, començava la història. Després de remades i remades, de permisos que no arriben, de forats a la barca, i de quintos per gaudir, ja hauria pogut anar intuint que el camí ni seria pla, ni seria curt. I certament, no ha estat ni pla ni curt, ha estat més aviat una muntanya russa, amb passatges emocionants i paisatges fins aleshores desconeguts, ple de neguitoses llumetes card-fishianes, amb girs de noranta graus que llençaven molt material a la paperera, amb baixades en picat que m'han tallat l'aire més d'una vegada, però per sobre de tot, ha estat agraït. Potser agre-dolç, fins al punt que la dolçor s'ha menjat el sabor agre del camí. I en això, en aquesta realitat que només pot ser dialèctica, han pres una partida important les companyies que, volent o sense voler, s'han sumat al trajecte. Un trajecte en (des)equilibri constant entre el món científic, social i personal.

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LIST OF PUBLICATIONS

Part of the results of this PhD Thesis has been published in scientific journals:

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
16S rRNA	16S ribosomal ribonucleic acid gene
AclB	ATP citrate lyase subunit B
AOA	Ammonia-Oxidizing Archaea
AprA	Adenosine-5′-phosphosulfate reductase subunit A
ATP	Adenosine triphosphate
bp	Base pair
CARD-FISH	Catalyzed reported deposition-Fluorescence *in situ* hybridization
cDNA	Complementary Deoxyribonucleic acid
CT	Cycles threshold
CTAB	Cetyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindole
dbRDA	Distance-based redundancy analysis
DGGE	Denaturing gradient gel electrophoresis
DIC	Dissolved Inorganic Carbon
DistLM	Distance based linear model
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
d.p.m.	Disintegrations per minute
DSMZ	Deutsche Sammlung won Mikroorganismen und Zellkuturen (German collection of microorganisms and cell cultures)
DsrB	Dissimilatory sulfite reductase subunit B
Fcc	Flavocytochrom c sulfide dehydrogenase
FISH	Fluorescence *in situ* hybridization
GSB	Green Sulfur Bacteria
HRP	Horseradish peroxidase
IAC	Internal amplification control
LGT	Lateral gene transfer

ABBREVIATION	DESCRIPTION
LOD	Limit of detection
MAR-CARD-FISH	Microautoradiography-Catalyzed reported deposition-Fluorescence *in situ* hybridization
NCBI	National Center for Biotechnology Information
NifH	Nitrogenase subunit involved in the nitrogen fixation
OMZ	Oxygen minimum zone
Oor	2-oxoglutarate-ferrodoxin oxidoreductase (ketoglutarate synthase)
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
Por	Pyruvate:ferrodoxin oxidoreductase
Psr	Polysulfide reductase
qPCR	Quantitative Polymerase Chain Reaction
RDP	Ribosomal Database Project II
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rTCA cycle	reductive Tricarboxylic acid cycle (Arnon cycle)
SOB	Sulfur-Oxidizing Bacteria
SOP	Sulfur-Oxidizing Prokaryotes
SoxB	Hydrolytic enzyme from the sulfur-oxidizing multienzyme Sox system
Sqr	Sulfide:quinone oxidoreductase
SRB	Sulfate-Reducing Bacteria
SRP	Sulfate-Reducing Prokaryotes
TOC	Total Organic Carbon
TP	Total phosphorous
TQ	TaqMan hydrolysis probe

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SUMMARY

Karstic lakes exhibit contrasting physicochemical gradients along depth where microbial populations distribute according to their metabolic and physiological requirements. In these environments, oxic-anoxic interfaces are hot spots of microbial diversity and activity where to study the links between community structure and function. Sulfidic redoxclines offer a suitable niche for the development of photo- and chemotrophic populations that obtain energy from the oxidation of reduced sulfur compounds. Among them, members of the class *Epsilonproteobacteria* have long been considered key players in biogeochemical cycling of C and S in aquatic environments where oxygen and sulfide occur. It is then somehow surprising that no studies have yet been addressed to elucidate their presence and activity in the Banyoles Karstic System.

To resolve whether or not members of the class *Epsilonproteobacteria* constitute stable populations in Lake Banyoles and to determine their abundance, diversity, seasonal dynamics and activity we carried out a molecular survey during three year cycles in a meromictic basin of Lake Banyoles (basin C-III). We applied a complementary array of molecular techniques (CARD-FISH, MAR-FISH, cloning, qPCR and high-throughput sequencing) to resolve the identity and ecological role of Epsilonproteobacteria in the system, with special focus on the contribution of these microorganisms on linking C and S cycles. Our results pointed to a clear seasonality of Epsilonproteobacteria, with maximal abundances at the redoxcline and upper monimolimnion in winter. Furthermore, clone libraries and high-throughput sequencing datasets revealed the predominance of sequences affiliated to genus *Arcobacter*. *In situ* incubations using radiolabeled bicarbonate and MAR-CARD-FISH measurements clearly indicated that Epsilonproteobacteria actively assimilated CO_2 in the dark thus being responsible of the high rates of dark carbon fixation measured at the redoxcline in winter. Molecular analyses targeting key genes of the Arnon Cycle (C fixation) and the multienzyme Sox System (Sulfur oxidation) provided additional evidence that the dominant member of the epsilonproteobacterial community is a chemolithotrophic, sulfide-oxidizing member of the genus *Arcobacter*, distantly related to its marine counterpart *Candidatus* Arcobacter sulfidicus.

Altogether, our data support the key role of Epsilonproteobacteria in linking C and S cycles and extend their influence to freshwater systems characterized by sharp oxic-anoxic interfaces and euxinic waters. Besides, this study clarifies the ecological role of Epsilonproteobacteria in such systems and identifies the main microorganism responsible for the high dark carbon fixation activity measured at the redoxcline depth by different authors thus solving an intriguing question that has been a matter of debate in recent years.

RESUMEN

La columna de agua de los lagos cársticos se estratifica siguiendo unos gradientes físico-químicos, a menudo abruptos, que generan diferentes nichos donde las poblaciones microbianas se distribuyen de acuerdo con sus necesidades metabólicas y fisiológicas. En estos ambientes, las interfases óxico-anóxicas presentan una diversidad y actividad microbiana enorme, siendo hábitats ideales en los que estudiar la conexión entre la estructura de la comunidad y su papel ecológico. Estas interfases, donde coinciden gradientes opuestos de oxígeno y sulfhídrico, ofrecen un nicho adecuado para el desarrollo de poblaciones foto- y quimiotróficas que obtienen energía de la oxidación de compuestos reducidos del azufre. Entre éstas últimas destacan las Epsilonproteobacteria, cuya ubicuidad y versatilidad metabólica les permite jugar un papel clave en la biogeoquímica del C y del S. Resulta sorprendente pues que hasta la fecha ningún estudio se haya centrado en investigar la presencia y actividad de las Epsilonproteobacteria en el Sistema Cárstico de Banyoles.

En este trabajo se ha investigado la abundancia, diversidad, dinámica estacional y actividad de las Epsilonproteobacteria en una cubeta meromíctica del lago de Banyoles (cubeta C-III) durante tres ciclos anuales. El estudio ha combinado varias técnicas moleculares (CARD-FISH, MAR-FISH, clonación, qPCR y secuenciación masiva) para resolver la identidad y función ecológica de las Epsilonproteobacteria en el sistema, con especial atención a su contribución en los ciclos del C y del S. Los resultados mostraron una clara estacionalidad en la abundancia de estos microorganismos, con máximos invernales en la redoxclina y las capas superiores del monimolimnion. Tanto las genotecas como los datos de secuenciación masiva revelaron el predominio de secuencias afiliadas al género *Arcobacter*. Además, los resultados de incubaciones *in situ* utilizando bicarbonato marcado radioactivamente y los análisis de MAR-CARD-FISH demostraron que las Epsilonproteobacteria asimilan activamente CO_2 en la oscuridad, siendo las responsables de las elevadas tasas de fijación oscura de C medidas en la redoxclina en invierno. Análisis moleculares complementarios sobre genes clave del ciclo de Arnon (fijación de C) y del sistema multienzimático Sox (oxidación de sulfuro) permitieron resolver que el miembro predominante de la comunidad estudiada es quimiolitótrofo y oxidador de compuestos reducidos de azufre del género *Arcobacter*, filogenéticamente emparentado con la especie marina *Candidatus* Arcobacter sulfidicus.

En conjunto, nuestros datos confirman el papel clave de las Epsilonproteobacteria en los ciclos del C y el S, extendiendo su influencia a los sistemas de agua dulce caracterizados por interfases óxico-anóxicas y euxínia. Este estudio no solo esclarece el papel ecológico de las Epsilonproteobacteria en estos sistemas sino que además identifica al principal responsable de las elevadas tasas de fijación oscura de C medidas en la redoxclina y reseñadas en estudios previos, resolviendo así una cuestión que ha sido objeto de debate en la literatura especializada en los últimos años.

RESUM

La columna d'aigua dels llacs càrstics s'estratifica seguint uns gradients físico-químics, sovint abruptes, que generen diferents nínxols on les poblacions microbianes es distribueixen en funció de les seves necessitats metabòliques i fisiològiques. En aquests ambients, les interfases òxico-anòxiques presenten una diversitat i activitat microbiana enorme, essent hàbitats ideals en els que estudiar la connexió entre l'estructura de la comunitat i el seu paper ecològic. Aquestes interfases, on coincideixen gradients oposats d'oxigen i sulfhídric, ofereixen un nínxol apropiat pel desenvolupament de poblacions foto- i quimiotròfiques que obtenen energia de l'oxidació de compostos reduïts de sofre. Entre aquestes últimes destaquen els Epsilonproteobacteria, la ubiqüitat i diversitat metabòlica dels quals els permet jugar un paper clau en els cicles biogeoquímics del C i del S. Resulta sorprenent doncs, que fins al moment cap estudi s'hagi centrat en investigar la presència i activitat d'Epsilonproteobacteria al Sistema Càrstic de Banyoles.

En el present treball s'ha estudiat l'abundància, diversitat, dinàmica estacional i activitat dels Epsilonproteobacteria en una cubeta meromíctica de l'Estany de Banyoles (cubeta C-III) durant tres cicles anuals. L'estudi ha combinat diferents tècniques moleculars (CARD-FISH, MAR-FISH, clonatge, qPCR i seqüenciació massiva) per resoldre la identitat i funció ecològica d'aquests microorganismes al sistema, amb especial atenció a la seva contribució als cicles del C i del S. Els resultats van mostrar una clara estacionalitat en l'abundància d'Epsilonproteobacteria amb màxims hivernals a la redoxclina i les capes superiors del monimolimnion. Tant les genoteques com les dades de la seqüenciació massiva van revelar un predomini de seqüències afiliades al gènere *Arcobacter*. A més, els resultats d'incubacions *in situ* utilitzant bicarbonat marcat radioactivament i les anàlisis de MAR-CARD-FISH van demostrar que els Epsilonproteobacteria assimilen activament CO_2 en la foscor, essent els responsables de les elevades taxes de fixació fosca de C mesurades a la redoxclina a l'hivern. Anàlisis moleculars complementaries sobre gens clau del cicle d'Arnon (fixació de C) i del sistema multienzimàtic Sox (oxidació del sofre), van permetre resoldre que el membre predominant de la comunitat estudiada és un microorganisme quimiolitotròfic i oxidador de compostos reduïts de sofre del gènere *Arcobacter*, filogenèticament emparentat amb l'espècie marina *Candidatus* Arcobacter sulfidicus.

Amb tot, els nostres resultats confirmen el paper clau dels Epsilonproteobacteria en els cicles del C i el S, estenent la seva influència als sistemes d'aigua dolça caracteritzats per interfases òxico-anòxiques i euxínia. Aquest estudi no només clarifica el paper ecològic dels Epsilonproteobacteria en aquests sistemes, sinó que a més identifica el principal responsable de les elevades taxes de fixació fosca de C mesurades a la redoxclina i ressenyades en estudis previs, resolent així una qüestió que ha estat objecte de debat en la bibliografia especialitzada en els darrers anys.

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INTRODUCTION

In 1987, Carl Woese established this grouping [Proteobacteria], calling it informally the "purple bacteria and their relatives". Because of the great diversity of forms found in this group, the Proteobacteria are named after Proteus, a Greek god of the sea, capable of assuming many different shapes. The groups corresponding to the immediate separations within the Proteobacteria are arbitrarily designated with the Greek letters: α-, β-, γ-, δ-, ε-Proteobacteria.

(Woese, 1987; Stackebrandt et al., 1988; Gupta, 2000)

1.1. Epsilonproteobacteria: its context in the bacterial world

In the last decade, several studies focused on the *Epsilon* subdivision of *Proteobacteria* provided a better understanding of the distribution and role of members of this class in natural ecosystems (Nakagawa et al., 2005; Grote et al., 2007, 2008; Porter and Engel, 2008; Gray and Engel, 2013; Rossmassler et al., 2012; Wright et al., 2012). Currently, Epsilonproteobacteria are recognized as ubiquitous in marine and terrestrial ecosystems, and their abundances and metabolic capabilities suggest they play an important role in biogeochemical cycles (Campbell et al., 2006).

1.1.1. Taxonomy of the class Epsilonproteobacteria

Until few years ago, little was known about *Epsilonproteobacteria*, one of the five classes within the extremely diverse phylum *Proteobacteria* (Gupta, 2000). Originally, class *Epsilonproteobacteria* was defined by few pathogenic bacteria; in particular, species of the genus *Campylobacter,* which colonize mucosal tracts of birds and animal hosts, and the genus *Helicobacter,* which members thrive in the epithelial surface of the gastrointestinal tract of various animal hosts, including humans (Dworkin et al., 2006). Later on, 16S rRNA phylogenetic surveys carried out in different habitats overcame the well-known limitations of culture-dependent techniques, revealing a large diversity within the class (Nakagawa et al., 2005; Grote et al., 2007, 2008; Porter and Engel, 2008; Gray and Engel, 2013; Rossmassler et al., 2012; Wright et al., 2012).

Considering the currently accepted taxonomy based on the List of Prokaryotic names with Standing in Nomenclature (LPSN) (http://www.bacterio.net) and National Center of Biotechnology Information (NCBI) (phylogeny based on 16S rRNA), the class *Epsilonproteobacteria* comprises two valid orders, the *Campylobacterales,* which includes 11 genera (*Arcobacter, Campylobacter, Dehalospirillum, Helicobacter, Hydrogenimonas, Sulfuricurvum, Sulfurimonas, Sulfurospirillum, Sulfurovum, Thiovulum, Wolinella*), and the *Nautiliales*, which comprises 6 genera (*Caminibacter, Lebetimonas, Nautilia, Nitratifractor, Nitratiruptor, Thioreductor*).

Despite two of these genera (*Campylobacter* and *Helicobacter*) are well studied because their members are well recognized human pathogens, most genera within the class *Epsilonproteobacteria* are poorly characterized and contain single or double validly published species names (Buchanan and Gibbons, 1974). Moreover, many genera within this class are solely composed by environmental 16S rRNA gene sequences (Campbell et al., 2006). Further efforts are then needed to fill the existing gaps on the diversity and ecological role of many of the uncultured Epsilonproteobacteria.

1.1.2. Ecophysiological diversity

Current knowledge shows that members of the class *Epsilonproteobacteria* are ubiquitous and metabolically versatile (Table 1.1), especially within the order *Campylobacterales*. The order *Nautiliales* are recovered exclusively from hydrothermal vents and they also possess a distinctly metabolic diversity.

Many of the Epsilonproteobacteria studied so far have the capability to growth chemolithoautotrophically: although chemoorganotrophic representatives are also abundant, especially within the genera *Campylobacter* and *Helicobacter* (Table 1.1). Interestingly, a high metabolic versatility is associated to some genera such as *Arcobacter* and *Nautilia*, which contains chemoautotrophic, chemoorganotrophic and organoheterotrophic representatives (e.g. *Arcobacter anaerophilus* IR-1 (Roalkvam et al., 2015)).

A wide range of substrates can be used as electron donors and acceptors (Table 1.1), especially for chemolithoautotrophic Epsilonproteobacteria, which are especially ubiquitous in euxinic (sulfide-rich and anoxic) waters, marine oxygen minimum zones, marine sediments and sulfidic caves (Grote et al., 2007, 2008; Porter and Engel, 2008). Several authors have pointed out that the metabolic versatility of these microorganisms allows them to colonize a wide range of ecological niches (Lin et al., 2006; Wright et al., 2012; Meyer and Huber, 2014; Roalkvam et al., 2015), thus explaining their prevalence in highly dynamic environments such as freshwater and marine sulfidic redoxclines (Vanessa M Madrid et al., 2001; Grote et al., 2008; Glaubitz et al., 2010).

The distinct environmental location of different epsilonproteobacterial genus among marine and terrestrial habitats, with few exceptions (*Arcobacter* spp. and *Sulfurospirillum* spp.) strongly correlates with their phylogeny, suggesting a fine-scale ecotype clade association that segregated marine and terrestrial lineages along the course of evolution (Campbell et al., 2006). Recent studies, however, has questioned this environmental driven evolutionary history, suggesting that phylogenetic clades do not fully agree with their habitat and geochemistry (Porter and Engel, 2008).

Table 1.1a. Main characteristics of genera within the order *Campylobacterales*.

Genus (Type specie[1])	Habitat	Descriptive characters[2]	Conditions	Physiology and metabolism[3]			References
				Metabolism	e⁻ donor	e⁻ acceptor	
Arcobacter (*A. nitrofigilis*)	Some pathogens for humans and other animals, production water, oil field, marine environments	Slender, spiral-shaped, curved rods; motile, polar flagella	Microaerobic	Chemoorganotrophic Chemolithotrophic Organoheterotrophic	S^{2-},H_2OM	NO_3^-, O_2 S^0, Mn	(Gevertz et al., 2000; Wirsen et al., 2002; Vandieken et al., 2012; Roalkvam et al., 2015)
Campylobacter[4] (*C. fetus*)	Human and other animal organs, pathogenic	Slender, spiral curved rods; motility, unipolar or bipolar flagella	Microaerobic	Chemoorganotrophic			(Dworkin et al., 2006)
Dehalospirillum (*D. multivorans*)	Activated sludge	Spirillum	Anaerobic	Chemoorganotrophic	H_2, C_2Cl_4, OM	C_2Cl_4, NO_3^-, OM	(Scholz-Muramatsu et al., 1995)
Helicobacter[4] (*H. pylori*)	Intestinal tract and oral cavity of humans and other animals; pathogenic	Rods to tightly spiral (helical shape); some species with tightly coiled periplasmic fibers	Microaerobic	Chemoorganotrophic			(Dworkin et al., 2006)
Hydrogenimonas (*H. thermophila*)	Hydrothermal vent	Highly motile short rods, single polar flagellum	Microaerobic-to-anaerobic	Chemolithotrophic	H_2	CO_2, O_2 NO_3^-, S^0.	(Takai et al., 2004)
Sulfuricurvum (*S. kujiense*)	Underground crude-oil storage cavity	Motile, curved rods, single polar flagellum.	Microaerobic-to-anaerobic	Chemolithotrophic	S^{2-}, $S_2O_3^{2-}$,H_2	O_2 NO_3^-	(Kodama and Watanabe, 2004)
Sulfurimonas (*S. autotrophica*)	Deep sea sediments, hydrothermal vents, marine oxygen depletion zones	Short rods, motile, single polar flagellum.	Microaerobic	Chemolithotrophic	S^0, H_2 reduced S compounds	O_2 NO_3^-, NO_2^-	(Inagaki et al., 2003; Takai et al., 2006; Grote et al., 2012)

[1] Type species retrieved from LPSN.
[2] All species of the class *Epsilonproteobacteria* are Gram-negative.
[3] Electron donors and acceptors detailed, indicate that almost one member of the genus can use it.
[4] There is a wide range of information regarding these two genera (*Campylobacter* and *Helicobacter*). However, we do not mention them due to their interest in clinical microbiology but not in microbial ecology.
Chemical abbreviations: (H_2) hydrogen; (S^2) sulfide; (NO_3^-) nitrate; (S^0) elemental sulfur; (O_2) oxygen; (C_2Cl_4) perchloroethylene; (CO_2) carbon dioxide; ($S_2O_3^{2-}$) thiosulfate; (NO_2^-) nitrite; (SO_3^{2-}) sulfite; (SeO_4^{2-}) selenate; (Mn) manganese; (OM) organic matter.

Table 1.1a. Main characteristics of genera within the order *Campylobacterales* (continued).

Genus (Type specie)[1]	Habitat	Descriptive characters[2]	Conditions	Physiology and metabolism[3]			References
				Metabolism	e⁻ donor	e⁻ acceptor	
Sulfurospirillum (*S. deleyianum*)	Sulfidic freshwater and marine habitats, anoxic mud, deep-sea hydrothermal vent polychaete	Vibrioid to spiral-shaped cells; motile, polar flagella	Microaerobic to anaerobic	Chemolithotrophic Chemoorganotrophic	H_2, S^{2-}, OM	NO_3^-, NO_2^-, SO_3^{2-}, $S_2O_3^{2-}$, S^0, OM	(Campbell et al., 2001; Dworkin et al., 2006; Sikorski, Munk, et al., 2010)
Sulfurovum (*S. lithotrophicum*)	Hydrothermal vent and sediments, deep-sea hydrothermal vent polychaete	Coccoid-oval or rod-shaped Non-motile cells	Microaerobic	Chemolithotrophic	S^0, $S_2O_3^{2-}$, H_2	NO_3^-, O_2, S^0, SO_3^{2-}	(Inagaki et al., 2004; Mino et al., 2014)
Thiovulum (*T. majus*)	Freshwater and marine habitats containing sulphur	Cells contain orthorhombic S^0 granules; motile, peritrichous flagella	Microaerobic	Chemolithotrophic	S^{2-}, H_2, S^0	O_2, S^0	(Fenchel, 1994; Dworkin et al., 2006; Marshall et al., 2012)
Wolinella (*W. succinogenes*)	Bovine rumen	Rod-shaped, motile, polar Flagellated	Anaerobic	Chemolithotrophic Chemoorganotrophic	H_2, OM	NO_3, O_2, OM	(Buchanan and Gibbons, 1974; Tanner et al., 1981; Dworkin et al., 2006)

[1] Type species retrieved from LPSN.
[2] All species of the class *Epsilonproteobacteria* are Gram-negative.
[3] Electron donors and acceptors detailed, indicate that almost one member of the genus can use it.
Chemical abbreviations: (H_2) hydrogen; (S^{2-}) sulfide; (S^0) elemental sulfur; (NO_3^-) nitrate; (O_2) oxygen; (C_2Cl_4) perchloroethylene; (CO_2) carbon dioxide; ($S_2O_3^{2-}$) thiosulfate; (NO_2^-) nitrite; (SO_3^{2-}) sulfite; (SeO_4^{2-}) selenate; (Mn) manganese; (OM) organic matter.

Table 1.1b. Main characteristics of the genera within the order *Nautiliales*.

Genus (Type specie[1])	Habitat	Descriptive characters[2]	Conditions	Physiology and metabolism[3]			References
				Metabolism	e⁻ donor	e⁻ acceptor	
Caminibacter (*C. hydrogeniphilus*)	Hydrothermal vent, *A.pompejana*	Non-motile rods or motile with polar flagella	Microaerobic to anaerobic	Chemolithotrophic Mixotrophic	H_2	NO_3^-, O_2, S^o	(Alain et al., 2002; Miroshnichenko et al., 2004; Giovannelli et al. 2011)
Lebetimonas (*L. acidiphila*)	Hydrothermal vent	Motile, short rod, single polar flagellum	Anaerobic	Chemolithotrophic	H_2	S^o	(Takai, Hirayama, et al., 2005)
Nautilia (*N. lithotrophica*)	Hydrothermal vent, *A.pompejana*	Motile rods, single polar flagellum	Anaerobic	Chemolithotrophic Heterotrophic Mixotrophic	H_2, OM	NO_3^-, S^o, $S_2O_3^{2-}$, S^{2-}, SeO_4^{2-}	(Miroshnichenko et al., 2002; Smith et al., 2008; Alain et al., 2009; Pérez-Rodríguez et al., 2010)
Nitratifractor (*N. salsuginis*)	Hydrothermal vent	Short rods, non-motile, bipolar flagella	Facultatively anaerobic	Chemolithotrophic	H_2	NO_3^-, O_2	(Nakagawa, Takai, Inagaki, Horikoshi, et al., 2005)
Nitratiruptor (*N. tergarcus*)	Hydrothermal vent	Short rods, non-motile, bipolar flagella	Facultatively anaerobic	Chemolithotrophic	H_2	NO_3^-, O_2	(Nakagawa, Takai, Inagaki, Horikoshi, et al., 2005)
Thioreductor (*T. micantisoli*)	Hydrothermal sediments	Motile rods	Anaerobic	Chemolithotrophic	H_2	NO_3^-, S^o	(Nakagawa, Inagaki, et al., 2005)

[1] Type species retrieved from LPSN.
[2] All species of the class *Epsilonproteobacteria* are Gram-negative.
[3] Electron donors and acceptors detailed, indicate that almost one member of the genus can use it.
[4] There is a wide range of information regarding these two genera (*Campylobacter* and *Helicobacter*). However, we do not mention them due to their interest in clinical microbiology but not in microbial ecology.
Chemical abbreviations: (H_2) hydrogen; (S^{2-}) sulfide; (NO_3^-) nitrate; (S^o) elemental sulfur; (O_2) oxygen; (C_2Cl_4) perchloroethylene; (CO_2) carbon dioxide; ($S_2O_3^{2-}$) thiosulfate; (NO_2^-) nitrite; (SO_3^{2-}) sulfite; (SeO_4^{2-}) selenate; (Mn) manganese; (OM) organic matter.

1.2. Epsilonproteobacteria in natural habitats

Habitats colonized by members of the class *Epsilonproteobacteria* range from deep-sea vents to glacial deposits. In marine systems, they are found in hydrothermal vents (Meyer and Huber, 2014), vent fauna (Grzymski et al., 2008), deep-sea marine subsurface (Inagaki et al., 2003), surface seawater (Suh et al., 2015), oxygen minimum zones (Wright et al., 2012) and sulfidic oxic-anoxic marine interfaces (Lin et al., 2006; Grote et al., 2007). Investigations focused on terrestrial habitats are less exhaustive, but some studies revealed the presence of Epsilonproteobacteria in groundwater and karstic systems (Gray and Engel, 2013), sulfidic caves and springs (Porter and Engel, 2008), freshwater lakes (Biderre-Petit et al., 2011), glacial deposits (Wright et al., 2012), gas hydrate mounds (Mills et al., 2003) and deltaic muds (Todorov et al., 2000).

1.2.1. Anoxic marine basins

Most of the studies focused on the ecology of Epsilonproteobacteria focused on marine ecosystems, especially hydrothermal vents and in oxic-anoxic transition zones of anoxic marine basins such as the Cariaco Basin and the Black and Baltic seas (Fig. 1.1). Several studies have also reported the presence of Epsilonproteobacteria in surface seawater, with special contribution of the genus *Arcobacter* (Suh et al., 2015).
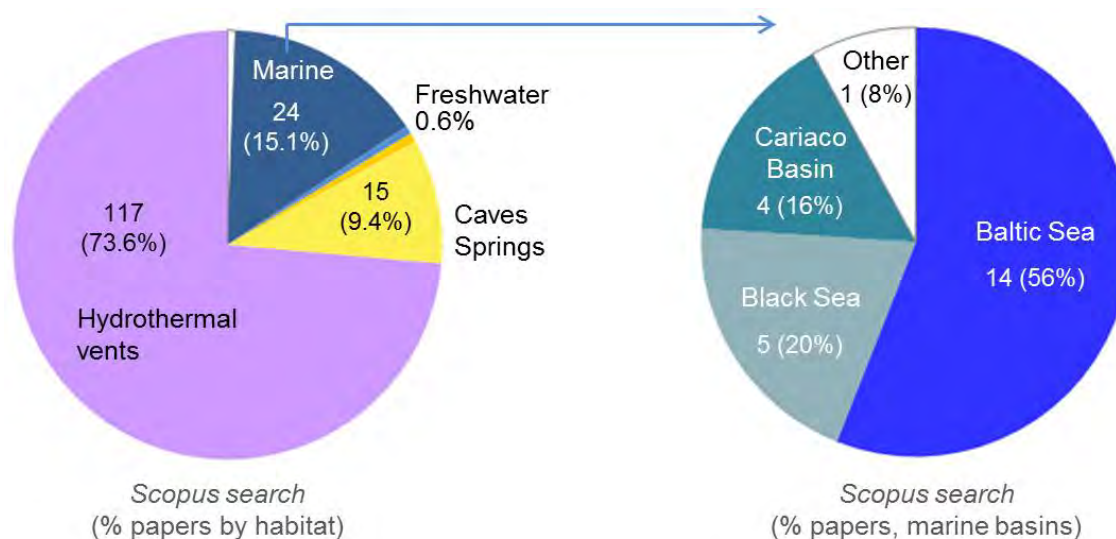
Figure 1.1. Relative contribution of papers studying the ecology of Epsilonproteobacteria in relation with the habitat where the study was carried out (left) and relative contribution of papers focused on marine Epsilonproteobacteria (right). Source: Scopus (search carried out in May 2014).

In deep marine basins, the stratification of the water column is mainly caused by differences in water salinity and temperature along depth, thus preventing vertical mixing of the water layers above and below the halocline or the thermocline, respectively. Oxygen depletion in deep waters is usually accompanied by accumulation of hydrogen sulfide due to dissimilatory sulfate reduction in both the plankton and sediment. This situation typically occurs in coastal and marginal seas with permanent or temporal stratification or in oxygen minimum zones (OMZ) near upwelling areas. The transition zone between upper oxygenated water and the anoxic, sulfidic bottom waters is known as the pelagic redoxcline. Many biogeochemical transformations mediated by microbes occur within this water compartment rapidly changing redox conditions (Leibniz Institute for Baltic Sea Research; Glaubitz, 2010). In these aquatic systems, the stratification of the water column is reflected by a pronounced stratification of the microbial community, including a shift in physiological features (Garcia et al., 2013). Pelagic redoxclines are hot spots of microbial diversity and activity and constitute interesting sites where to study the links between community structure and function. Most studies dealing on the microbial ecology of these zones have been carried out in the Black and Baltic Seas and the Cariaco Basin (Fig. 1.1).

Studies centered on pelagic redoxclines of Black Sea, Baltic Sea and Cariaco Basin reported large abundances of Epsilonproteobacteria, and pointed out that these microbes may have an important contribution to biogeochemical processes at these oxic-anoxic interfaces (Lin et al., 2006, 2008; Grote et al., 2007, 2008). 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). In the Black Sea and the Cariaco Basin epsilonproteobacterial communities are more diverse and composed of phylotypes related to *Sulfurimonas* sp., *Arcobacter nitrofigilis* 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).

In these systems, Epsilonproteobacteria are nearly absent in upper oxygenated water layers, reaching maximal abundances at the sulfidic redoxcline depth (between 130-205 m, 100 m and 320 m, in the Gotland Deep (Baltic Sea), Black Sea and the Cariaco Basin, respectively (Madrid et al., 2001; Vetriani et al., 2003; Grote et al., 2007, 2008). Maximal abundance of Epsilonproteobacteria in the redoxcline of Baltic Sea was 2×10^5 cells mL^{-1} (Grote et al. 2007, 2008). In this compartment, chemolithotrophic Epsilonproteobacteria highly contribute in the biogeochemical carbon cycle (Grote et al., 2008; Glaubitz et al., 2009; Glaubitz et al., 2010; Bruckner et al., 2013). Notwithstanding this, an acetate-utilizing Epsilonproteobacteria of the genus *Arcobacter* thriving in the oxic-anoxic interface in the Baltic Sea has recently been described (Berg et al., 2013).

Epsilonproteobacteria also inhabit deep-sea sediments (Li et al., 1999; Inagaki et al., 2002; Reed et al., 2006) and hydrothermal habitats (Teske et al., 2007).

1.2.2. Hydrothermal vents

Data published so far pointed to deep-sea hydrothermal environments as one of the largest reservoirs of Epsilonproteobacteria in this planet (Campbell et al., 2006). In these habitats, niches for micro- and macroorganisms that require low amounts of oxygen are generated when hot anoxic fluids emerging from hydrothermal vents mix with cold oxygenated seawater (Reysenbach et al., 2000). During the last decade, several studies have expanded our view of how deep-sea vent microbes gain energy from reduced chemical compounds and trace elements from ocean basalts, seafloor sulfide deposits and hydrothermal plumes (Campbell et al., 2006). Microbial life alters the chemistry of hydrothermal vents and provides nourishment for vent fauna (Holden et al., 2012).

In these environments, epsilonproteobacterial populations exhibit a wide range of physiological and metabolic features (chemolithoautotrophic, mixotrophic and heterotrophic metabolisms) and intrinsic properties in cellular structure and capabilities (motility, chemotaxis, intracellular storage of elemental sulfur, etc.) Also, a wide array of potential electron donors (molecular hydrogen, formate, elemental sulfur, thiosulfate, dicarboxylic acids, aspartate, formate, pyruvate, and other reduced sulfur compounds) and electron acceptors (carbon dioxide, molecular oxygen, nitrate, elemental sulfur, thiosulfate, sulfide, selenite) are available to provide energy for autotrophic carbon fixation (Campbell et al., 2006).

In hydrothermal vents, epsilonproteobacterial communities are usually composed of members of the genus *Sulfurimonas*, *Hydrogenimonas* and *Sulfurovum* (Takai et al., 2004; Nakagawa et al., 2007). All these representatives are chemolithoautotrophs, acting as primary producers and thus favoring energy transfer from hydrothermal fluids to higher trophic levels (Hügler et al., 2010).

Most of these studies revealed an overwhelming dominance of Epsilonproteobacteria in deep-sea vents either as free living populations or as symbionts of vent fauna (i.e. polychaetes). These studies suggest that Epsilonproteobacteria are key players in the cycling of carbon, nitrogen and sulfur, and that they have important roles in symbiotic associations with vent metazoan (Campbell et al., 2006; Zinger et al., 2011; Gaudron et al., 2012; Meyer and Huber, 2014).

Epsilonproteobacteria have been found in intimate associations at least in two vent animals: the polychaete *Alvinella pompejana* and vent crustaceans (*Rimicaris* spp.) being the predominant vent-associated microorganisms in both animals (Haddad et al., 1995; Polz and Cavanaugh, 1995). In *Rimicaris* spp. they growth as a monoculture (Polz and Cavanaugh, 1995). Episymbiont community of *A. pompejana* is a multispecies biofilm consortium of Epsilonproteobacteria (Grzymski et al., 2008). Interestingly, these Epsilonproteobacteria are phylogenetically related to *Sulfurovum* sp NBC37-1, a free-living bacterium from deep sea vents (Grzymski et al., 2008). Current data suggests that epibiotic Epsilonproteobacteria contribute to host nutrition or, alternatively, they

detoxifies the host environment from toxic sulfide and metals (Gaill et al., 1988; Grzymski et al., 2008). Furthermore, investigations pointed out that the success of many Epsilonproteobacteria as symbionts is probably related to their adaptive capabilities (proteins optimally adapted to specific temperature ranges within $10^{\circ}C$–$65^{\circ}C$), broad metabolic capacity, high levels of strain variability, virulent traits in common with pathogens and biofilms benefits (Haddad et al., 1995; Grzymski et al., 2008; Lee et al., 2008; Gulmann et al., 2015). It has also been reported a chemoautotrophic endosymbiosis between a member of the class *Epsilonproteobacteria* and the gastropod *Alviniconcha* aff. *hessleri* (Suzuki et al., 2005), and also other endosymbiosis with a marine urchin (*Lytechinus variegatus*) where genus *Arcobacter* dominated (Hakim et al., 2015). It seems that a novel nutritional strategy relying on chemoautotrophy in the epsilonproteobacterial endosymbiont is utilized by the hydrothermal-vent gastropod (Suzuki et al., 2005).

1.2.3. Freshwater habitats

Remarkably, very little information is available for the distribution, abundance and activity of Epsilonproteobacteria in stratified lakes, especially considering that these waterbodies are usually characterized by sharp O_2/H_2S interfaces that might provide an optimal niche for the growth and activity of epsilonproteobacterial sulfide-oxidizers (Fig. 1.2). Very recently, a study carried out in Lake Pavin, a low-sulfate, freshwater meromictic crater lake, identified *Sulfurimonas* and *Sulfuricurvum* as main sulfide-oxidizing prokaryotes (Biderre-Petit et al., 2011). However, further investigations are needed to assess the distribution and activity of Epsilonproteobacteria in stratified, freshwater lakes.
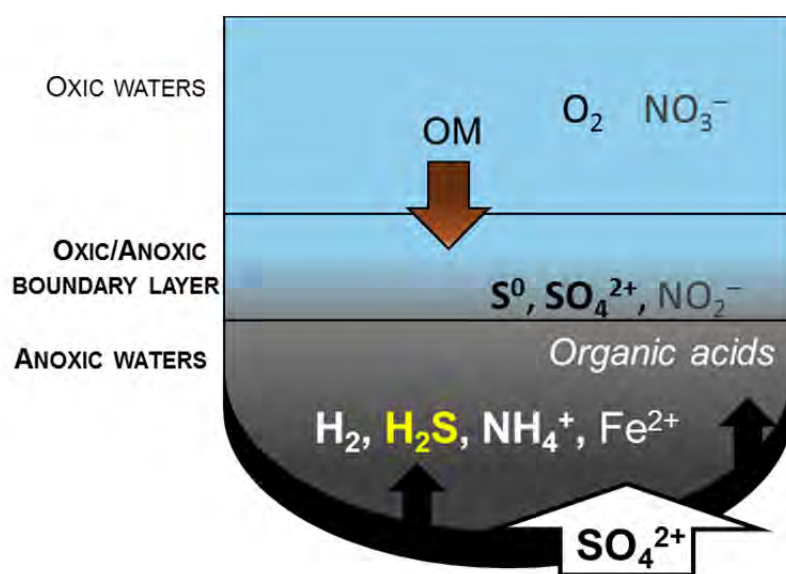
Figure 1.2. Suboxic-anoxic transition zones are areas where there are numerous biogeochemical transformations and rapidly changes in redox conditions.

1.2.4. Terrestrial habitats

Epsilonproteobacteria have mainly been reported in sulfidic limestone caves with both photic and aphotic springs (Angert et al., 1998; Elshahed et al., 2003; Engel et al., 2003; Pedersen et al., 2004; Rudolph et al., 2004; Barton and Luiszer, 2005; Porter and Engel, 2008; Porter et al., 2009; Rossmassler et al., 2012). They have also been identified in other natural terrestrial habitats such as saline wells of a karst aquifer (Gray and Engel, 2013), deltaic muds (Todorov et al., 2000), and other sulfur-rich environments such as an elemental sulfur sub-aerial arctic glacial deposits (Wright et al., 2013), oil field brines (Voordouw et al., 1996; Gevertz et al., 2000), hydrocarbon-contaminated groundwater (Watanabe et al., 2000; Kodama and Watanabe, 2003) and gas hydrate mounds (Mills et al., 2003).

Many genera identified in terrestrial environments are closely related to their marine counterparts. Epsilonproteobacteria thriving in sulfidic caves, karstic systems and at glacial sulfur deposits are diverse, including *Sulfuricurvum, Sulfurovum* and *Sulfurospirillum* (Porter and Engel, 2008; Porter et al., 2009; Gray and Engel, 2013; Wright et al., 2013), *Wolinella* (Barton and Luiszer, 2005), and *Thiomicrospira denitrificans* (Angert et al., 1998) later described as *Sulfurimonas denitrificans* (Takai et al., 2006). Sequences related to genus *Arcobacter, Campylobacter* and *Thiomicrospira denitrificans* have also been reported in oil brines and contaminated groundwater (Voordouw et al., 1996; Gevertz et al., 2000; Watanabe et al., 2000; Kodama and Watanabe, 2003).

In sulfidic caves, probably the best-studied terrestrial habitat for Epsilonproteobacteria, the community composition is influenced by environmental variables such as temperature, pH and salinity, while dissolved oxygen concentration and bicarbonate concentration has less influence, with no apparent effect of sulfide concentration (Rossmassler et al., 2012). In turn, the distribution of Epsilonproteobacteria in karstic systems correlate well with dissolved oxygen and sulfide concentration (Gray and Engel, 2013).

Very few information is available regarding the existence of Epsilonproteobacteria symbionts in terrestrial animals, with the exception of few epsilonproteobacterial sequences identified in the gut of termites (Hongoh et al., 2003; Nakajima et al., 2005; Rosengaus et al., 2011).

1.3. Contribution of Epsilonproteobacteria in biogeo-chemical cycles

Several studies demonstrate that Epsilonproteobacteria have an important role in nutrient cycling and ecosystem functioning (Grote et al., 2008; Grote et al., 2012; Wright et al., 2012). The wide metabolic versatility of this group and their adaptability towards dynamic physico-chemical environments, allows them to act as primary colonizers, primary producers or as key partners in symbiotic associations (Gaill et al., 1988; Taylor et al., 1999; Alain et al., 2004; Grzymski et al., 2008; Grote et al., 2008; Gulmann et al., 2015). This key role in biogeochemical cycling is well-recognized in sulfidic redoxclines where they are major contributors to carbon, sulfur and nitrogen cycles (Grote et al., 2008; Glaubitz et al., 2009; Grote et al., 2012; Bruckner et al., 2013).

1.3.1. Contribution to Carbon cycle

Most Epsilonproteobacteria isolated are true chemolithoautotrophs, acting as primary producers in both marine and terrestrial ecosystems (Madrid et al., 2001; Campbell et al., 2003; Grote et al., 2008; Glaubitz et al., 2009; Wright et al., 2013). Current data indicate that all autotrophic Epsilonproteobacteria fix CO_2 using the reductive tricarboxylic acid cycle (rTCA cycle or Arnon cycle, Fig. 1.3) (Hügler et al., 2005).

The rTCA cycle has been described only in a few taxa, including all members of the obligate photoautotrophic and anaerobic Green Sulfur Bacteria (GSB) (Evans et al., 1966; Fuchs et al., 1980; Tang and Blankenship, 2010), a few members of the class *Deltaproteobacteria* (Schauder et al., 1987), some members of the thermophilic order *Aquificales* (Hall et al., 2008) and groups of the archaeal family *Thermoproteaceae* (Hügler et al., 2003).

Candidatus Arcobacter sulfidicus, a mesophilic, chemolithoautotrophic sulfide-oxidizer isolated from a coastal marine sediment was the first epsilonproteobacterium able to assimilate inorganic carbon using rTCA cycle (Wirsen et al., 2002). Subsequently, two fosmid libraries containing genome fragments of epsilonproteobacterial episymbionts revealed the presence of a key gene of the rTCA cycle, the ATP citrate lyase (*aclBA*) (Campbell et al., 2003). Further studies based on the detection of this gene as biomarker for the rTCA cycle spread the evidence that most, if not all, autotrophic Epsilonproteobacteria use this pathway to fix CO_2 (Hügler et al., 2005; Takai, Campbell, et al., 2005; Moussard et al., 2006).

Despite the presence of several autotrophic representatives, many Epsilonproteobacteria are heterotrophs. Organotrophic Epsilonproteobacteria have been reported in both aquatic and terrestrial habitats (Scholz-Muramatsu et al., 1995; Campbell et al., 2001). In this regard, an acetate-oxidizing *Arcobacter* sp. from a marine sediment allowed the description of a novel metabolic capability of members of this genera which couples acetate oxidation to manganese reduction (Vandieken et al., 2012)). Recently, Glaubitz and co-workers identified an autotrophic *Sulfurimonas* sp. able to use pyruvate to

replenish chemolithoautotrophic growth and ensure survival in nutrient-limited habitats (Glaubitz et al., 2014).
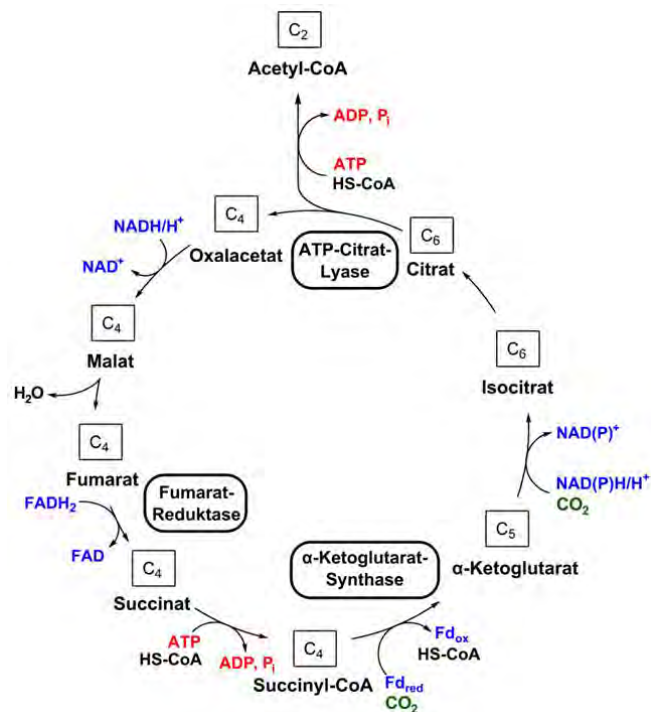
Figure 1.3. Schematic diagram of the reductive tricarboxylic acid cycle (rTCA or Arnon cycle) showing its three key enzymes. Source: Boundless (Boundless.com).

1.3.2. Contribution to Sulfur cycle

Sulfur transformations and fate in the environment are critically dependent on microbial activities of metabolically and phylogenetically diverse microorganisms (Sievert, Kiene, et al., 2007; Klotz et al., 2011) (Fig. 1.4). In addition to assimilatory sulfur reactions intended to synthesize organic compounds, many bacteria and archaea use reduced sulfur compounds as energy source in dissimilatory metabolic reactions (Sievert, Kiene, et al., 2007). Sulfur compounds can be used either as electron acceptors or as electron donors by sulfate-reducing prokaryotes (SRP) and sulfur-oxidizing prokaryotes (SOP), respectively (Sievert, Kiene, et al., 2007). All metabolic transformations, such as the oxidation of reduced sulfur compounds and dissimilatory sulfate reduction, are catalyzed by distinct enzymes that can be used as biomarkers for these processes (Petri et al., 2001; Geets et al., 2006; B Meyer and Kuever, 2007) (Fig. 1.5).

Most chemolithotrophic Epsilonproteobacteria are able to use reduced inorganic sulfur compounds such as H_2S as energy source (Wirsen et al., 2002; Inagaki et al., 2003, 2004; Takai et al., 2003; Takai, Campbell, et al., 2005). Assuming that H_2S is a well-known toxic compound to organisms in higher trophic levels (Copenhagen, 1953), the ability of some

sulfur-oxidizing Epsilonproteobacteria to oxidize sulfide can be viewed as a detoxifying activity, thus creating a sulfide-free zone that might have important ecological implications in certain areas, such as in oxygen minimum zones (Lavik et al., 2009; Grote et al., 2012).

Most epsilonproteobacterial sulfur-oxidizers, oxidize sulfur compounds via the Sox system (Yamamoto et al., 2010; Yamamoto and Takai, 2011; Akerman et al., 2013) (Fig. 1.6). Particularly, *Sulfurovum* NBC37-1, *Nitratiruptor* SB155-2 and *Sulfurimonas denitrificans* DSM1251 possess the *sox* multigene system (Nakagawa et al., 2007; Sievert et al., 2008). Besides, genes encoding for the enzyme sulfide-quinone oxidoreductase (*Sqr* gene) which catalyze sulfide oxidation have been identified in genomes of several species of *Epsilonproteobacteria*. Recently Sqr activity has been reported for *Sulfurimonas denitrificans* (Han and Perner, 2016).
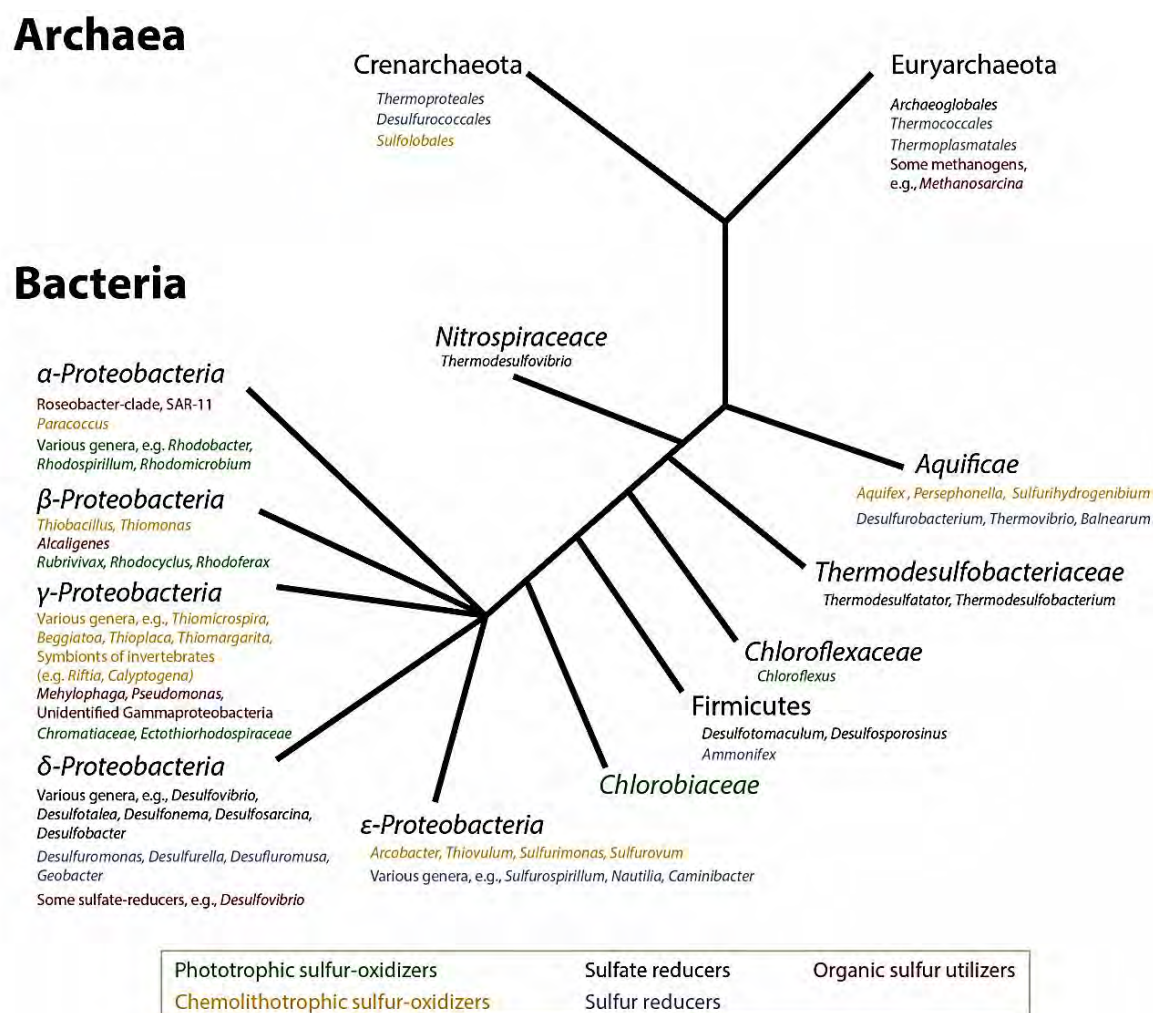
Figure 1.4. Sulfur-metabolizing microorganisms among major phylogenetic lineages. Modified from Sievert et al.(Sievert, Kiene, et al., 2007).
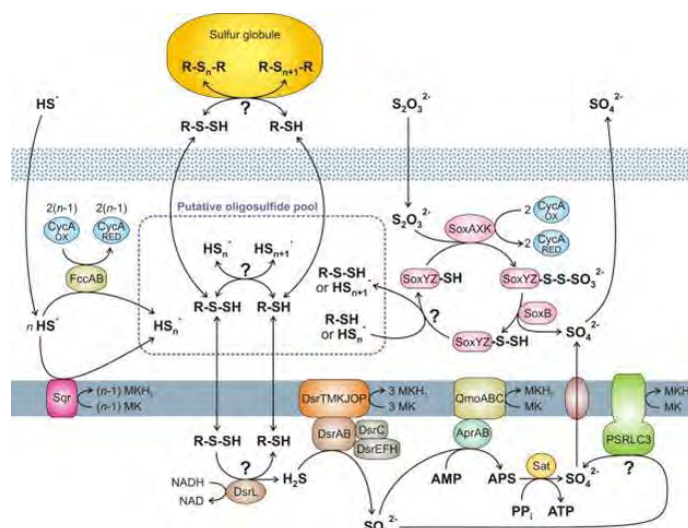
Figure 1.5. Different putative metabolic pathways of oxidative sulfur metabolism in Green Sulfur Bacteria. Source: Gregersen et al. (Gregersen et al., 2011).

Recent investigations revealed that the deep-sea epsilonproteobacterium *Sulfurovum* sp. NBC37-1 use sulfur compounds not only as electron-donors but also as electron acceptors, thus operating in two different types of sulfur-related energy metabolism: *i)* an hydrogen-oxidizing sulfur respiration using polysulfide reductase gene families (*Psr* genes), and *ii)* a thiosulfate-oxidizing nitrate/oxygen respiration using the *sox* multienzyme system (Yamamoto et al., 2010) (Fig. 1.6). Bacteria associated to both sulfur reduction and oxidation have been identified within the class *Epsilonproteobacteria* and the phylum *Aquificae*. This capacity may be considered as an ecophysiological strategy that extends their metabolic versatility and ecological distribution of Epsilonproteobacteria (Yamamoto et al., 2010; Yamamoto and Takai, 2011).
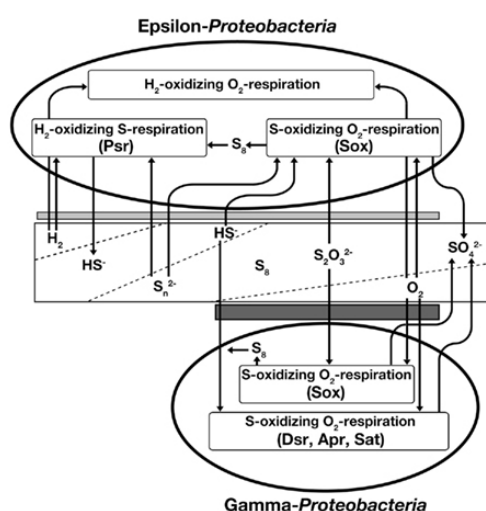
Figure 1.6. Energy metabolic pathways identified in members of classes *Epsilon- and Gammaproteobacteria* in deep-sea hydrothermal fields. Psr, polysulfide reductase; Sox, Sox multienzyme system; Dsr, dissimilatory sulfite reductase; Apr, adenosine 5'-phosphosulfate reductase; Sat, sulfate adenylyltransferase. Source: Yamamoto and Takai (Yamamoto and Takai, 2011)

1.3.3. Contribution to Nitrogen cycle

Although the contribution of Epsilonproteobacteria in the biogeochemical nitrogen cycle is less documented than that in both C and S cycles, recent studies have revealed unexpected links between members of this class and the cycling of N (Kern and Simon, 2009; Grote et al., 2012; Bruckner et al., 2013; Hanson et al., 2013; Meyer and Huber, 2014; Vetriani et al., 2014).

The most known nitrogen pathway among the class *Epsilonproteobacteria* is the use of nitrate as electron acceptor during nitrate respiration (Kern and Simon, 2009; Grote et al., 2012; Bruckner et al., 2013; Hanson et al., 2013; Vetriani et al., 2014). Several species within the class *Epsilonproteobacteria* are able to reduce nitrate and nitrite using either respiratory nitrate ammonification (*Nautilia profundicola*) or denitrification (*Sulfurimonas gotlandica* GD1) (Kern and Simon, 2009; Grote et al., 2012; Hanson et al., 2013).

Recently, the first evidence of nitrogen fixation in Epsilonproteobacteria from deep-sea hydrothermal vents has been reported in *Lebetimonas* (Meyer and Huber, 2014). *Lebetimonas* expressed *nifH*, a gene encoding a nitrogenase subunit involved in the nitrogen fixation, during growth with N_2 as the sole nitrogen source, suggesting that at least some Epsilonproteobacteria representatives are able to carry out nitrogen fixation under nitrogen limiting conditions (Meyer and Huber, 2014).

1.4. Methodological molecular approaches for the study of the abundance, diversity, and activity of Epsilon-proteobacteria

Different molecular methods have been routinely used by different authors to investigate the abundance, diversity, and activity of Epsilonproteobacteria in terrestrial and aquatic environments (Labrenz et al., 2004; Hügler et al., 2005; Grote et al., 2007).

1.4.1. Abundance and diversity

Studies on the abundance of Epsilonproteobacteria in marine pelagic redoxclines have commonly used fluorescence *in situ* hybridization (FISH) and catalyzed reported deposition fluorescence *in situ* hybridization (CARD-FISH) (Lin et al., 2006; Grote et al., 2007, 2008). Thus, different FISH and CARD-FISH probes specifically targeting members of the class *Epsilonproteobacteria* have been designed and successfully applied in the recent years (Table 1.2).

Despite the well-known potential of whole-cell fluorescence *in situ* hybridization techniques for enumeration and distribution of specific microorganisms in natural samples, PCR-based techniques provide useful alternatives that could be more sensitive and save analytical time. In this regard, both PCR and qPCR protocols have been

optimized for the specific detection and enumeration of Epsilonproteobacteria (Labrenz et al., 2004; Grote et al., 2007) although they have not routinely been applied to ecological studies of this group. Otherwise, fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), clone and pyrotag libraries have also been used to study the relative abundances and diversity of Epsilonproteobacteria (Labrenz et al., 2007). Notwithstanding these considerations, PCR primers specifically targeting the 16S rRNA gene of the Epsilonproteobacteria have not been designed and protocols using universal bacterial primers are error prone due to well-known PCR biases that may led to the over- or underestimation of the actual Epsilonproteobacteria abundance (Campbell et al., 2003; Engel et al., 2003; Porter et al., 2009; Gray and Engel, 2013). To the best of our knowledge, very few PCR and qPCR primers that specifically target some species of the class *Epsilonproteobacteria* are currently available (Table 1.2).

1.4.2. Activity

The study of certain metabolic pathways or functional capabilities is particularly relevant to provide a proper explanation of the ecological function of a specific bacterial group. There are different strategies to address this purpose, for instance: *i)* the identification of biomarker genes encoding key enzymes involved in a known metabolic pathway, and *ii) in situ* or *in vitro* measures of the biological uptake rates for a given compound, usually labeled with either stable (*e.g.* ^{13}C) or radioactive isotopes (*e.g.* ^{14}C).

Among the first approach, several authors applied PCR for the specific detection of the genes coding for key enzymes of the rTCA cycle such as the ATP citrate lyase (*acl*), 2-oxoglutarate-ferrodoxin oxidoreductase (ketoglutarate synthase) (*oor*) and pyru-vate:ferrodoxin oxidoreductase (*por*) on samples from diverse environments where Epsilonproteobacteria were dominant (Campbell et al., 2003; Campbell and Cary, 2004; Hügler et al., 2005). Other genes encoding key enzymes involved in the sulfur metabolism have also been used as biomarkers in several studies. The use of *soxB* gene from the multienzyme Sox system allows the identification of the putative sulfur-oxidizing capability among members of the class *Epsilonproteobacteria.* Accordingly, specific primers to target epsilonproteobacterial *soxB* gene sequences has also been designed (Akerman et al., 2013).

Regarding the second approach, the application of *in situ* incubations using radiolabeled inorganic carbon has routinely been used to ascertain the potential autotrophic capabilities of planktonic populations of Epsilonproteobacteria. Besides, combination of autoradiography techniques with FISH or CARD-FISH (MAR-FISH) have helped to identify those cells able to uptake the radiolabeled substrate (Labrenz et al., 2005; Grote et al., 2007; Porter et al., 2009). Alternatively, stable isotope probing has also been used to overcome the risk of handling radioactive compounds (Glaubitz et al., 2009; Berg et al., 2013). On the other hand, the determination of sulfide oxidation and nitrate reduction rates have only been obtained for epsilonproteobacterial isolates such as the recently

isolated chemolithotrophic, sulfide-oxidizing marine *Sulfurimonas gotlandica* GD1 (Bruckner et al., 2013).

Table 1.2. Oligonucleotide probes targeting environmental-relevant members of the class *Epsilonproteobacteria* used in FISH, CARD-FISH, PCR and qPCR protocols. Oligonucleotide probes targeting pathogenic members of the class and used for clinical analysis are not included.

	Oligonucleotide sequence (5′→3′)	Target microorganism	Reference
FISH AND CARD-FISH PROBES[1]			
ARC1430	TTAGCATCCCCGCTTCGA	*Arcobacter*	(Snaidr et al., 1997)
ARC94	TGCGCCACTTAGCTGACA	*Arcobacter*	
EPSY549	CAGTGATTCCGAGTAACG	*Epsilonproteobacteria*	(Lin et al., 2006)
EPSY914[2]	GGTCCCCGTCTATTCCTT	*Epsilonproteobacteria*	(Loy 2003)
FB648	ACCTCTCCCATGGTCTAGTT	*Candidatus* Thioturbo danicus and strain DK4	(Muyzer et al., 2005)
FB741	CCTCAGCGTCAGCTATGTTC	*Candidatus* Thioturbo danicus and strain DK4	
FB842	ACTGTGTTACTGCAGCCTCT	strain DK4	
LKC1006	CTCCAATGTTTCCATCGG	Epsilonproteobacterial group II from Lower Kane Cave	(Engel et al., 2003)
LKC59	TCCTCTCATCGTTCGACT	Epsilonproteobacterial group II from Lower Kane Cave	
SUL90[2]	CGTGCGCCACTAATCATA	*Sulfurimonas gotlandica* GD17	(Grote et al., 2007)
EPS682[3]	CGGATTTTACCCCTACAC	*Epsilonproteobacteria*	(Lin et al., 2007)
PCR PRIMERS			
EPS685R	TCTACGGATTTTACCCCTAC	*Epsilonproteobacteria*	(Rodriguez-Mora et al., 2013)
qPCR PRIMERS			
OST1F	TCAGATGTGAAATCCAATGGCTCA		
OST1R	CTTAGCGTCAGTTATGTTCCAGG	*Thiomicrospira denitrificans*-like	(Labrenz et al., 2004)
Com1	CAGCAGCCGCGGTAATAC		
Com2ph	CCGTCAATTCCTTTGAGTTT		

[1]FISH and CARD-FISH probes are recovered from probeBase (http://131.130.66.201/probebase/), searching for *Epsilonproteobacteria* as target organisms, and restricting the search to probes used for FISH.
[2] FISH and CARD-FISH probes are recovered from probeBase (http://131.130.66.201/probebase/), searching for *Epsilonproteobacteria* as target organisms, with any restriction in the search.
[3] This FISH probe does not appear in probeBase.

1.4.3. Genomics of Epsilonproteobacteria

A total of 27 epsilonproteobacterial genomes or nearly-completed genomes, excluding Epsilonproteobacteria of clinical relevance and animal pathogens, are currently available in public databases (National Center for Biotechnology Information, NCBI) although not all of them are fully annotated. Current sequenced genomes include species of the genera *Sulfurimonas* (Sievert et al., 2008; Sikorski, Munk, et al., 2010; Grote et al., 2012), *Sulfuricurvum* (Han et al., 2012; Tan and Foght, 2014; Hamilton et al., 2015), *Sulfurospirillum* (Sikorski, Lapidus, et al., 2010; Goris et al., 2014; Tan and Foght, 2014; Tatusova et al., 2014; Ross et al., 2015), *Sulfurovum* (Nakagawa et al., 2007; Park et al., 2012; Tatusova et al., 2014; Hamilton et al., 2015), *Lebetimonas* (Meyer and Huber, 2014; Tatusova et al., 2014), *Arcobacter* (Pati et al., 2010; Tatusova et al., 2014; Roalkvam et al., 2015), *Nautilia* (Campbell et al., 2009), *Thiovulum* (Marshall et al., 2012), *Nitratiruptor* (Nakagawa et al., 2007), *Nitratifractor* (Anderson et al., 2011), *Caminibacter* (Giovannelli et al., 2011) and *Wolinella* (Baar et al., 2003).

Different approaches such as the construction and sequencing of large-insert DNA libraries (fosmid libraries) have been employed to reveal metabolic features of different Epsilonproteobacteria (Campbell et al., 2003). Nowadays, metagenomics has become an important tool for the characterization of microorganisms, providing information on the structure, composition, and gene functions of environmental microbial assemblages. Several studies have applied metagenomics to unveil potential gene content of poorly characterized organisms such as the Epsilonproteobacteria (Grzymski et al., 2008; Wright et al., 2013; Llorens-Marès et al., 2015; Keller et al., 2015).

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2

AIMS AND OUTLINE OF THE THESIS

The main goal of this thesis is to gain a better insight into the ecology of Epsilonproteobacteria in the sulfidic redoxcline of basin C-III, a meromictic basin of Lake Banyoles. Our research has been planned to achieve the following objectives:

I) To determine the abundance and seasonal dynamics of Epsilonproteobacteria in basin C-III.

II) To assess the phylogenetic diversity of the planktonic community of Epsilonproteobacteria and to identify the main species that composes it.

III) To evaluate the contribution of Epsilonproteobacteria to dark carbon fixation at the redoxcline and the monimolimnetic waters of the studied system.

IV) To study the functional diversity of genes involved in the sulfur cycle in the water column of the studied basin C-III with special focus to genes related to Epsilonproteobacteria sulfide-oxidizers.

To determine the abundance and seasonal dynamics of Epsilonproteobacteria in the studied system (**objective I**), we applied and optimized a CARD-FISH protocol using the epsilonproteobacterial specific probe EPSY914 on samples collected at the redoxcline and upper monimolimnion of basin C-III along different seasons and year cycles (2011–2014) (**Chapter 4**).

The phylogenetic composition of the planktonic bacterial assemblage, and particularly the identification of the main taxa within the class *Epsilonproteobacteria* (**objective II**), was conducted by using high-throughput sequencing (pyrosequencing by Roche454®) to DNA extracted from selected samples collected at different dates and depths of the studied year cycles (**Chapter 4**).

To evaluate the contribution of Epsilonproteobacteria to dark carbon fixation (**objective III**) we applied microautoradiography coupled to CARD-FISH (MAR-CARD-FISH) to identify the main bacteria involved in uptaking CO_2 in the dark. Moreover, *in situ* incubation experiments using radiolabeled bicarbonate were carried out to estimate the dark carbon fixation rates that were then used to evaluate the contribution of Epsilonproteobacteria to the overall C fixation at ecosystem level. Furthermore, clone libraries targeting the *aclB* gene were analyzed to assess if the dominant epsilonproteobacterial population in basin C-III incorporates CO_2 via the Arnon cycle (**Chapter 4**).

During this first experimental work, we detected unspecificities of the probe EPSY914 that seriously compromised the results and overestimated the actual abundance of Epsilonproteobacteria. Thus, an alternative approach based on the design of specific qPCR primers for the quantification of the dominant epsilonproteobacterial species in the studied system was optimized to overcome this limitation. The application of this qPCR protocol allowed a proper quantification of these species and allowed a better interpretation of their distribution and seasonal dynamics (**Chapter 5**).

Bacterial groups participating in the cycling of sulfur in the studied system were identified using high-throughput sequencing (Illumina chemistry) of several genes involved in different metabolic pathways related to the S cycle. Particularly, the epsilonproteobacterial contribution to sulfur cycle (**objective IV**) was evaluated using the *soxB* gene as an indicator of sulfur oxidation via the multienzyme Sox System (**Chapter 6**).

3

MATERIAL & METHODS

3.1. 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 Garcia-Berthou, 1989). Water enters the lake by subterranean bottom springs creating strong chemical gradients along the water column due to the high concentration of dissolved sulfate (~13 mM, Guerrero et al., 1985). This sulfate-rich water together with anoxic conditions at deep layers allows an active sulfate-reduction both in bottom 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. 3.1). Sampling was carried out in different seasons between winter 2011 and winter 2014. All dates and depths sampled during the study period are detailed in the Table 3.1.

Depth profiles of temperature, conductivity, pH, Redox potential (E_H), and oxygen concentration were determined *in situ* using a multiparametric probe OTT-Hydrolab MS5 (Hatch Hydromet, Loveland, CO, USA). Photosynthetically active radiation (PAR) profiles were measured using a submersible PAR sensor (Li-250 Light Meter; Li-Cor Inc.).
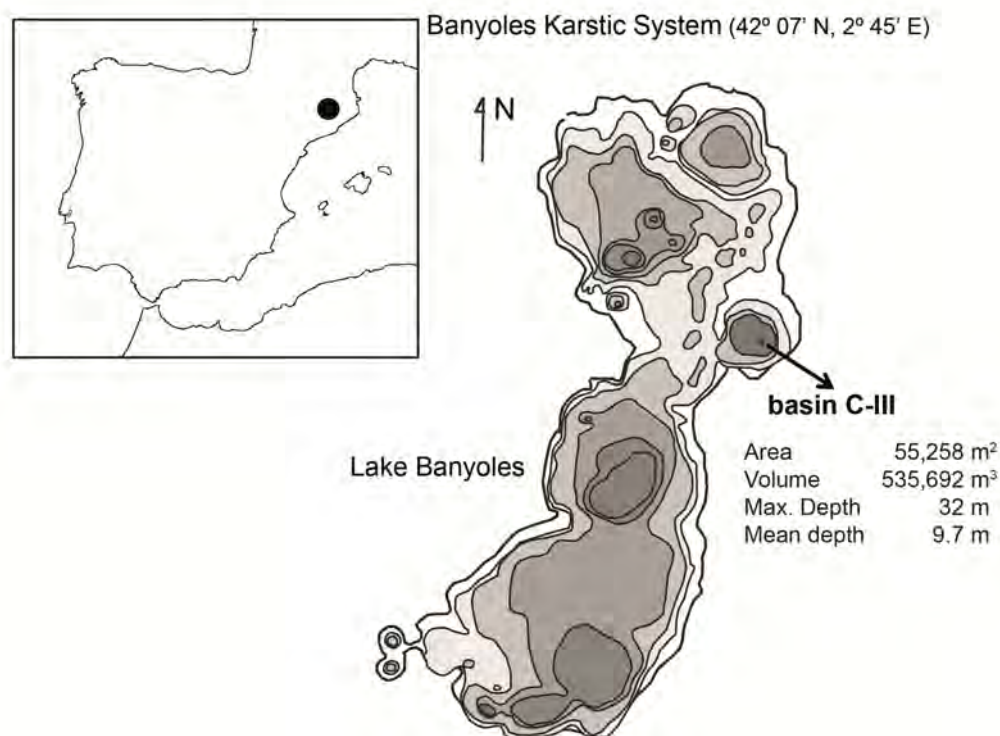
Figure 3.1. Geographic location of the Banyoles Karstic System and map of Lake Banyoles showing the location of basin C-III and main morphometric characteristics. Modified from Moreno-Amich and Garcia-Berthou (Moreno-Amich and Garcia-Berthou, 1989).

Table 3.1. Dates and depths sampled in the basin C-III of Lake Banyoles between winter 2011 and winter 2014.

Date	Depth (m)
winter 2011 (25th January)	21, 21.75, 22, 22.5, 25
summer 2011 (5th July)	18.5, 19.5, 19.75, 20, 24
fall 2011 (19th October)	18, 18.5, 24
spring 2012 (27th March)	21.5, 21.75, 22, 22.5, 23.5
summer 2012 (12th July)	19.75, 20, 20.25, 20.3, 20.5, 20.75, 21, 22.5
fall 2012 (7th November)	16, 17, 18, 18.5, 19, 19.25, 19.5, 20, 20.5, 28.5
winter 2013 (15th January)	3, 12, 20, 20.5, 20.75, 21, 21.25, 21.5, 21.75, 22, 22.25, 23, 24
summer 2013 (16th July)	3, 7, 10, 18, 19, 19.25, 19.5, 19.75, 20, 20.25, 20.75, 21, 21.5, 22.5
fall 2013 (26th November)	6, 16, 16.5, 16.75, 17, 17.25, 17.5, 17.75, 18, 21
winter 2014 (13th January)	4, 19, 19.25, 19.5, 19.75, 20, 24

Water samples for biological and chemical analyses were collected at selected depths with special focus on the oxic–anoxic interface (18m to 22 m depth depending on the season that the sampling took placed) 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 et al., 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 were collected in sterile screw-capped glass tubes, alkalinized by adding NaOH (0.1 M final concentration) and sulfide immediately fixed by adding zinc acetate (0.1 M final concentration). Sulfide was later analyzed by the leucomethylene-blue method (Trüper 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 sulfate 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 (4x250 mm) with the AG Guard column (4x50 mm), using 30 mM MSA for the production of the mobile phase for cations and 22 to 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 analyzed in a Total Organic Carbon analyzer (TOC-V CSH, Shimadzu). Total phosphorous (TP) was measured according to UNE-EN ISO 6878.

3.2. Catalized Reported Deposition-Fluorescence *In Situ* Hybridization (CARD-FISH)

CARD-FISH is an improvement of the Fluorescence *In Situ* Hybridization (FISH) techniques, which is especially suitable for the quantification of microorganisms in aquatic habitats and complex environmental matrices since it provides enhanced fluoresce signal, thus improving the detection and enumeration of target cells (Pernthaler et al., 2002). In CARD-FISH, the oligonucleotide probe is bound to a large horseradish peroxidase (HRP) enzyme, which catalyzes the deposition of many fluorescent tyramide molecules, enhancing the fluorescence intensity signal (Fig. 3.2). Notwithstanding this advantage, cell permeabilization protocols need to be adjusted to enable the larger enzyme-labeled oligonucleotides to penetrate into the cells (Pernthaler et al., 2002).
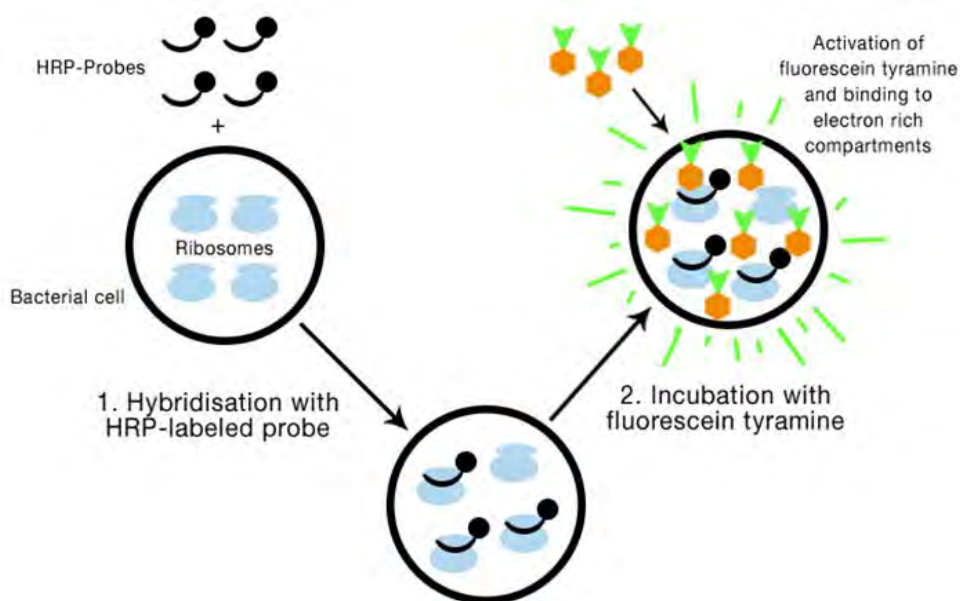
Figure 3.2. Principle of Catalyzed Reported Deposition-FISH. From SILVA webpage (http://www.arb-silva.de/fish-probes/fish-protocols/)

In the current study we made use of CARD-FISH to quantify the abundance of members of the Class *Epsilonproteobacteria* and the Phylum *Chlorobi* using the specific probes EPSY914 (Loy et al., 2007) and GSB-532 (Tuschak et al., 1999), respectively (Table 3.2). Filter sections were counter-stained with 4'-6-diamidino-2-phenylindole (DAPI) (1 µg mL^{-1}) to enumerate total cells. Between 250 and 600 DAPI-stained cells were counted in 20 randomly selected microscopic fields using an Axioskop epifluorescence microscope (Zeiss, Germany).

3.3. Biological uptake of inorganic carbon

The bulk uptake of inorganic carbon was measured by *in situ* incubations carried out in winter (15th January, 2013) and summer (16th July, 2013) as previously described (Camacho and Vicente, 1998). For each sample, a 60-mL plastic flask fully filled with water to minimize oxygenation was used. Samples were spiked with radiolabeled bicarbonate (NaH^{14}CO$_3$; specific activity 4 µCi, DHI, Denmark) to a final concentration of 0.07 µCi mL^{-1}. The *in situ* incubations lasted 4 h (noon period) under at depth light conditions. At every sampled depth, 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 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 (0.5M) 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 et al., 1993). Almost identical results were obtained from duplicate subsamples and the results are presented as mean values. Values of inorganic carbon assimilation for each sample were obtained using the follow conversion factors (Wetzel and Likens, 2000):

$$^{12}\text{C assimilated} = \frac{^{14}\text{C assimilated} \cdot 1.06^{a} \cdot \quad ^{12}\text{C available}^{b} \cdot \text{Sample volume}}{^{14}\text{C added} \cdot \text{Incubation time} \cdot \text{Filtered volum}} \cdot 1000^{c}$$

[a] Correction for $^{12}C/^{14}C$ isotopic discrimination
[b] mg C L^{-1} available
[c] Factor for converting mg C L^{-1} h^{-1} to μC L^{-1} h^{-1}

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 Garcia-Berthou, 1989; Casamitjana et al., 2006).

Table 3.2. PCR/qPCR primers targeting 16S rRNA gene sequences and CARD-FISH probes used in this study.

Target	Primer/Probe[a,b]	Sequence 5'-3'	PCR or qPCR thermal gradient	qPCR R²	Efficiency (%)	Reference
Bacteria	Bac27F	AGAGTTTGATCMTGGCTCAG	35cycles: 30'' at 94°C 60'' at 52°C 2' at 72°C			(Lane, 1991; Weisburg et al., 1991)
	Univ1492R	CGGTTACCTTGTTACGACTT				
	1048F	GTGSTGCAYGGYTGTCGTCA	35 cycles: 3' at 95°C 15'' at 95°C 60'' at 60°C	0.99	99	(Maeda et al., 2003)
	1194R	ACGTCRTCCMCACCTTCCTC				
Epsilonproteobacteria[c]	**EPSY914**	**GGTCCCCGTCTATTCCTT**				(Loy, 2003)
Green Sulfur Bacteria[c]	**GSB532**	**TGCCACCCTGTATC**				(Tuschak et al., 1999)
Arcobacter spp.	76F	GTGGCGCACGGGTGAGTAATA	50 cycles: 10' at 95°C 15'' at 95°C 1' at 60°C	0.99	88	This study
	243R	GCCATTACCCACCAACTATCTG				
	Arco Probe	**6FAM-TCTTTCCCTTTACGAACTTATGTTCAAAAGGCA-TAMRA**				
DNA IAC[d,e]	IAC F	TACGGATGAGGAGGACAAAGGA				(Lopez-Siles et al., 2014)
	IAC R	CACTTCGCTCGATCCATTGG				
	IAC Probe	**VIC-CGCCGCCTATGGGCATCGCA-TAMRA**				

[a] Probe sequences are in bold.
[b] Primers and probes were used in the different chapters, as follows: All Bacteria primers and probes targeting Epsilonproteobacteria and Green Sulfur Bacteria (Chapter 4); qPCR primers targeting Bacteria and *Arcobacter* spp. as well as primers and probe for the Internal Amplification Control (Chapter 5); qPCR primers targeting Bacteria (Chapter 6).
[c] The percentage of formamide used for hybridization step was 55% for EPSY914 and 20% for GSB532.
[d] IAC, Internal Amplification Control; DNA IAC sequence (5'-3'): TACGGATGAGGAGGACAAAGGACAGCGCCGCTATGGGCATCGCACCAATGGATCAGAGCGAAGTG.
[e] qPCR thermal gradient and properties specified refer to the *Arcobacter* spp. qPCR, in which we use an Internal Amplification Control (IAC) (Lopez-Siles et al., 2014).

Table 3.3. Primers targeting functional genes used in the current study.

Target	Primer[a]	Sequence 5′-3′	PCR thermal gradient	Reference
aclB gene	aclB892F	TGGACMATGGTDGCYGGKGGT	2 cycles: 2′ at 94°C 2′ at 37°C 3′ at 72°C 35 cycles: 30′′ at 94°C 30′′ at 54°C 1′ at 72°C	(Campbell et al., 2003)
	aclB1204R	ATAGTTKGGSCCACCTCTTC		
aprA gene	aps1F	TGGCAGATCATGATYMAYGG	30 cycles: 30′′ at 94°C 30′′ at 58°C 40′′ at 72°C	(Meyer and Kuever, 2007)
	aps4R	GCGCCAACYGGRCCRTA		
dsrB gene	DSRp2060F	CAACATCGTYCAYACCCAGGG	30 cycles: 30′′ at 94°C 30′′ at 54°C 40′′ at 72°C	(Geets et al., 2006) (Wagner et al., 1998)
	DSR4R	GTGTAGCAGTTACCGCA		
soxB gene	soxB432F	GAYGGNGGNGAYACNTGG	30 cycles: 30′′ at 94°C 30′′ at 58°C 40′′ at 72°C	(Petri et al., 2001)
	soxB693B	TANGGRAANGCYTGNCCGAT		

[a] *aclB* gene primers were used in Chapter 4 whereas the remaining primers were used in Chapter 6.

3.4. Microautoradiography-CARD-FISH (MAR-CARD-FISH)

CARD-FISH combined with microautoradiography (MAR-CARD-FISH) is used to obtain qualitative information on substrate uptake by individual members of specific prokaryotic communities (Sintes and Herndl, 2006). Although the technique cannot be used to quantify the rate at which each cell incorporates the substrate, MAR-CARD-FISH may provide valuable quantitative information about the fraction of target cells that uptake the radiolabeled substrate.

MAR-CARD-FISH was carried out as previously described (Llirós et al., 2011) with the following modifications according to the conditions of this particularly study. Briefly, 350 µL of 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.

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. Incubation periods of 4, 8, 15 and 28 days were tested. The optimal exposure time was determined and resulted in 15 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 in emulsion fixation (KODAKT_{max}; diluted1:4 in Milli-Q water), and two final 30-s consecutive rinsing steps with distilled and tap water.

Finally, 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.

3.5. 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 –80ºC 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 (Llirós et al., 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.

DNA extracts for the amplification of sulfur related functional genes, and subsequent Illumina sequencing (Chapter 6), were purified and concentrated with the commercial kit GFX™ PCR DNA and Gel Purification Kit (GE Healthcare). This purification step was carried out to get rid of residual components of PCR amplification (e.g. PCR enzymes, salts, dNTPs, primers).

3.6. Cloning

PCR amplification of 16S rRNA gene from aquatic bacteria and the *Arcobacter* spp. thriving in basin C-III was carried out using specific primers Bac27F/Univ1492R (Suzuki and Giovannoni, 1996; Engberg et al., 2000) and primer pair 76F/243R (Chapter 5), respectively. Similarly, PCR amplification of the beta subunit of the ATP citrate lyase (*aclB*) was done using specific primer pair aclB892F/aclB1204R (Campbell et al., 2003). Details for each PCR amplification are compiled in Tables 3.2 and 3.3. The bacterial 16S rRNA clone library was constructed using PCR amplicons obtained from a sample

collected at the O_2/H_2S interface (Table 3.4). The *aclB* gene amplicons were obtained from samples where maximal dark fixation rates were measured during both winter and summer *in situ* incubations (Table 3.4). The *Arcobacter* spp. 16S rRNA gene clone libraries were constructed to test the specificity of primers set 76F/243R. *Arcobacter* spp. 16S rRNA gene amplicons were obtained from samples corresponding to different depths and seasons.

Table 3.4. List of different samples (date and depth) used for the construction of bacterial 16S rRNA gene, ATP citrate lyase (*aclB*) gene and the *Arcobacter* spp. 16S rRNA gene clone libraries.

Clone library	Date	Depth (m)
Bacterial 16S rRNA gene	winter 2011 (25th January)	21.75
aclB gene	winter 2013 (15th January)	22
	summer 2013 (16th July)	21
Arcobacter spp. 16S rRNA gene	winter 2013 (15th January)	22
	summer 2013 (16th July)	20.75
	fall 2012 (7th November)	19

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, United Kingdom), 0.16 mM of $MgCl_2$ (QIAGEN, Valencia, USA), 0.26 mM of dATP (PROMEGA, Madison, USA) and Taq polymerase (QIAGEN, Valencia, USA)). Polyadenylation reaction mixtures were incubated 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, 250, 96 clones were randomly picked for *Bacteria* 16S rRNA library, *aclB* gene libraries and *Arcobacter* spp. 16S rRNA 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 and *Arcobacter* spp. 16S rRNA genes sequences were 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 were 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 et al., 1992) and bootstrapping (1,000 replicates).

3.7. Design of specific primers set and TaqMan hydrolysis probe targeting the 16S rRNA gene of *Arcobacter* spp. by qPCR

The use of TaqMan hydrolysis probes is widely extended among different disciplines, especially in medical research, to increase the specificity of quantitative PCR (qPCR). The principle of the method was first reported in 1991 by Holland *et al.* (Holland et al., 1991) (Fig. 3.3).
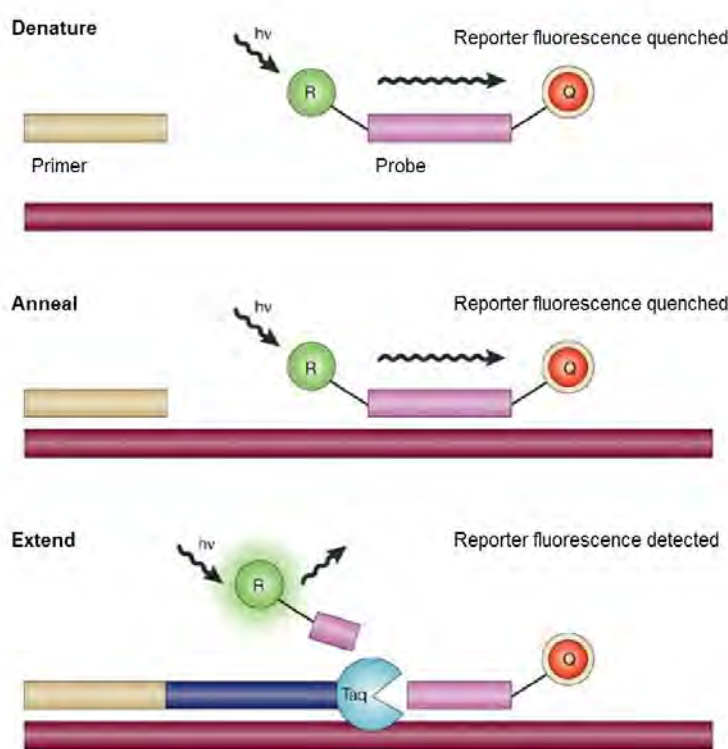
Figure 3.3. Principles of TaqMan probe system. TaqMan probes are designed such that they anneal with the DNA region comprised between a specific set of primers. During the elongation step, the 5′ to 3′ exonuclease activity of the Taq polymerase degrades the probe, previously annealed tot the template. The hydrolysis of the probe relieve the quenching effect achieved by the quencher (e.g. tetramethylIrhodamine: TAMRA) and allows the fluorescence of the fluorophore (e.g. 6-carboxyfluorescein –FAM-). Fluorescence emitted at each qPCR cycle is proportional to the amount of product formed. Modified from Koch (Koch, 2004).

3.7.1. Design of qPCR primers (76F/243R) and hydrolysis TaqMan probe (Arco-TQ probe)

A consensus 16S rRNA gene sequence of the freshwater strain of *Arcobacter* spp. was constructed from a dataset of 104 sequences obtained after curation and alignment of sequences recovered in the clone library from a sample collected at the redoxcline of basin C-III in winter 2011 (25th January, 21.75m). Primer design was conducted in Primer-BLAST (National Center for Biotechnology Information, http://www.ncbi.-nlm.nih.gov/tools/primer-blast) (Table 3.2). Design of the Arco-TQ probe was carried out using Primer Express version 3.0 software (Applied Biosystems, Foster City, CA, USA) (Table 3.2). Thermodynamic characteristics such as G+C content, melting temperature, secondary structure and primer-dimer free energy formation were considered for primer selection after analysis with NetPrimer software (http://premierbiosoft.com/netprimer, PREMIER Biosoft International, California). The Internal Amplification Control (IAC) used for the inhibition control consisted in the combination of a set of primers and a hydrolysis probe as previously described (Lopez-Siles et al., 2014) (Table 3.2).

3.7.2. Optimization of the qPCR

Experiments to evaluate optimal primer and probe concentrations were performed as previously described by Lopez-Siles and co-workers (Lopez-Siles et al., 2014). The addition of 10^4 target copies for the IAC did not affect the efficiency of the freshwater *Arcobacter* spp. qPCR assay. Therefore, this IAC's DNA quantity was further used in all qPCR reactions as inhibition control.

3.7.3. Quantification of standards

A plasmid containing an insert of an environmental 16S rRNA gene sequence of the target *Arcobacter* spp. (previously isolated from a clone library constructed from DNA extracted from winter redoxcline samples) was used as standard in qPCR assays. Plasmids were extracted from a pure culture of a positive clone using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). Purified plasmids were linearized with the restriction enzyme NotI and quantified with Qubit (Invitrogen). Initial target concentration was calculated using the following equation (Whelan et al., 2003):

$$\text{DNA gene copies (copy)} = \frac{6.023 \times 10^{23} \left(\frac{\text{copy}}{\text{mol}}\right) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 \left(\frac{\frac{\text{g}}{\text{mol}}}{\text{bp}}\right)}$$

Standard curves were obtained from 10-fold serial dilutions of the titrated suspension of linearized plasmids, and ranged from 10^3 to 10^8 copies/reaction, which correspond to the linear range span for the reaction.

3.7.4. Specificity tests

Specificity of primers (76F/243R) and the Arco-TQ probe was tested *in silico*, using the TestPrime tool in Silva (http://www.arb-silva.de/search/testprime/) and comparing against the Ribosomal Database Project II (RDP) through Probe Match tool (http://rdp.cme.msu.edu/probematch/search.jsp), respectively. Specificity of the oligonucleotides was also tested empirically. A cloning and subsequent sequencing of qPCR products amplified with 76F/243R primer pair (without Arco-TQ probe) in DNA and cDNA extracts of several redoxcline samples (15th January 2013; 16th July 2013 and 7th November 2012) was carried out to check potential unspecificities of primers used. The combination of primer pair (76F/243R) and the Arco-TQ probe was also tested using 40 clones obtained in clone libraries from winter samples (see above) and DNA extracts from *Arcobacter* spp. (*A. nitrofigilis* DSM-7299, *A .marinus* DSM-24769) and *Sulfurimonas autotrophica* (DSM-16294). The quantitative PCR assay was carried out as described above.

3.7.5. Sensitivity test: confident quantification range and detection limit

To determine the confident quantification range of the assay, ten-fold dilutions of a linearized plasmid containing a single copy of the 16S rRNA gene of *Arcobacter* spp. was used. Two replicas of each dilution were assayed, ranging from 10^9 to 1 gene copies per reaction.

Data was analyzed by a Probit test (Minitab® 14 Statistical Software, Pennsylvania, USA), in which the ratio of positive/negative amplification events was plotted against the amount of target genes present per reaction. The theoretical minimum number of 16S rRNA genes of *Arcobacter* spp. per reaction to have a 95% of probability to obtain a positive detection was 1×10^3 16S rRNA gene copies.

3.8. Quantification of microbial abundance

Quantitative PCR (qPCR) is a highly sensitive method for the quantification of microorganisms from different samples (Walker, 2002). A total of 59 samples collected from basin C-III (Lake Banyoles) at different seasons and depths during different year cycles were quantified for *Bacteria* and *Arcobacter* spp. using this technique (see Table 3.5. for a complete list of samples analyzed).

Table 3.5. List of samples (date and depth) analyzed by qPCR.

Date	Depth (m)
winter 2011 (25th January)	21, 21.75, 22, 22.5, 25
summer 2011 (5th July)	18.5, 19.5, 19.75, 20, 24
fall 2011 (19th October)	18, 18.5, 24
spring 2012 (27th March)	21.5, 21.75, 22, 22.5, 23.5
summer 2012 (12th July)	20, 20.5, 20.75, 21
fall 2012 (7th November)	16, 17, 18, 18.5, 19, 19.25, 19.5, 20, 20.5, 28.5
winter 2013 (15th January)	3, 12, 21, 21.75, 22, 22.25,
summer 2013 (16th July)	3, 10, 19.25, 19.75, 20.25, 21, 22.5
fall 2013 (26th November)	6, 16.5, 16.75, 17, 17.25, 17.75, 18, 21
winter 2014 (13th January)	4, 19.25, 19.5, 19.75, 20, 24

Samples were quantified in duplicate. For data analysis, the mean of duplicate reactions was used. Duplicates were considered valid if the standard deviation between the quantification cycles (C_T) was <0.34. A no-template control consisting of a reaction without target DNA template as well as a no-amplification control which did not contain any DNA template (either bacterial or IAC) were also included in each run. Each measurement was verified and standardized by its own domain-specific standardization derived from a dilution series of a corresponding genomic or environmental target 16S rRNA gene.

3.8.1. *Quantification of bacterial 16S rRNA gene copies*

Copy numbers of bacterial 16S rRNA gene were determined by qPCR from DNA extracts using primers and conditions compiled in Table 3.2. Quantifications were performed in a Mx3005P system (Agilent Technologies) using SYBR Green detection chemistry. Every reaction was prepared for a final volume of 30 µL containing 15 µL of 2x Brilliant III ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies), 1.2 µL of each forward and reverse primer (final concentration of 400 nM), 1 µL of template DNA (10 ng µL^{-1}), and molecular biology grade water up to 30 µL volume. Standards for bacterial quantification were made using genomic DNA of *Escherichia coli* DSM-30083. Primer sequences, reaction temperatures, R^2 values and amplification efficiencies are compiled in Table 3.2.

3.8.2. Quantification of Arcobacter spp. *16S rRNA gene copies*

The abundance of the *Arcobacter* spp. was determined by using a novel assay following guidelines of the RT PCR system for probe design (Applied Biosystems, Foster city, CA, USA) (See Chapter 5). Amplification reactions were carried out in a total volume of 20 µL containing: 1x TaqMan Universal PCR Master Mix 2x (Applied Biosystems, Foster City, CA, USA), 900 nM of 76F and 243R of each primer, 300 nM of the internal amplification control (IAC) primers set, and 250 nM of each probe, 10^4 copies of an IAC template and 1µL of genomic DNA template (10 ng µL^{-1}). Primer sequences, reaction temperatures, R^2 value and amplification efficiency are compiled in Table 3.2. All qPCR runs were performed using a 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Data was collected and analyzed with the 7500 SDS system software version 1.4 (Applied Biosystems, Foster City, CA, USA).

3.9. Conversion of 16S rRNA gene copy number to cell concentration

The 16S rRNA gene copies were transformed to cell numbers assuming average values of 4.02 and 4.80 ribosomal RNA operons per cell for members of the Domain *Bacteria* and for *Arcobacter* spp., respectively (The Ribosomal RNA Database under the NCBI taxonomy, Center for Microbial Systems, University of Michigan, http://rrndb.umms.med.umich.edu/search/) (Stoddard et al., 2014).

3.10. High throughput sequencing and data processing

3.10.1. Pyrosequencing of bacterial 16S rRNA gene

Pyrosequencing of bacterial 16S rRNA genes was carried out in DNA extracted from selected depths and seasons (Table 3.6). Pyrosequencing was performed at Research and Testing Laboratory facilities (RTL) (Texas, USA) using Roche 454 FLX-Titanium technology (Dowd et al., 2008). Amplification reactions were carried out 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 Operational Taxonomic Units (OTUs) (97% cutoff) using MOTHUR (Schloss et al., 2009). OTUs with less than 4 members were removed from downstream analyses to avoid the inflation of diversity estimates by spurious OTUs. 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.mo-

thur.org). Alpha-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 1,600 sequences from each sample to minimize bias due to different sampling efforts.

Table 3.6. Samples (date and depths) used for high throughput sequencing.

Date	Depth (m)
winter 2011 (25th January)	21.75
spring 2012 (27th March)	22.5
summer 2012 (12th July)	19.75, 21
winter 2013 (15th January)	22
summer 2013 (16th July)	10, 19.75, 20.25, 22.5
winter 2014 (13th January)	4, 19.5, 19.75, 20, 24

3.10.2. Functional genes sequencing using Illumina and data processing

Genes encoding three key enzymes of sulfur cycle, namely: the adenosine-5'-phosphosulfate reductase subunit A (*aprA*), the dissimilatory sulfite reductase subunit B (*dsrB*), and the hydrolytic enzyme soxB from the sulfur-oxidizing multi-enzyme Sox system (*soxB*), were amplified with the sets of primers aps1F/aps4R (Birte Meyer and Kuever, 2007), DSRp2060F/DSR4R (Wagner et al., 1998; Geets et al., 2006) and soxB432F/soxB693B (Petri et al., 2001), respectively. Amplification of sulfur genes was carried out in DNA extracts obtained from selected depths and seasons (Table 3.7).

Table 3.7. Samples (date and depth) used for Illumina high throughput sequencing.

Date	Depth (m)
spring 2012 (27th March)	22.5
summer 2013 (16th July)	10, 19.75, 22.5
winter 2014 (13th January)	4, 19.5, 19.75, 20, 24

Amplifications were carried out in an automatic thermocycler (9700 GeneAmp) with cycling programs described in Table 3.3. The PCR reactions were prepared in a final volume of 50 µL containing 2 µL of DNA extraction of the environmental sample, 0.2 µM of each primers used, 0.4 mM of dNTPs, 2 mM of $MgCl_2$, 1U of Taq polymerase (Eurobio) and its reaction buffer 1x final concentration. Verification of PCR amplicons was done with gel electrophoresis using an agarose gel 1% at 100 volts.

To determine the composition of sulfate reducing and sulfur-oxidizing bacteria communities, the aforementioned amplicons were further amplified using specific primers containing the proper adapters (CTTTCCCTACACGACGCTCTTCCGATCT and GGAG-TTCAGACGTGTGCTCTTCCGATCT for forward and reverse primers respectively). Amplicons were then sequenced using the MiSeq Technology 300, 250, and 150 paired-end technology (Illumina) depending on the size of amplicons, as described Aubé (Aubé, 2014).

Sequence datasets obtained were analyzed using a ng6 NGS platform genefunc pipeline specially designed for this type of analyses (Mariette et al., 2012) and processed as previously described (Aubé, 2014). Briefly, Flash (Magoč and Salzberg, 2011) was used to concatenate sequences and to reduce the number of N at the end of sequences. The analysis was performed with minimum and maximum overlaps defined in Table 3.8 and with a maximum mismatch ratio of 10%. Sequences were quality filtered according to their size, number of ambiguities (any N permitted) and presence of chimeras using the *uchime-denovo* algorithm implemented in USEARCH (Edgar et al., 2011). Taxonomic affiliation and translation to aminoacid sequences were carried out using databases specially constructed for the selected genes using reference sequences available at NCBI (Aubé, 2014). Databases for *aprA*, *dsrB*, *soxB* contained respectively 161, 250 and 96 protein sequences representing most of the known genera harboring these genes (Aubé, 2014). Sequences were translated using Framebot (Wang et al., 2013). Protein sequences containing a stop codon were discarded. Curated peptide sequences obtained were then clustered into operational taxonomic units (OTUs) with CD-HIT (Li and Godzik, 2006) using a cut-off of 91, 95 and 94% for aprA, dsrB and soxB respectively as previously defined (Hügler et al., 2010; Colin et al., 2013; Watanabe et al., 2013). Singletons were discarded to avoid the presence of spurious OTUs generated during amplification steps and minimize the overestimation of the actual diversity. Iterative random subsampling (100 iterations) to the minimum number of sequences was carried out for each sample to minimize bias caused by different sampling effort across samples (Table 3.8). Taxonomic assignment of sequence clusters (*i.e.* OTUs) was done using blasp (Camacho et al., 2009) allowing a maximum e-value of 1.10 10^{-5}. Representative sequences for each OTU were then aligned together with reference sequences for each gene using ClustalW.

Table 3.8. Parameters used in the analysis of functional genes.

Gene	Size of the database	Length of reads (bp)	Minimum overlap (bp)	Maximum overlap (bp)	Identity threshold (%)	Number of subsampled sequences
aprA	161	300	190	230	91	2000
dsrB	250	250	200	240	95	2000
soxB	96	250	120	160	94	670

3.11. Statistical analyses

The non-parametric Spearman's rank correlation (Spearman's rho) was used to test statistical dependence between Epsilonproteobacteria and GSB abundance (CARD-FISH counts) for winter and summer samples (Chapter 4).

The non-parametric Kruskal-Wallis test was used for comparing abundances of *Arcobacter* spp. between seasons (qPCR data, Chapter 5). The non-parametric Wilcoxon signed-rank test was run to compare the related samples of *Arcobacter* spp. abundance measured by qPCR and Epsilonproteobacteria abundance measured by CARD-FISH, to assess whether their abundances mean ranks differ for each water compartment among different seasons (Chapter 5).

In all tests, data on Epsilonproteobacteria, GSB, and *Arcobacter* spp. abundance (in cells mL^{-1}) was logarithmically transformed. All statistical tests were carried out in SPSS software package (v21, SPSS Chicago, IL, U.S.A.).

3.12. Sequence submission and accession numbers

Pyrosequencing datasets (Chapter 4) have been deposited in the NCBI database via the Biosample Submission Portal (http://www.ncbi.nlm.nih.gov/biosample/) under accession number PRJNA282702. 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 & DISCUSSION

4

DIVERSITY OF FRESHWATER EPSILONPROTEOBACTERIA AND DARK INORGANIC CARBON FIXATION IN THE SULFIDIC REDOXCLINE OF BASIN C-III

In freshwater systems, the distribution of epsilonproteobacterial sulfide oxidizers has only been reported in terrestrial caves and springs, where they largely contribute to the development of sulfide-oxidizing streambed biofilms (Elshahed et al., 2003; Porter and Engel, 2008; Porter et al., 2009; Rossmassler et al., 2012). Remarkably, very little information is available for the distribution, abundance and activity of Epsilon-proteobacteria in stratified lakes (Biderre-Petit et al., 2011; Hamilton et al., 2014). This is especially contradictory considering that most of these waterbodies are characterized by sharp O_2/H_2S interphases that provide an optimal niche for the growth and activity of epsilonproteobacterial sulfide-oxidizers.

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 et al., 2008, Casamayor, 2010). Similar results were obtained in other stratified lakes with sulfidic redoxclines (Cloern et al., 1983; Camacho et al., 2001; Hadas et al., 2001; Casamayor et al., 2012) pointing out the

ecological significance of chemolithotrophic processes even in the presence of oxygenic and anoxygenic autotrophs.

The aim of this chapter is to investigate whether or not Epsilonproteobacteria constitute stable populations at the redoxcline of a meromictic basin in Lake Banyoles where Green Sulfur Bacteria (GSB) seasonally bloom, providing data on their diversity, activity and contribution to the (dark) inorganic carbon fixation in the system. Accordingly, the composition of the planktonic bacterial community was studied by analyzing 16S rRNA gene pyrotag libraries from different depths and seasons. Abundances of Epsilonproteobacteria and Green Sulfur Bacteria were determined by CARD-FISH using specific probes for both groups. The contribution of different autotrophic metabolisms to total inorganic carbon fixation was measured via *in situ* incubations of radiolabeled bicarbonate (see M&M for more details).

4.1. Results

4.1.1. *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. 4.1A). 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. 4.1B). 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. 4.2). Oxygen peaks of up to 14.3 mg L^{-1} were measured at the mixolimnion (8–10 m depth) in spring and summer as 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. 4.2). Redox potential dropped at the chemocline depth coinciding with oxygen extinction and sulfide diffusion from anoxic bottom waters, generating a sharp redoxcline (Fig. 4.1). 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, sulfate concentrations in this euxinic bottom water layer were similar in both winter and summer periods (average of 12.16±0.80 mM 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 µM 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. 4.2). Nitrite was always below detection limit in all samples analyzed 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).
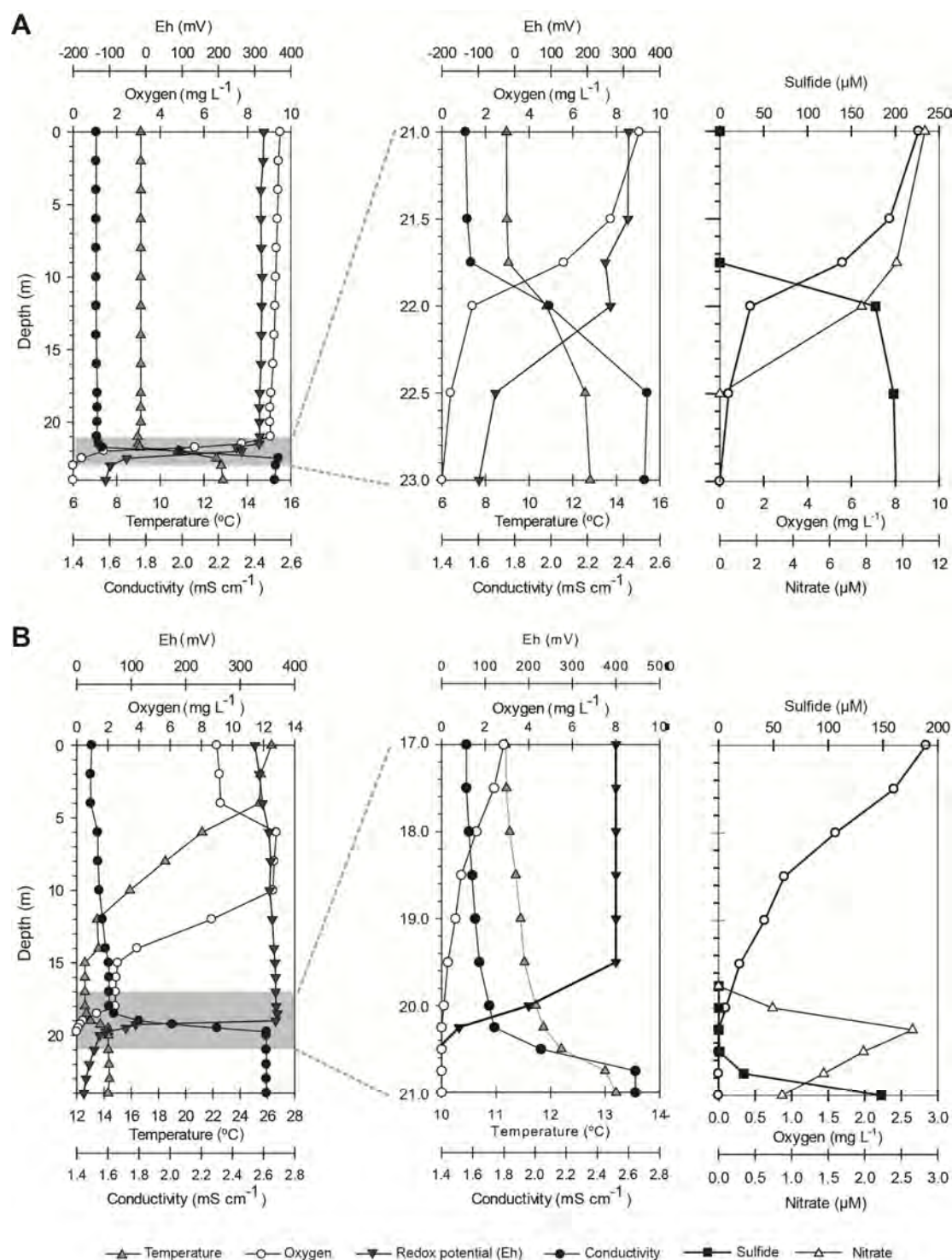
Figure 4.1. Physicochemical profiles of the water column of basin C-III in (A) winter 2011 (25th, January) and (B) summer 2012 (12th, July). Right panels show an enlarged view of the physicochemical gradient at the sulfidic redoxcline (shaded in grey) and the profiles of potential electron donors (*i.e.* sulfide) and acceptors (*i.e.* oxygen and nitrate) for Epsilonproteobacteria.
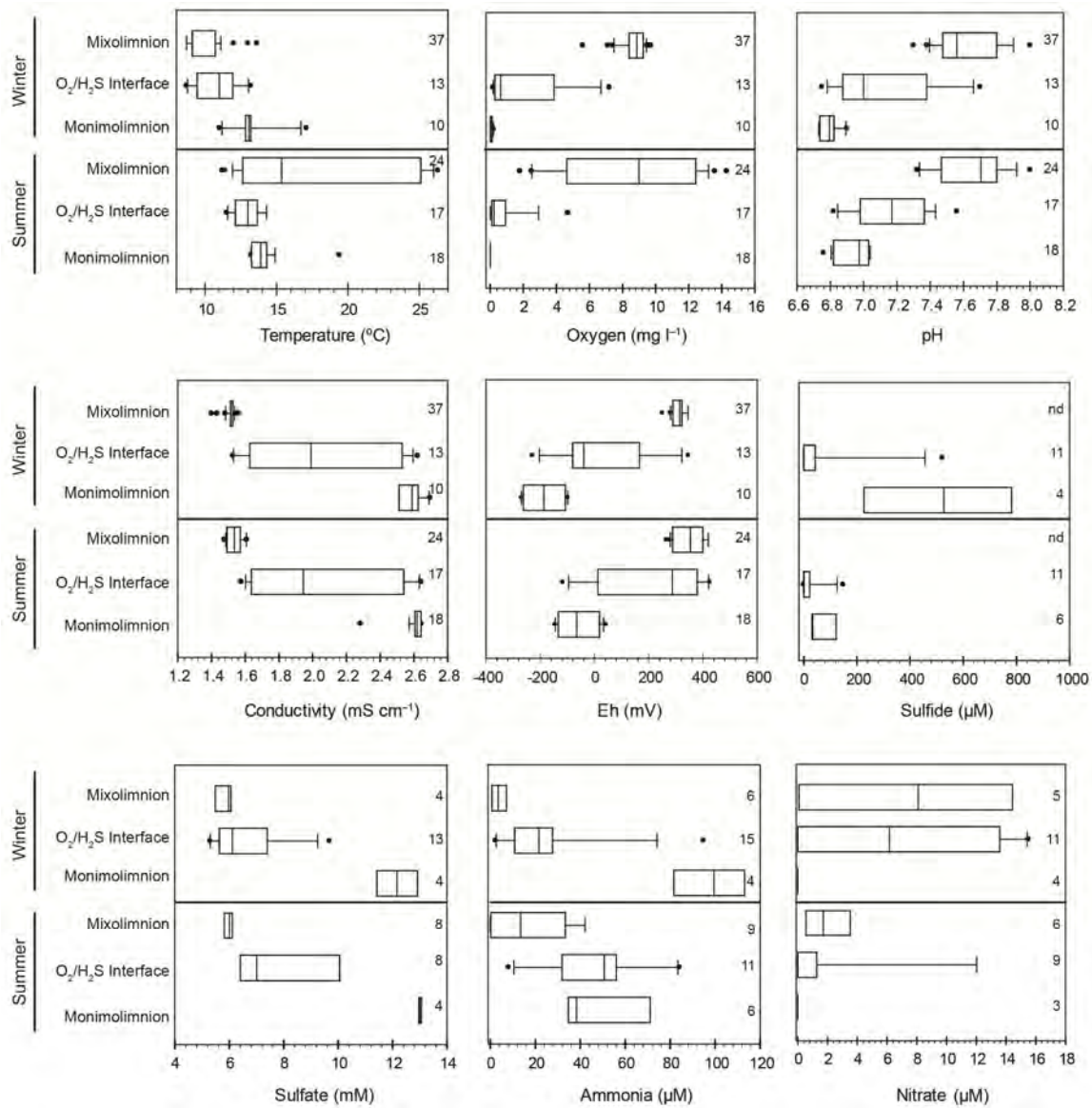
Figure 4.2. Boxplots for the physicochemical variables measured in different water compartments of basin C-III during winter and summer sampling periods. Boxplots show median, first and third quartiles, and range. The number of samples included in each case (n) is indicated at the right side of each boxplot.

4.1.2. 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 25th January, 2011; 22.5 m on 27th March, 2012; 19.75 m and 21 m on 12th July, 2012; 22 m on 15th January, 2013; and 20.25 m on 16th July, 2013) (Fig. 4.3). Sequences affiliated to Class *Epsilonproteobacteria* were predominant in pyrotag libraries from the two winter samples analyzed (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 *Desulfomonile*, 63.6% of total delta-proteobacterial 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 analyzed, reaching their maximal contributions at the upper layers of the redoxcline in summer 2012 (19.75 m, Fig. 4.3). 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 4.1).

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. 4.3). These sequences grouped into two OTUs (97% cutoff) that clustered together and were closely related to *Arcobacter butzleri* (Fig. 4.4). To better resolve the taxonomic assignment of the dominant 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, 25th January 2011). Sequences affiliated to class *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, sulfide-oxidizer marine *Epsilonproteobacteria* (Wirsen et al., 2002) (Fig. 4.5). Of the remaining 5 clones, 3 of them grouped into unique OTUs also showing high sequence identity to *Candidatus* Arcobacter sulfidicus whereas 2 of them (OTUs 15 and 17) clustered with *Sulfuricurvum kujiense* 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. 4.4).

Table 4.1. Coverage and richness and diversity indices calculated from pyrotag datasets obtained from analyzed samples. Winter samples are shaded in grey.

Sample (date/depth)	Coverage	Richness		Diversity
		Sobs	Chao1	Shannon
25/01/2011 (21.75 m)	0.99	66	98	1.8
27/03/2012 (22.5 m)	0.99	35	136	1.0
12/07/2012 (19.75 m)	0.99	66	99	1.9
12/07/2012 (21 m)	0.99	45	100	1.2
15/01/2013 (22 m)	0.99	60	114	1.2
16/07/2013 (20.25 m)	0.99	75	110	2.2

4.1.3. 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 analyzed 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. 4.3), 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 sulfide co-occurred 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. 4.3). This general trend of seasonal distribution was also confirmed after analysis of pyrotag libraries from selected depths (Fig. 4.3). 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 per genome, Klappenbach et al., 2001). Particularly, whereas the relative abundance of Epsilonproteobacteria ranged between 9 to 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. 4.3).
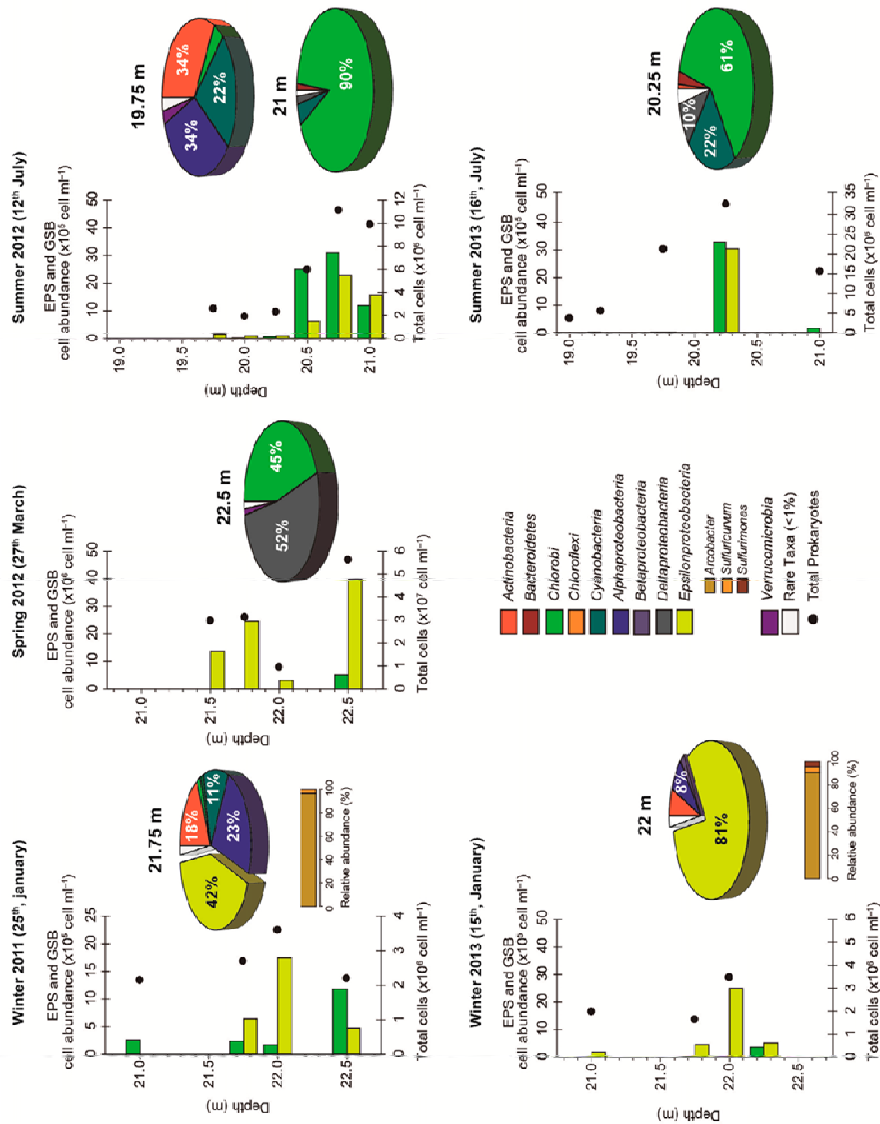
Figure 4.3. Abundance (in cells/mL) of total cells (black dots), Epsilonproteobacteria (yellow bars) and Green Sulfur Bacteria (green bars) measured by CARD-FISH at the O_2/H_2S interface (i.e. redoxcline) of basin C-III on selected dates of different annual cycles. Pie charts show the phylogenetic composition (relative abundance of different phyla –classes for *Proteobacteria*–) of planktonic communities at selected depths. The relative composition of the epsilonproteobacterial community (% of total epsilonproteobacterial-assigned sequences) is also indicated for winter samples.
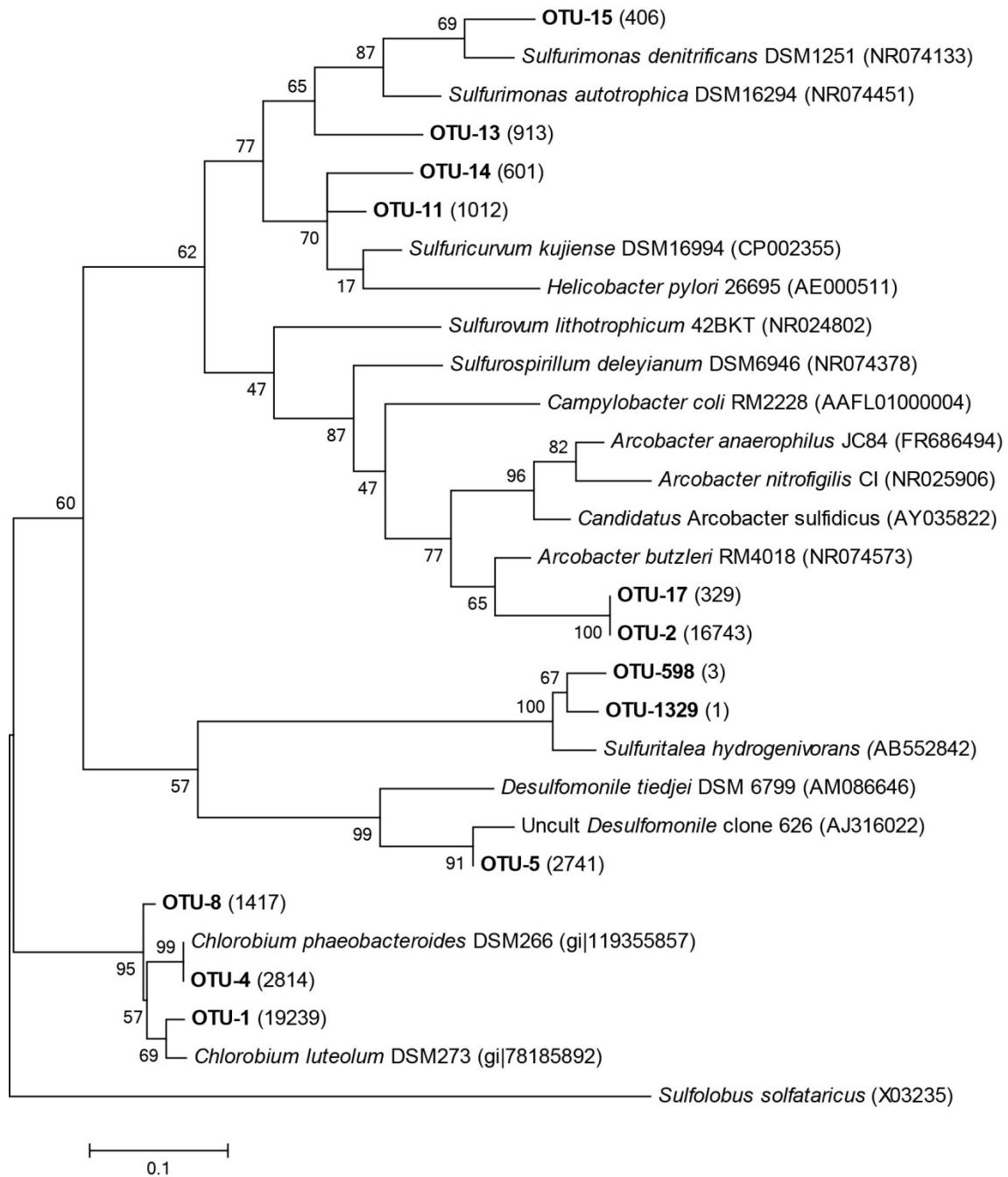
Figure 4.4. Maximum likelihood phylogenetic tree showing the affiliation of OTUs identified in pyrotag 16S rRNA gene libraries from basin C-III at different dates. OTUs were delineated at 97% cutoff. The number of sequences for each OTU is indicated between brackets. Node numbers refer to bootstrap support in percentage (1,000 replicates).
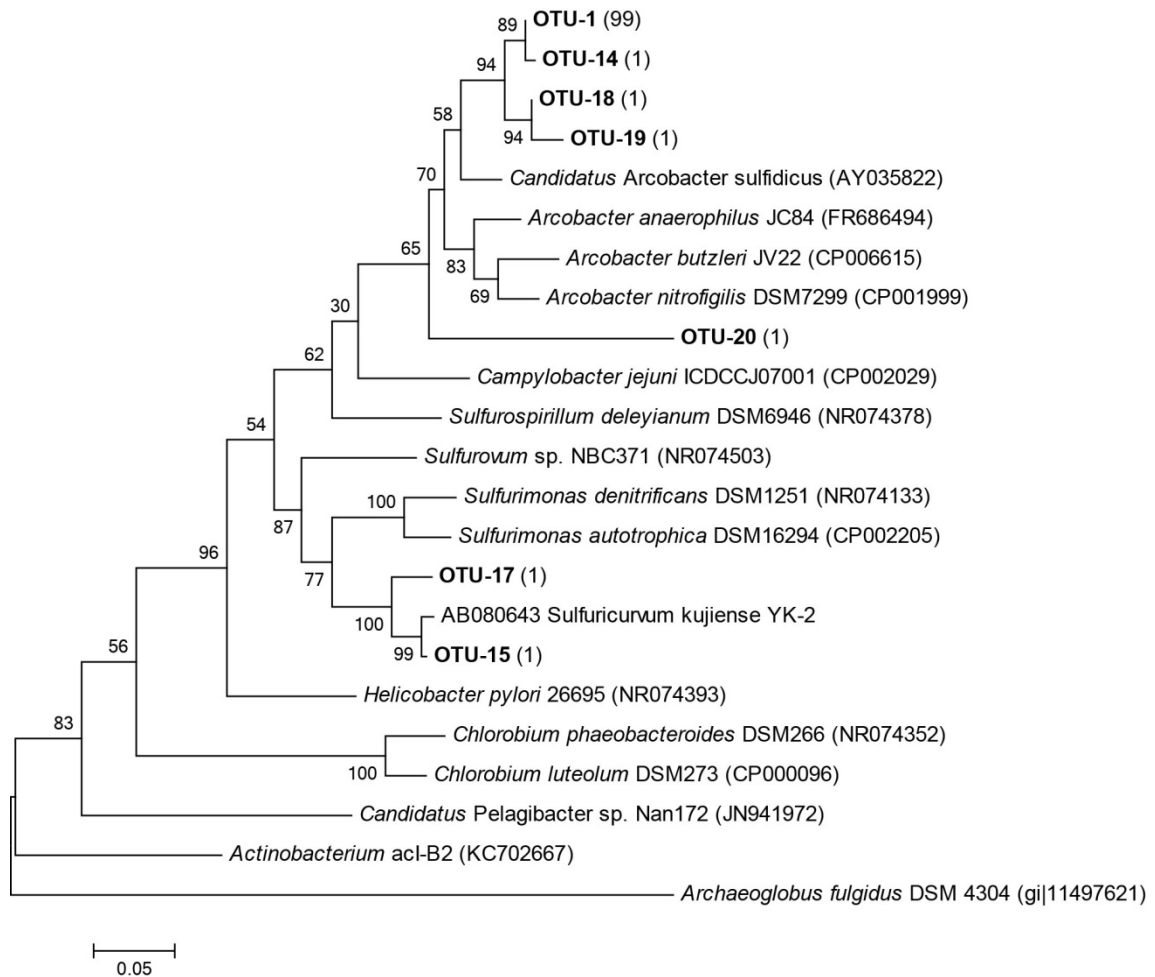
Figure 4.5. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences obtained in the clone library of a sample collected at the O_2/H_2S interface in winter (21.75m; 25th January, 2011). The number of sequences for each OTU is indicated in parentheses. Numbers at nodes indicate bootstrap support in percentage (1,000 replicates).

A specificity check of probe EPSY914 using the TestProbe tool available at the SILVA database (http://www.arb-silva.de/search/testprobe/) confirmed inespecificities of this probe against members of the phylum *Chlorobi* (*Chlorobium limicola* and *Chl. phaeobacteroides*) and the Class *Deltaproteobacteria* (*Desulfomonile tiedjei*) with 2 mismatches (Table 4.2). Both phyla were prevalent in the water column of basin C-III in summer and spring, respectively (Fig. 4.3). 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 *Chlorobium* cells on the quantification of Epsilonproteobacteria by CARD-FISH using probe EPSY914 (Fig. 4.6). 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. 4.3). 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).

Table 4.2. Unspecificities of probe EPSY914 against different bacterial species (SILVA_SSU_r121 database).

Strain	Accession number	Mismatches	Position[1]	EPSY914 sequence match[2] (5'-AAGGAAT**A**GACGGGG**A**CC-3')
Chlorobium limicola DSM 246	AJ290824	2	913	TGAAACTCA ······T ······G · CGCACAAGC
Chlorobium phaeobacteroides DSM 1855	AJ290832	2	913	TGAAACTCA ······T ······G · CGCACAAGC
Desulfomonile tiedjei DSM 6799	AM086646	2	913	TAAAACTCA ······T ······G · CGCACAAGC

[1] According to *E. coli* position.
[2] Reverse complement of EPSY914 probe sequence
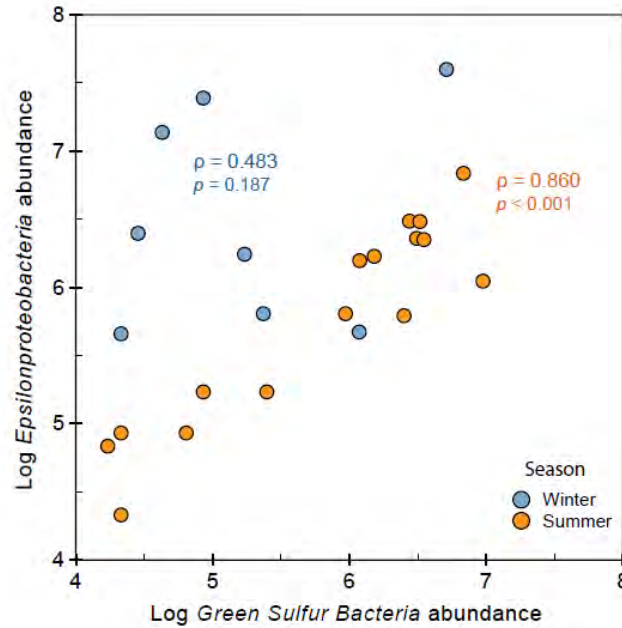
Figure 4.6. Scatter plot between log counts of Green Sulfur Bacteria (GSB), using probe GSB532) and Epsilonproteobacteria (EPS, using probe EPSY914) in summer and winter samples collected at the O_2/H_2S interface of basin C-III. Spearman correlation coefficients and *p* values for winter and summer samples are indicated.

4.1.4. 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 sulfide-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. 4.7A). In winter these rates were almost six-fold higher than maximum rates of 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. 4.8A). 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 where performed in summer, coinciding with the minimum abundance of Epsilon-

proteobacteria 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. 4.7B). 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, 16th January 2013). Deposition of silver granules around cells hybridized with probe EPSY914 was clearly visible in MAR-CAR-FISH images (Fig. 4.9). 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 4.3). Being aware of the potential overestimation of Epsilonproteobacteria due to non-specific binding of probe EPSY914 (see above), we analyzed a cDNA-based pyrotag library from the same sample. This dataset revealed the prevalence of epsilonproteobacteria-assigned reads (88.2% of total sequences, Table 4.1) and provided additional evidence that epsilonproteobacterial cells were active members of the planktonic assemblage at this depth.
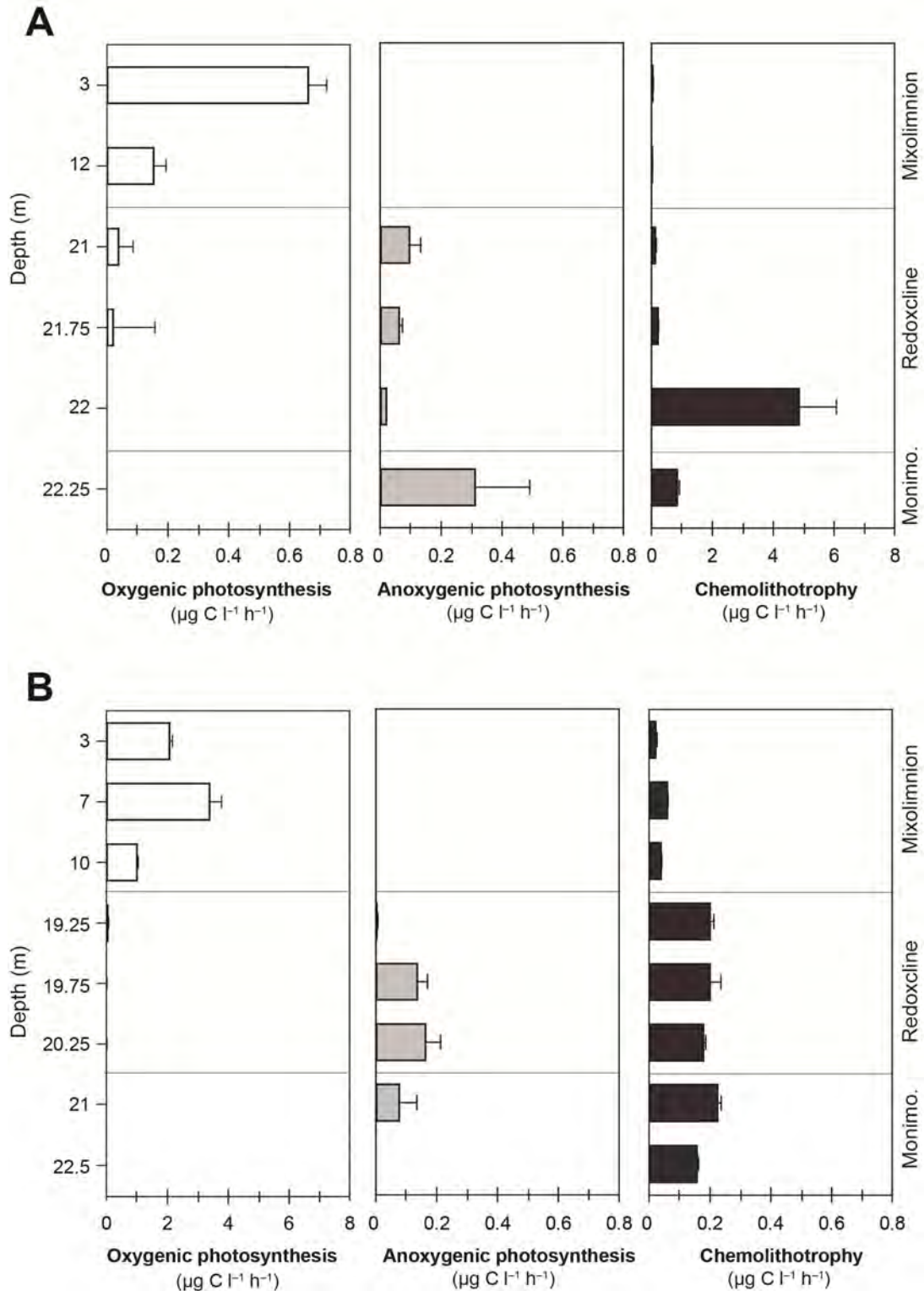
Figure 4.7. ^{14}C fixation rates by oxygenic and anoxygenic phototrophs and by chemolithotrophic microorganisms at different water compartments (mixolimnion, redoxcline, monimolimnion) of basin C-III calculated from (A) winter and (B) summer *in situ* incubation data. Values are the average of measurements made on sample duplicates (± standard error of the mean). Note the differences in the scale of X-axis.
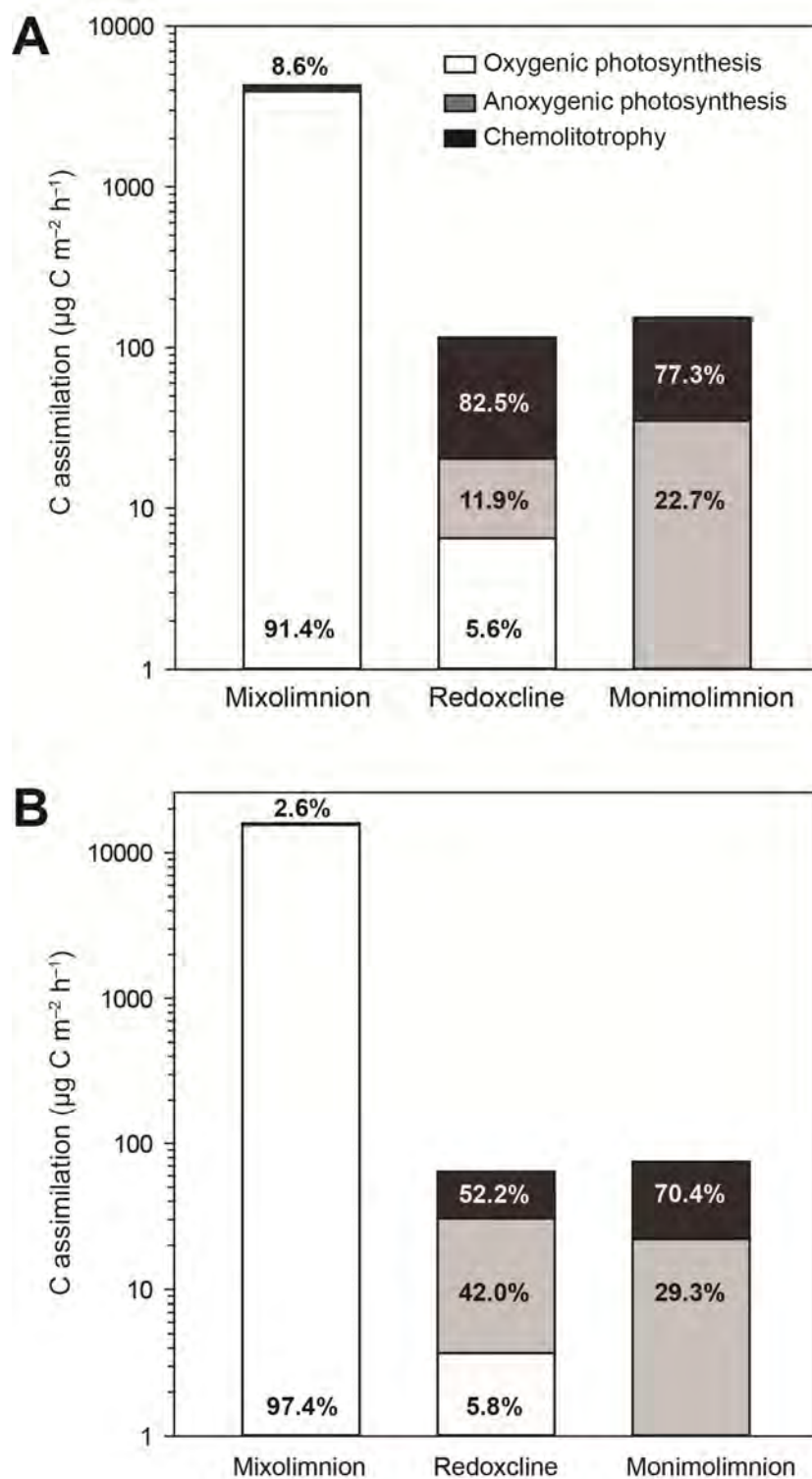
Figure 4.8. Cumulative contribution (total inorganic carbon assimilation and % contribution at each water layer) of oxygenic and anoxygenic photosynthesis, and chemolithotrophy, to inorganic C fixation in different water compartments of basin C-III estimated from (A) winter and (B) summer *in situ* incubation data and considering the lake hypsography. Note that the Y-axis scale is logarithmic.
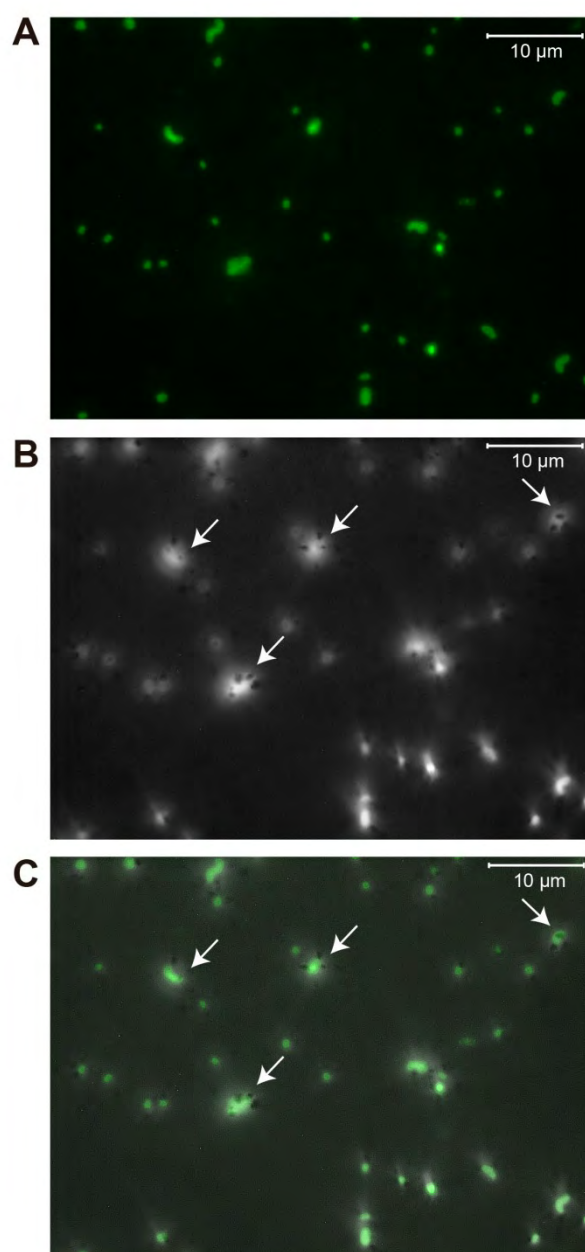
Figure 4.9. Micrographs obtained after MAR-CARD-FISH analysis of the winter sample where the maximal dark carbon fixation rates were measured (16th January, 2013, 22m). (A) epsilonproteobacterial cells showing positive hybridization with probe EPSY914, (B) Deposition of silver granules at the periphery of cells actively uptaking radiolabeled bicarbonate. (C) Merged image showing that most active cells hybridized with probe EPSY914.

Table 4.3. Abundance of Bacteria and Epsilonproteobacteria (EPS), contribution of EPS to total *Bacteria*, percentage of active EPS uptaking CO_2 and percentage of cDNA-based pyrotag reads assigned to EPS in the planktonic sample where maximum inorganic CO_2 fixation rate was measured (22 m, 16th January 2013).

Treatment	Target	Abundance[1] (cells mL^{-1})	EPS[2] (%)	$^{14}CO_2$-positive EUB[3] (%)	$^{14}CO_2$-positive EPS[4] (%)	EPS-as-signed reads (%)[5]
Light	Bacteria	3.4×10^6	85.9	17.6	35.2	88.2
	EPS	2.9×10^6				
Dark	Bacteria	5.6×10^6	77.8	18.5	39.6	
	EPS	4.3×10^6				

[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 Bacteria (positive hybridization with probe EUB I-II-III) that showed deposition of silver granules around the cell periphery with respect to total Bacteria cell counts.

[4] 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.

[5] Percentage of pyrotags assigned to Class *Epsilonproteobacteria* in the cDNA pyrotag library obtained from the same sample (22 m, 16/01/2013). Data were corrected according to the average number of rRNA operons in genomes of *Epsilonproteobacteria* (4.8, (Klappenbach et al., 2001)).

Assuming that chemolithoautotrophic Epsilonproteobacteria fix CO_2 *via* the reductive tricarboxylic acid cycle (rTCA) (Hügler et al., 2005), further evidences of the potential capacity of planktonic Epsilonproteobacteria in basin C-III to assimilate CO_2 were provided after cloning the *aclB* gene in samples collected at selected depths and dates using specific primers (Campbell et al., 2003). The gen *aclB* encodes for the beta subunit of ATP citrate lyase, a key enzyme in the rTCA (Antranikian et al., 1982; Campbell et al., 2003; Hügler et al., 2005). A total of 193 valid *aclB* clones were finally sequenced, aligned and translated to protein sequences (Table 4.4). These environmental sequences clustered into 5 OTUs (97% cutoff) with high sequence identity at amino acid level to reference *aclB* sequences from other Epsilonproteobacteria (Table 4.5). 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 *Thiovulum* spp. than to *aclB* from *Candidatus* Arcobacter sulfidicus and relatives (Fig. 4.10).

Table 4.4. Number of total and valid clones recovered in *aclB* clone libraries constructed from O_2/H_2S interface samples of basin C-III.

Clone library	Number of clones	High-quality sequences (%)[1]	*aclB* sequences (%)[2]	Number of OTUs
Winter 2013 (21.75 m / 22 m)	125	113 (90)	110 (97)	
Summer 2013 (20.75 m / 21 m)	96	89 (97)	83 (93)	
Total	221	202 (91)	193 (96)	5

[1] Percentage calculated in relation to total clone sequences.
[2] Percentage calculated in relation to high-quality sequences.

Table 4.5. First Blast Hit against the reference proteins database at the NCBI for each of the representative translated *aclB* OTU sequences in the analyzed clone libraries (see Table 4.4).

OTU ID (number of sequences)	First Blast Hit	Identity (%)	Accession Number
1 (157)	ATP-Citrate lyase (*Thiovulum* spp.)	88	WP008352685
2 (28)	Hypothetical protein (uncultured *Sulfuricurvum* sp RIFRC-1)	86	WP015653104
3 (4)	ATP-Citrate lyase (*Sulfuricurvum kujiense*)	93	WP013459529
4 (3)	Hypothetical protein (uncultured *Sulfuricurvum* sp. RIFRC-1)	86	WP015653104
5 (1)	ATP-Citrate lyase (*Sulfurovum* sp.)	90	WP008245184
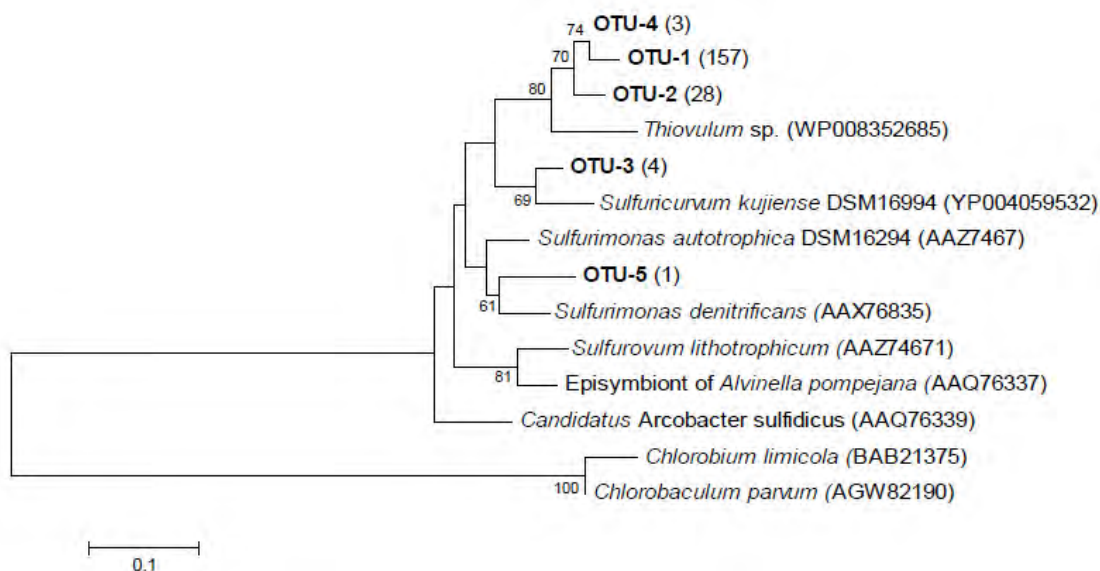
Figure 4.10. Neighbor-Joining phylogenetic tree of aclB aminoacid sequences obtained in clone libraries constructed from samples collected at the O_2/H_2S interface in winter (15th January, 2013) and summer (16th July, 2013). The number of sequences for each OTU is indicated between brackets. Numbers at nodes indicate bootstrap support (1,000 replicates).

4.2. Discussion

Sulfidic redoxclines in both marine and freshwater habitats are suitable niches for the growth and activity of chemo- and photolithotrophic sulfur-oxidizing microorganisms, which actively contribute to sulfur and carbon biogeochemical cycles. Several studies have reported the outstanding role of chemolithotrophic, sulfide-oxidizing Epsilonproteobacteria in marine pelagic redoxclines (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 sulfidic redoxcline located at photic depths, a shallow location rarely occurring in marine anoxic basins. Our work provides evidences 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 both marine and freshwater systems due to global change and local human pressure (Diaz and Rosenberg, 2008; Jeppesen et al., 2011; Wright et al., 2012; Jenny et al., 2016).

4.2.1. 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 redoxclines 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 were 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 unfavorable light conditions caused by the deeper location of the O_2/H_2S interface (22 m 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 sulfide-rich water layers stimulated, however, anoxygenic sulfur phototrophs (Borrego et al. 1999, 1997). Despite the seasonal differences in the abundance of Epsilonproteobacteria and GSB co-occurrence of both populations at the redoxcline depth is probably the rule rather than the exception in basin C-III. Accordingly, a potential competition for electron donors (*i.e.* sulfide) between both populations probably exists at least in late spring and summer. The dominance of GSB in late spring and summer suggest that phototrophs outcompeted Epsilonproteobacteria during warm seasons. However, further research is needed to confirm this extent and to elucidate the exact terms and the temporal dynamics of this interaction.

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 phylotypes 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 redoxclines chemolithoautotrophic planktonic assemblages also comprise gammaproteobacterial sulfur-oxidizers of the SUP05 clade (Glaubitz et al. 2009, 2010, 2013).

In freshwater lakes, Biderre-Petit and co-workers identified *Sulfurimonas* and *Sulfuricurvum* as main sulfide-oxidizing prokaryotes in the low-sulfate Lake Pavin through phylogenetic analysis of 16S rRNA and *aprA* gene markers (Biderre-Petit et al., 2011). Moreover, conspicuous populations of *Sulfuritalea hydrogenivorans* (*Betaproteobacteria*) have recently been identified as the major planktonic sulfur-oxidizing prokaryote in Lake Mizugaki, a meromictic Japanese lake where Epsilonproteobacteria were absent (Kojima et al., 2014).

In clear contrast, the chemolithoautotrophic planktonic assemblage in sulfate-rich, meromictic basin C-III is composed of Epsilonproteobacteria sulfur-oxidizers whereas no sequences affiliated to gammaproteobacterial SUP05 clade were identified in any of the pyrotag libraries analyzed. Besides, the epsilonproteobacterial community was dominated by a phylotype closely related to *Candidatus* Arcobacter sulfidicus, an autotrophic, marine sulfide-oxidizing Epsilonproteobacteria (Wirsen et al., 2002; Sievert, Wieringa, 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 et al., 1983; Vandamme et al., 1991; Llobet-Brossa et al., 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, sulfide-oxidizing member of this genus. In this regard, and considering that hypolimnetic waters of Lake Banyoles are slightly brackish (conductivity ≈ 2,000–2,500 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 sulfur compounds similarly to its marine counterpart (Wirsen et al., 2002). No evidences of filamentous sulfur-formation (Wirsen et al. 2002; Sievert et al. 2007) or autotrophic denitrification or chemoorganotrophic growth that are characteristic of other epsilonproteobacterial sulfide-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* (*Epsilon-proteobacteria*) after metagenomic analyzes of samples collected at oxic-anoxic interphases and euxinic water layers of basin C-III (Llorens-Marés et al., 2015) suggest that planktonic Epsilonproteobacteria might be able to couple sulfide oxidation to autotrophic denitrification in this habitat.

4.2.2. Dark carbon fixation by freshwater Epsilonproteobacteria in basin C-III

The co-occurrence of hydrogen sulfide and potential electron acceptors such as oxygen and nitrate at the redoxcline depth offers a suitable niche for the growth and activity of chemolithoautotrophic sulfide-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 responsible of dark inorganic carbon fixation rates measured in marine pelagic redoxclines is well documented for the Cariaco Basin (Taylor et al., 2001), the Black Sea (Jørgensen et al., 1991; Sorokin et al.,

1995; Grote et al., 2008; Glaubitz et al., 2010), 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 et al., 2008) or planktonic thiobacilli (Casamayor, 2010). Although centered 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 CO_2. 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 analyzes 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 sulfide 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 et al. 2003; Campbell and 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 *Thiovulum* spp. The lack of congruent phylogenies between 16S rRNA and protein-coding genes is a well-known consequence of lateral gene transfer (LGT) 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 and co-workers 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 (Moussard et al., 2006). In this regard, further research is needed to identify whether or not LGT 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.

4.2.3. *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 CO_2 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; Saccà 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 et al., 2008, 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 4.6).

Overall, the major relative contribution of dark fixation to total inorganic carbon fixation in stratified lakes has been found in small, doline type, sulfide-rich lakes, like lakes Arcas, Cisó and Vilar (Table 4.6), where anoxygenic photosynthesis or chemolithotrophic metabolisms can even dominate over oxygenic photosynthesis. High relative

contribution of dark CO_2 fixation processes have 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 sulfide-oxidizers accumulate taking advantage of the simultaneous presence of both oxidized and reduced compounds at the oxic-anoxic transition zone (*i.e.*, the redoxcline).

Table 4.6. Maximum dark carbon fixation rates measured at the O_2/H_2S interface (i.e. redoxcline) of different marine and freshwater environments.

	System	Depth (m)	Max. depth (m)	Maximum dark CO_2 fixation rate ($\mu g\ CO_2\ L^{-1}\ h^{-1}$)	Reference
Marine basins	Black Sea	115	2,212	0.3	(Grote et al., 2008)
	Baltic Sea	142	459	1.1	(Grote et al., 2008)
	Cariaco Basin	350	1,400	1.3	(Taylor et al., 2001)
	Mariager Fjord	13.5	30	43.2	(Zopfi et al., 2001)
Karstic lakes	Cisó	0–0.2	6.5	12.4	(Casamayor et al., 2008)
	Vilar	5.5	9	22.8	(Casamayor et al., 2008)
	Estanya	12	22	17.6	(Casamayor et al., 2008)
	Arcas	8.3	14.2	41.2	(Camacho and Vicente, 1998)
	Cadagno	11.4	21	94.1	(Camacho et al., 2001)
	Lagunillo del Tejo	5.5	6	1.8	(Casamayor et al., 2012)
	La Cruz	19.5	20	23	(Casamayor et al., 2012)
	El Tobar	13	18	18.1	(Casamayor et al., 2012)
	Banyoles Basin C-III	22	32	4.9	This study
Coastal lagoon	La Massona	5.5	10.5	0.6	(Casamayor et al., 2008)

Besides, the measurement of high rates of dark inorganic carbon fixation in sulfide-poor environments such as Lake La Cruz and Lake El Tobar (Casamayor et al., 2012) poses the question of whether this activity is fuelled by the trace amounts of sulfide available or if

it is carried out by microorganisms not involved in the sulfur cycle such as ferrolithotrophic 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 inorganic carbon fixation measured in deep anoxic layers —with very low redox potentials and paucity of oxidized substances— deserve further investigation.

5

DISTRIBUTION AND SEASONAL DYNAMICS OF A FRESHWATER ARCOBACTER USING q-PCR

In the previous chapter we confirmed the presence of an epsilonproteobacterial community mainly composed of an autotrophic, sulfide-oxidizing, freshwater member of the genus *Arcobacter* in basin C-III of Lake Banyoles. During the experimental work carried out it became evident that CARD-FISH overestimated the abundance of Epsilonproteobacteria in samples collected during spring and summer, where sequences affiliated to this class were rarely identified in pyrotag datasets (Chapter 4). These observations suggested that probe EPSY914, traditionally considered specific to Epsilonproteobacteria, also hybridize with members of the phylum *Chlorobi* and class *Deltaproteobacteria* thus interfering with epsilonproteobacterial counts. Accordingly, we advise against the use of probe EPSY914 in habitats where Green Sulfur Bacteria and sulfate-reducing bacteria (*i.e.* Deltaproteobacteria) may co-occur with sulfide-oxidizing Epsilonproteobacteria.

The lack of alternative molecular tools to properly quantify Epsilonproteobacteria in these habitats, compelled us to design qPCR primers specifically targeting the *Arcobacter* species thriving in basin C-III of Lake Banyoles and to optimize a qPCR protocol to properly determine its abundance. The application of this new molecular tool allows the

proper quantification of the main members of the epsilonproteobacterial community and provides an unbiased insight into their distribution and seasonal dynamics.

5.1. Results

5.1.1. Design of specific primers and optimization of the qPCR assay

The design of the primers set (76F-243R) and the Arcobacter Taqman probe (Arco-TQ probe) was done using the Primer-BLAST tool of the NCBI (section 3.7.1 of Chapter 3: Material&Methods). Primers and probe properties (length, melting temperature and GC content) are specified in Table 5.1.

Table 5.1. Properties of primers and the TaqMan probe designed to target *Arcobacter* spp. Thermodynamic characteristics and properties of the oligonucleotides were analyzed using NetPrimer software. Forward Primer: 76F, Reverse Primer: 243R, Arcobacter TaqMan Probe: Arco-TQ, Tm: melting temperature, GC: GC content.

	Oligonucleotide sequence (5'→3')	Length (bp)	Tm (°C)	GC (%)
76F	GTGGCGCACGGGTGAGTAATA	21	61.0	57.1
243R	GCCATTACCCCACCAACTATCTG	23	59.7	52.1
Arco-TQ	TCTTTCCCTTTACGAACTTATGTTCAAAAGGCA	33	65.6	36.4

The specificity of the new primer pair (76F-243R) was tested *in silico* and *in vitro*. *In silico* assays were carried out using the TestPrime tool available at the Silva website (http://www.arb-silva.de/search/testprime/). This *in silico* analysis yielded complete specificity, with all hits within the class *Epsilonproteobacteria* (coverage of 0.3%) and specifically matching some *Arcobacter* species (2.5% coverage within the genus *Arcobacter*). For empirical test of specificity, several DNA extracts corresponding to samples collected at different depths and seasons were used as template in qPCR assays using the new designed primer pair. Resulting amplicons were then cloned and sequenced to check for specificity of the primer pair used. Sequence analysis of resulting clones indicated the non-specific amplification of other bacterial targets across depths and seasons (Table 5.2).

To overcome these problems we designed a hydrolysis probe (TaqMan) that matched a specific region for the target strain within the amplicon generated with primer pair 76F-243R. The specificity of the *Arco*-TaqMan (*Arco*-TQ) probe was tested by comparing its sequence against the Ribosomal Database Project II (RDP) through Probe Match tool

(http://rdp.cme.msu.edu/probematch/search.jsp). This *in silico* analysis revealed a single unspecific match with an unclassified member of the family *Desulfobacteraceae* (coverage of 0.0001%). All the remaining hits affiliated within the class *Epsilon-proteobacteria* (coverage of 0.02%, 0.0009% and 0.0002% for genera *Arcobacter*, *Sulfuricurvum* and *Sulfurovum*, respectively).

Alignment of primers 76F-243R and the *Arco*-TQ probe against a dataset of the freshwater Arcobacter sequences recovered from basin C-III showed perfect match and specificity. Further qPCR assays using both positive and negatives controls (DNA isolated from 40 clones containing 16S rRNA gene inserts of the target *Arcobacter* strain and different aquatic bacteria, respectively) yielded amplification only for positive controls and no false-positives were obtained. It should be pointed out that no amplification was obtained in DNA extracts from other *Arcobacter* strains (*A. nitrofigilis* DSM-7299, *A. marinus* DSM-24769) thus demonstrating the specificity of the primer pair-probe combination used. A linear range for quantification was obtained between 10^8 to 10^3 16S rRNA gene copies (R^2=0.99). The average slope of the linear regression curve was –3.65 over 6-log quantification linear range (10^3 to 10^8), thus giving an efficiency of 87.7% on average (Fig. 5.1). The theoretical limit of detection (LOD) of the assay was 1×10^3 16S rRNA gene copies, which correspond to 1.3×10^3 16S rRNA gene copies mL^{-1}. Considering an average value of 4.8 rRNA operon copies per Arcobacter cell (Ribosomal RNA Database, http://rrndb.umms.med.umich.edu/) the calculated LOD corresponds to 271 Arcobacter cells mL^{-1}.

Table 5.2. Number of total and valid clones recovered in 16S rRNA clone libraries constructed from redoxcline samples of basin C-III to test the specificity of the primers set 76F-243R.

Sample	Valid sequences	*Arcobacter* sequences (%)[1]	Unspecific match
Winter sample (DNA) (15/01/2013)	11	10 (90.9)	*Actinobacteria*
Winter sample (cDNA) (15/01/2013)	13	13 (100)	
Summer sample (DNA) (16/07/2013)	8	1 (12.5)	*Dehalospirillum* *Firmicutes*
Fall sample (DNA) (07/11/2012)	8	0 (0)	*Actinobacteria* *Betaproteobacteria* *Spirochaetes*

[1] Percentage calculated in relation to valid sequences.
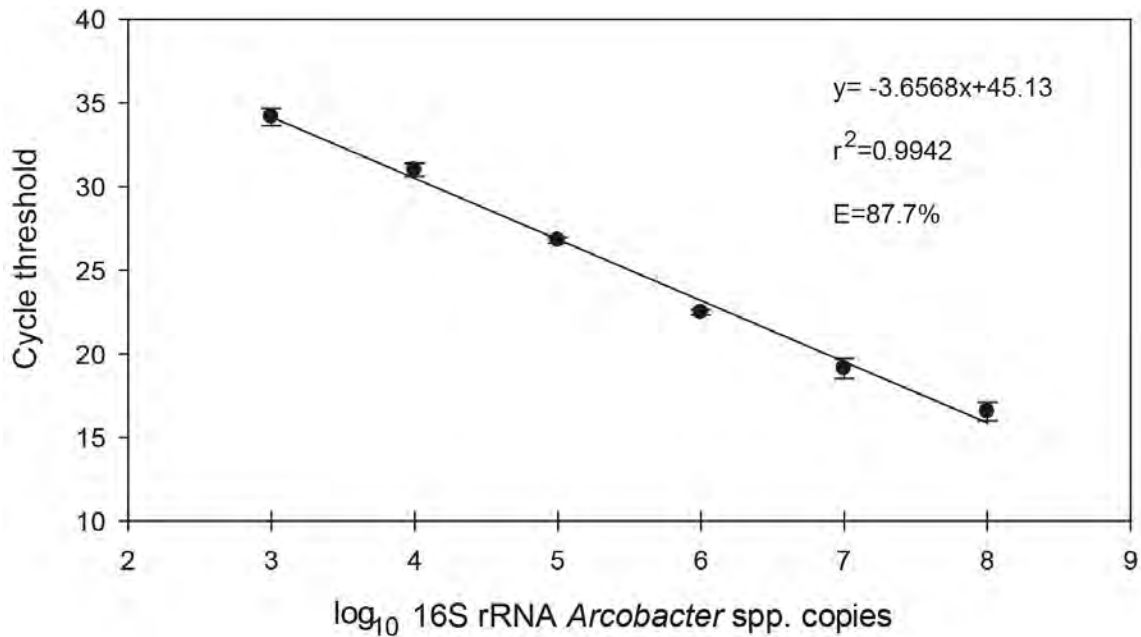
Figure 5.1. Standard curve for the qPCR assay using primer pair 76F/243R and Arco-TQ probe. The regression equation, the coefficient of determination (r²) and the amplification efficiency (E) are also given. Values are the means of four independent determinations ± standard error of the mean (SEM) (error bars).

5.1.2. *Distribution and abundance of* Arcobacter spp. *along the study period*

Quantification of the abundance of Arcobacter in DNA extracts from samples collected at different depths (mixolimnion, redoxcline and upper monimolimnion), seasons (winter, spring, summer and fall) and years (2011, 2012, 2013, 2014) was carried out by qPCR using the new primer-probe combination (Fig. 5.2). Abundance of Arcobacter was significantly different between seasons (Kruskal-Wallis, $\chi^2=28.1$, $p=3.5\times10^{-6}$), being winter samples those where concentrations were higher (average of $8.7\times10^5 \pm 6.1\times10^5$ cells mL^{-1} considering all sampling depths). Maximal abundance in winter were measured at the redoxcline (2.3×10^5, 9.9×10^6 and 4.4×10^4 cells mL^{-1} in winter samples from 2011, 2013 and 2014, respectively), and in samples from the upper monimolimnion (1.4×10^6 and 1.5×10^6 cells mL^{-1} in winter 2011 and 2014, respectively). In turn, the abundance of Arcobacter during summer was lower (average of $2.1\times10^4 \pm 1.1\times10^4$ cells mL^{-1} considering all sampling depths). In fall, Arcobacter was rarely detected in both redoxcline and monimolimnetic samples with the exception of samples collected during fall 2013, where a concentration of up to 4.5×10^5 cells mL^{-1} was measured at the suboxic redoxcline. In spring 2012, the abundance of Arcobacter was maximal at the redoxcline although values were lower than those calculated from winter samples (average of $6.0\times10^4 \pm 3.2\times10^4$ cells mL^{-1} considering all sampled depths).

5.1.3. Comparison between CARD-FISH and qPCR measurements

As expected, abundance values of Epsilonproteobacteria and Arcobacter measured by CARD-FISH (EPSY914 probe) and qPCR (76F-243R/Arco-TQ), respectively, showed significant differences (Wilcoxon test, Z= –5.3, p=9.5×10^{-8}) (Fig. 5.3). When partitioned across seasons, these differences were not significant for winter samples (Wilcoxon test, Z= –0.982; p=0.326), agreeing with the high correlation of CARD-FISH counts and results from high throughput sequencing datasets previously shown (Chapter 4, Fig. 4.2). In turn, significant differences were obtained between CARD-FISH counts and qPCR copy numbers in samples from spring (Z= –2.0, p=0.043), summer (Z= –3.5, p=4.4×10^{-4}), and fall (Z= –3.9, p=8.8×10^{-5}). These results confirmed the critical interference of planktonic bacteria other than Epsilonproteobacteria in CARD-FISH counts due to unspecificities of probe EPSY914 (see Chapter 4 for details).
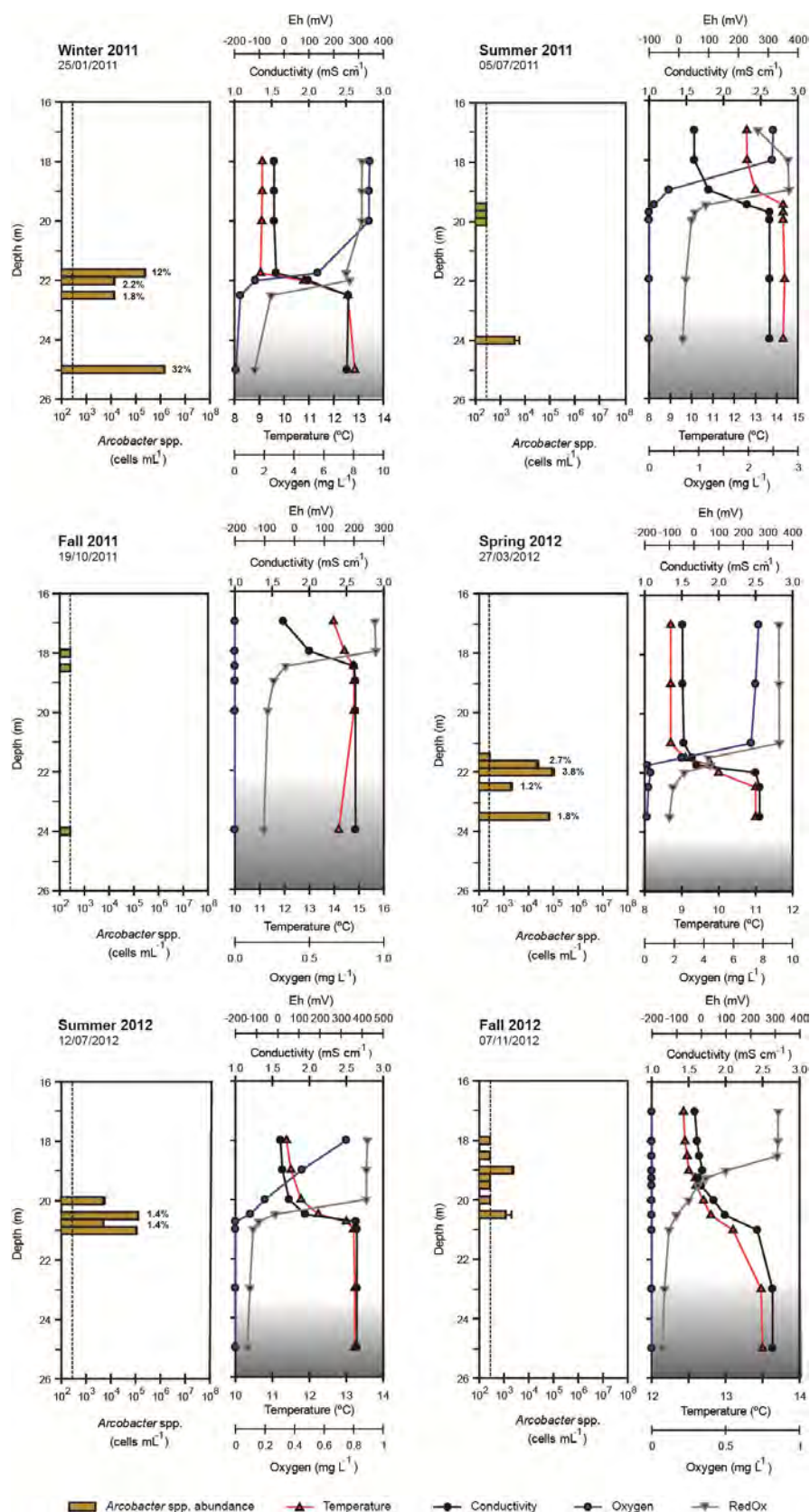
Figure 5.2. Abundance of *Arcobacter* spp. measured by qPCR at the redoxcline and upper monimolimnion of basin C-III on selected dates of different annual cycles. The relative contribution of Arcobacter at each depth is also indicated for the depths with a contribution higher than 1%. The dotted line indicates the detection limit of the qPCR analysis. Note that the X-axes of physicochemical profiles have different scales.
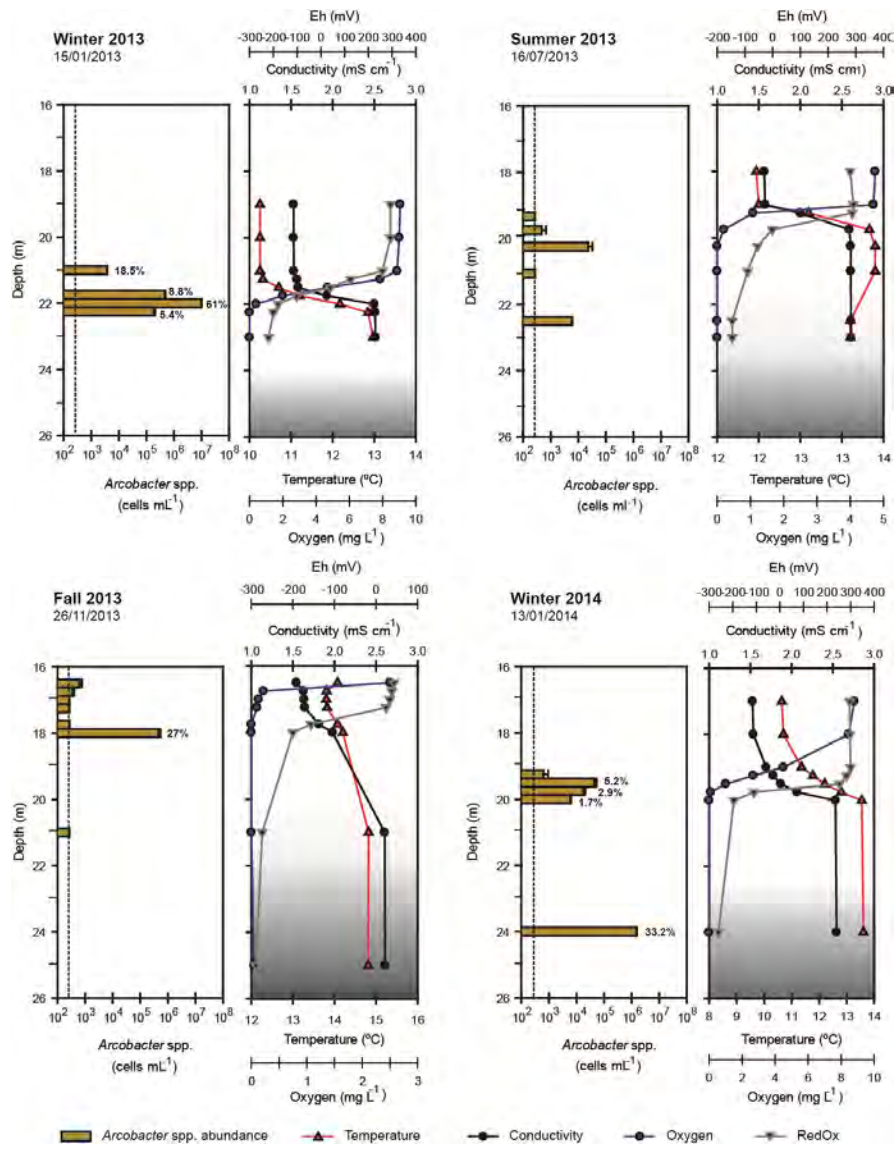
Figure 5.2. Continued.
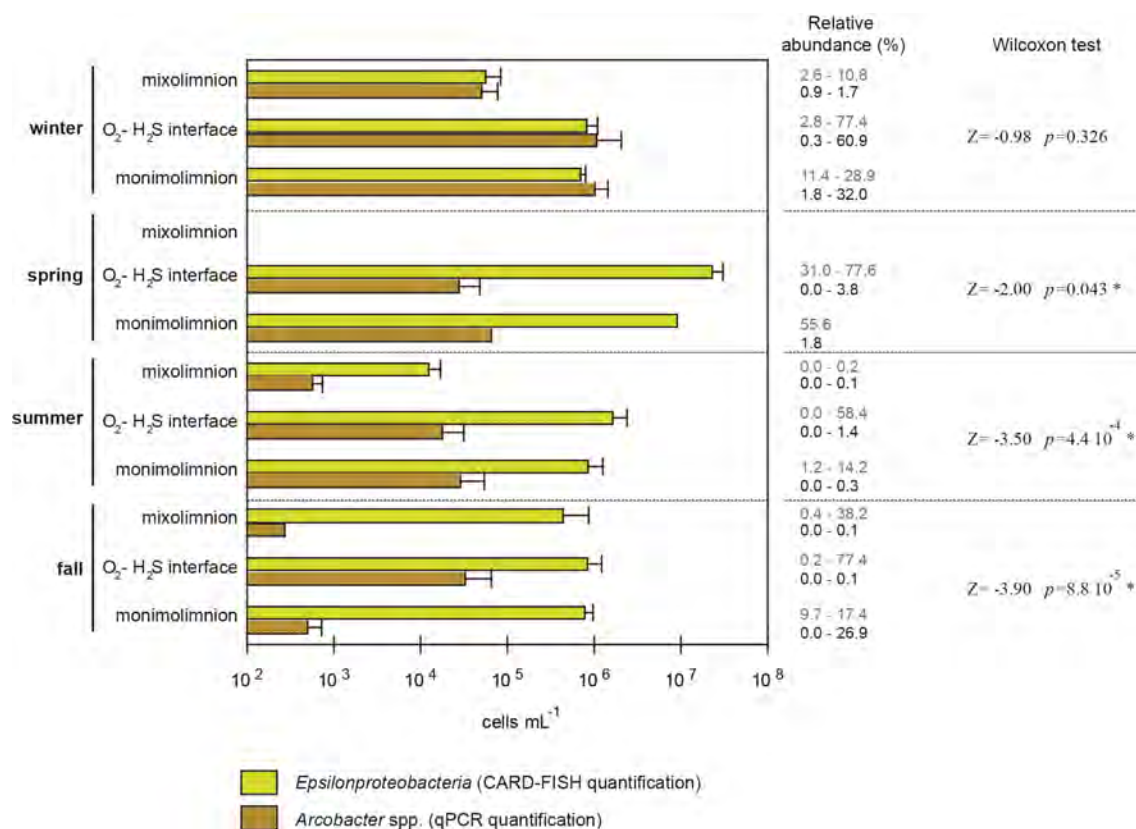
Figure 5.3. Abundance (cells mL^{-1}) of Epsilonproteobacteria (yellow bars) quantified by CARD-FISH (EPSY914 probe) and Arcobacter cells (brown bars) measured by qPCR (76F-243R/Arco-TQ) at different water compartments (mixolimnion, redoxcline and upper monimolimnion) of basin C-III of Lake Banyoles. Values are the average (±SEM) of different depths at each water compartment for the same season of different annual cycles. For spring 2012 only one data point is available for the monimolimnion whereas no data are available for the mixolimnion. The ranges of relative abundance (%) of Epsilonproteobacteria for both techniques used are shown. The standard normal distributed Z-value and the *p-value* from Wilcoxon test are indicated on the right side. Asterisks indicate significant differences at α=0.05.

5.2. Discussion

5.2.1. Evaluation of the qPCR approach

Results of the sensitivity tests indicated that the new qPCR method using primer pair 76F-243R in combination with the Arco-TQ probe is highly specific for the *Arcobacter* spp. thriving in basin C-III (Chapter 4) even when DNA from sulfide-oxidizing Epsilonproteobacteria (*Sulfurimonas autotrophica*) or *Arcobacter* species (*Arcobacter nitrofigilis* DSM-7299 and *A. marinus* DSM-24769) are used as controls. No cross-reaction was observed with DNA from planktonic bacteria isolated from basin C-III (data not shown).

Efficiency of the qPCR assay was of 87.7% on average. This efficiency is similar to that reported for the quantification of 16S rRNA genes of pathogenic members of the genus *Arcobacter* (*A. butzleri*, Efficiency = 86.36%, de Boer et al., 2013) but lower than those obtained in qPCR assays for the quantification of planktonic populations of *Sulfurimonas denitrificans* in the Baltic Sea (efficiencies ranging from 97% to 104 %, Labrenz et al., 2004). qPCR assays using TaqMan chemistry do not always reach 100% efficiency (*e.g.* 93.5% for the quantification of 16S rRNA genes of betaproteobacterial ammonia oxidizers) (Lim et al., 2008). The low efficiency might be probably related to both the annealing efficiency of the primer pair used and to the structure of the DNA target (Bustin et al., 2009).

The LOD of the current assay is closely similar to that obtained for the quantification of *Sulfurimonas denitrificans* (1.4x10^3 16S rRNA gene copies mL^{-1}) (Labrenz et al., 2004). In turn, the current LOD (2.7$\times 10^2$ cells mL^{-1}) is higher when compared to other Taqman-based qPCR protocols applied for the quantification of specific microbial species such as *Dehalococcoides* spp. in environmental samples (LOD of the qPCR=1 cell mL^{-1}) (Ritalahti et al., 2006). Our LOD is, however, one order of magnitude lower to that calculated for the CARD-FISH protocol (8.6$\times 10^3$ cells mL^{-1})[1]. In this regard, the conversion of gene copies mL^{-1} to cells mL^{-1} is not straightforward therefore most bacterial species possess more than one copy of the rRNA operon in their genomes (Farrelly et al. 1995). Besides, this number may vary according to the physiological status of the cell (Klappenbach et al., 2000; 2001). Assuming an efficient DNA extraction and quantification as well as a high specificity of primers and probes, gene copies should be equal to total target cells after multiplying them by the number of copies of 16S rRNA operons in genomes of the target species (Lloyd et al., 2013). Accordingly, and considering that members of the genus *Arcobacter* possess, on average, 4.8 rRNA operon copies in their genomes (http://rrndb.umms.med.umich.edu/), we were able to convert gene copies to cells to allow a direct comparison of qPCR results to CARD-FISH counts (see Material & Methods, Chapter 3).

5.2.2. Limitations of qPCR and CARD-FISH in the quantification of Arcobacter

The large discrepancies between the abundances obtained by CARD-FISH counts and qPCR results in planktonic samples containing diverse bacterial communities (spring, summer and fall) (Section 5.1.3), raised questions regarding which method offered most reliable results. In the same way, clear inconsistencies were observed between relative abundances of Epsilonproteobacteria calculated from CARD-FISH counts and high throughput sequencing datasets (Chapter 4). CARD-FISH has been successfully applied for the proper quantification of Epsilonproteobacteria in marine

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[1] For qPCR the detection limit (in cells mL^{-1}) has been calculated considering a filtered water volume of 200 mL, an average DNA concentration of 52 ng μL^{-1} and an average of 4.8 rRNA operons in *Arcobacter* genomes (The Ribosomal RNA Database, http://rrndb.umms.med.umich.edu/) For CARD-FISH, the detection limit (in cells mL^{-1}) has been calculated considering a filtered water volume of 25 μL and a minimum number of 1 probe-positive cell in a 20 microscopic fields using x1000 augment skills.

environments without any particular interference of other bacterial groups (Lin et al., 2006; Grote et al., 2007, 2008). In these marine pelagic redoxclines, Epsilonproteobacteria usually dominate the planktonic community whereas Green Sulfur Bacteria (GSB) are rarely observed due to the deep location of the sulfidic redoxcline (Lin et al., 2006; Grote et al., 2007; Jost et al., 2008). Under these conditions, unspecific binding of probe EPSY914 to GSB cells is not expected since the latter rarely reach conspicuous abundances. In lacustrine environments such as basin C-III in Lake Banyoles, the location of the sulfidic redoxcline at photic depths allows GSB to bloom thus leading to overestimation of Epsilonproteobacteria by unspecific binding of probe EPSY914 to members of the phylum *Chlorobi* (see Section 4.1.3, Chapter 4). These drawbacks might be overcame by using more specific probes against the target species or by the design of new primers (and probes if needed) for specific detection using qPCR (Labrenz et al., 2004; Grote et al., 2007). Considering the difficulties in designing and optimizing new CARD-FISH probes against a single species and the time constraints of CARD-FISH procedures ($\approx$48 h just to complete the hybridization protocol), we finally decided to use qPCR as alternative. Despite the analytical constraints stated above (high specificity towards an unique species, amplification efficiency <90%, and a high limit of detection), the optimized qPCR protocol allowed the proper quantification of the *Arcobacter* spp. without the need of time-consuming experimental protocols. Further work is needed to assess if the new qPCR protocol may be also useful to quantify Epsilonproteobacteria in other stratified lakes (*e.g.* in the neighboring lakes and ponds of the Banyoles Karstic System) since the high specificity of the TaqMan probe used makes it useless in habitats dominated by other epsilonproteobacterial species.

5.2.3. Re-analysis of the distribution and dynamics of Arcobacter in basin C-III

To our knowledge, quantification of Epsilonproteobacteria by qPCR in stratified aquatic systems has only been addressed by Labrenz and co-workers in the pelagic redoxcline of the Central Baltic Sea (Labrenz et al., 2004). These authors reported the presence of a dominant population of *Sulfurimonas denitrificans* (formerly known as *Thiomicrospira denitrificans* and finally described as *Sulfurimonas gotlandica* GD1) at average values of $7.08 \times 10^4 \pm 2.64 \times 10^4$ cells mL^{-1}. These figures are well below the abundance values measured for the *Arcobacter* strain in the redoxcline of basin C-III during winter ($1.78 \times 10^6 \pm 1.62 \times 10^6$ cells mL^{-1}) and illustrate the difference between both systems in terms of both diversity and nutrient status (see below).

Despite differences in absolute values, the abundance data obtained using the new qPCR protocol confirmed the winter dominance of *Arcobacter* sp. previously resolved using CARD-FISH (Chapter 4). The new approach provided a better resolution of the distribution and abundance of the target species during spring, summer and fall. In this regard, depth maxima of Arcobacter usually coincided with the O_2-H_2S interphase (*i.e.* the sulfidic redoxcline), where a dynamic gradient of oxygen and sulfide provides an optimal niche for its growth and activity (see Section 4.1.3, Chapter 4). This distribution agrees with previous studies on the ecology of sulfide-oxidizing chemolithotrophic Epsilonproteobacteria regarding the necessary co-occurrence of conspicuous

concentrations of H_2S and oxygen in redoxclines of stratified lakes and marine basins (Gevertz et al., 2000; Ahmad et al., 2002; Campbell et al., 2006). The unexpected detection of Arcobacter 16S rRNA genes in the sulfide-free, fully oxygenated bottom layers of the mixolimnion compelled us to consider alternatives to a strict chemolithotrophic metabolism for the target strain. Members of the class *Epsilonproteobacteria* are metabolically versatile (Campbell et al., 2006) and, particularly, the genus *Arcobacter* includes lithoautotrophic, lithoheterotrophic and organoheterotrophic members (Campbell et al., 2006; Carlström et al., 2013; Roalkvam et al., 2015). In this regard, some species of chemolithotrophic Epsilonproteobacteria (*Sulfurimonas gotlandica* GD1 and GD17) are able to use pyruvate to replenish their chemolithotrophic growth under conditions of nutrient limitation (Labrenz et al., 2013; Glaubitz et al., 2014). Besides, some *Arcobacter* spp. able to incorporate acetate have been detected in pelagic redoxclines of the Baltic Sea (Berg et al., 2013), in marine sediments (Wirsen et al., 2002; Vandieken et al., 2012) and in surface seawater (Suh et al., 2015), evidencing a metabolic versatility larger than expected. Assuming some versatility regarding carbon and energy sources for the dominant *Arcobacter* spp. in basin C-III, the dark carbon fixation rates measured in the mixolimnetic compartment (see Chapter 4, Section 4.1.4) point to the presence of other chemolithoautotrophic microbes able to fix carbon in the dark using energy sources other than sulfide (not present). In this regard, the occurrence of conspicuous populations of ammonia-oxidizing archaea (AOA) in the mixolimnion of basin C-III is a plausible explanation according to the identification of their molecular signatures in previous surveys (unpublished results) and the well-recognized capacity of AOA to thrive under extremely low ammonia concentrations in both marine and freshwater environments (Auguet et al., 2011; Könneke et al., 2014).

6

DISTRIBUTION AND DIVERSITY OF SULFUR-OXIDIZERS AND SULFATE-REDUCERS IN BASIN C-III BASED ON *soxB*, *aprA* AND *dsrB* FUNCTIONAL GENE ANALYSIS

The investigations presented in the two preceding chapters provide a better understanding of the planktonic community of sulfide-oxidizing chemolithotrophs in basin C-III of Lake Banyoles. These studies revealed that members of the genus *Arcobacter* might have a key role in cycling carbon in the dark at the sulfidic redoxcline and euxinic water layers. Several questions regarding the metabolic pathways that these chemolithotrophs use to oxidize sulfide and the genetic diversity related to the sulfur cycle remained, however, unanswered.

To fill this gap, we studied the diversity of three functional genes involved in S cycle in the water column of basin C-III, trying to resolve their phylogenies and their match with those built using phylogenetic markers (*i.e.* 16S rRNA gene). Accordingly, genes encoding three key enzymes in the microbial sulfur cycle, namely: the adenosine-5′-phosphosulfate reductase subunit A (*aprA*), the dissimilatory sulfite reductase subunit B (*dsrB*), and the hydrolytic enzyme soxB from the sulfur-oxidizing multienzyme Sox system (*soxB*), were amplified using specific primers to target both sulfur-oxidizing and sulfate-reducing bacteria. The diversity of these three gene biomarkers in samples

collected from the mixolimnion, the redoxcline and the monimolimnion at different time intervals were assessed through high throughput sequencing using Illumina chemistry. A sample from the upper monimolimnion collected in spring was also analyzed to resolve uncertainties related to unspecific matches of CARD-FISH probe EPSY914 towards members of the class *Deltaproteobacteria* (see Chapter 4 for details). Sequence datasets were analyzed to resolve phylogenies based on these functional genes and their match to 16S rRNA gene-based phylogenies. Moreover, we carried out a multivariate analysis to identify environmental drivers that better predict the community composition based on target functional genes markers.

6.1. Results

6.1.1. Composition of bacterial assemblage based on functional gene markers

The richness and diversity of three functional genes involved in the S cycle (*aprA*, *dsrB* and *soxB*) was assessed by Illumina sequencing using DNA extracts from selected depths and seasons (Section 3.10 in Material and Methods) using specific primers (Table 3.3 in Material and Methods). Composition of sulfur-oxidizing bacteria (SOB) and sulfate-reducing bacteria (SRB) was evaluated from high-quality sequences retrieved from *aprA/soxB* and *aprA/dsrB* iTag libraries, respectively (Table 6.1). It is important to note that the primer pair used to amplify *aprA* gene targets both SOB and SRB (Meyer and Kuever, 2007).

Table 6.1. Number of raw sequences, high-quality sequences (after quality filtering and chimera removal), and the total number of Operational Taxonomic Units (OTUs) obtained for each library.

Gene	Raw sequences	High-quality sequences (%)	OTUs
aprA	311,531	308,810 (99.1)	250
soxB	80,777	78,069 (96.6)	35
dsrB	673,349	667,800 (99.2)	1593

6.1.1.1. Sulfur-Oxidizing Bacteria

aprA gene

iTag libraries of *aprA* gene were mainly composed by sequences of SOB affiliated to *Beta-* and *Gammaproteobacteria* (Figs. 6.1 and 6.4B). The relative abundance of both groups decreased along depth, being less than 4% of total sequences in the anoxic monimolimnion. In winter, *aprA* sequences affiliated to the class *Betaproteobacteria* (mainly *Thiobacillus*, OTU-89) and the class *Gammaproteobacteria* (*Chromatium*, OTU-170; *Halochromatium*, OTU-171) (Fig. 6.4B). Sequences affiliated to other sulfur-oxidizing

prokaryotes such as *Beggiatoa* were scarce, with relative abundances rarely exceeding 1% of total sequences (data not shown). The maximum diversity was calculated for samples collected at the mixolimnion and the upper redoxcline in winter, and from the upper monimolimnion in summer (Table 6.2).

soxB gene

The taxonomic composition observed for *soxB* libraries was complementary to that obtained for *aprA* (Figs. 6.1 and 6.5B). All winter *soxB* libraries were dominated by sequences affiliated to class *Epsilonproteobacteria* and particularly to genus *Arcobacter* (mainly OTU-20, Fig. 6.5B). In these winter libraries, sequences affiliated to *Arcobacter* represented more than 99% of total sequences, being the winter monimolimnetic sample an exception (96.6% of sequences affiliated to *Arcobacter* and 1.9% to genus *Thiomicrospira* (*Gammaproteobacteria*)) (Fig. 6.1). A different scenario was observed in the summer redoxcline sample, where genera *Chlorobaculum* (OTU-21), *Arcobacter* (OTU-20) and *Allochromatium* (OTU-7) represented each one a third of the sequences retrieved (Figs. 6.1 and 6.5B). Same genera were present in the summer upper monimolimnion library. The Shannon diversity index calculated from these data reflected the low diversity in *soxB* libraries (Table 6.2).

Table 6.2. Richness (Chao1) and diversity (Shannon) indices calculated from 16S rRNA pyrotag and Illumina functional genes datasets analyzed. Redoxcline samples are shaded in grey.

Sample (date)	Depth (m)	16S rRNA		*aprA*		*soxB*		*dsrB*	
		Chao1	Shannon	Chao1	Shannon	Chao1	Shannon	Chao1	Shannon
Winter (13/01/2014)	4	262	3.1	60	3.4	12	0.3	358	2.8
	19.5	357	3.1	106	2.8	10	0.1	353	3.7
	19.75	329	3.0	74	1.7	13	0.1	333	2.8
	20	301	3.0	66	1.4	7	0.1	296	2.1
	24	519	2.3	109	2.0	15	0.1	242	1.9
Summer (16/07/2013)	10	87	2.7	-	-	-	-	-	-
	19.75	192	2.2	78	1.3	8	1.8	235	2.2
	22.5	215	2.4	109	2.9	7	1.3	293	1.9
Spring (27/03/2012)	22.5	48	1.0	53	1.1	-	-	212	1.5

6.1.1.2. Sulfate-Reducing Bacteria

aprA gene

aprA libraries, mainly those from the upper monimolimnion, were dominated by sequences affiliated to class *Deltaproteobacteria* (Figs. 6.1 and 6.4B). These sequences affiliated to genus *Desulfomonile* (OTU-89 and OTU-177), with relative abundances ranging from 2.9% to 26.9% of total sequences in winter and summer mixolimnion and redoxcline samples, and reaching their maximal contribution in samples collected at the upper monimolimnion (Fig. 6.4B). Remarkably, a high number of sequences in all samples from the redoxcline affiliated to unclassified groups within the class *Deltaproteobacteria*, suggesting that these samples harbor a large diversity of uncultured sulfate-reducing bacteria (Fig. 6.4A).

dsrB gene

Similarly, sequences affiliated to *Deltaproteobacteria* were also predominant in *dsrB* libraries (Fig. 6.1) although prevalent genera varied among samples (Fig. 6.6B). In winter, *Desulfocapsa* (OTU-4) and *Desulfomonile* (OTU-405 and OTU-577) were the main genera in mixolimnetic samples representing the 58.3% and the 22.7% of total bacterial sequences, respectively (Fig. 6.6B). In clear contrast, winter samples collected at deeper depths showed a progressive decrease in sequences affiliated to *Desulfocapsa* (<1% at 20 m) and a concomitant rise in the representativeness of sequences affiliated to *Desulfomonile* (≈83.7% in the upper monimolimnion). Similarly to results obtained for *aprA*, a high percentage of *dsrB* sequences (from 34.4% to 56%) remained unclassified at the genus level in winter redoxcline samples. This situation clearly contrasted that found for summer redoxcline and upper monimolimnion samples, where a clear dominance of *dsrB* sequences affiliated to genus *Desulfocapsa* and genus *Desulfomonile* were observed. In spring, sequences affiliated to this latter genus clearly outnumbered other groups (94% of total bacterial sequences). Diversity indices calculated from *dsrB* libraries showed higher values for samples from the redoxcline, compared to those from the upper monimolimnion (Table 6.2).
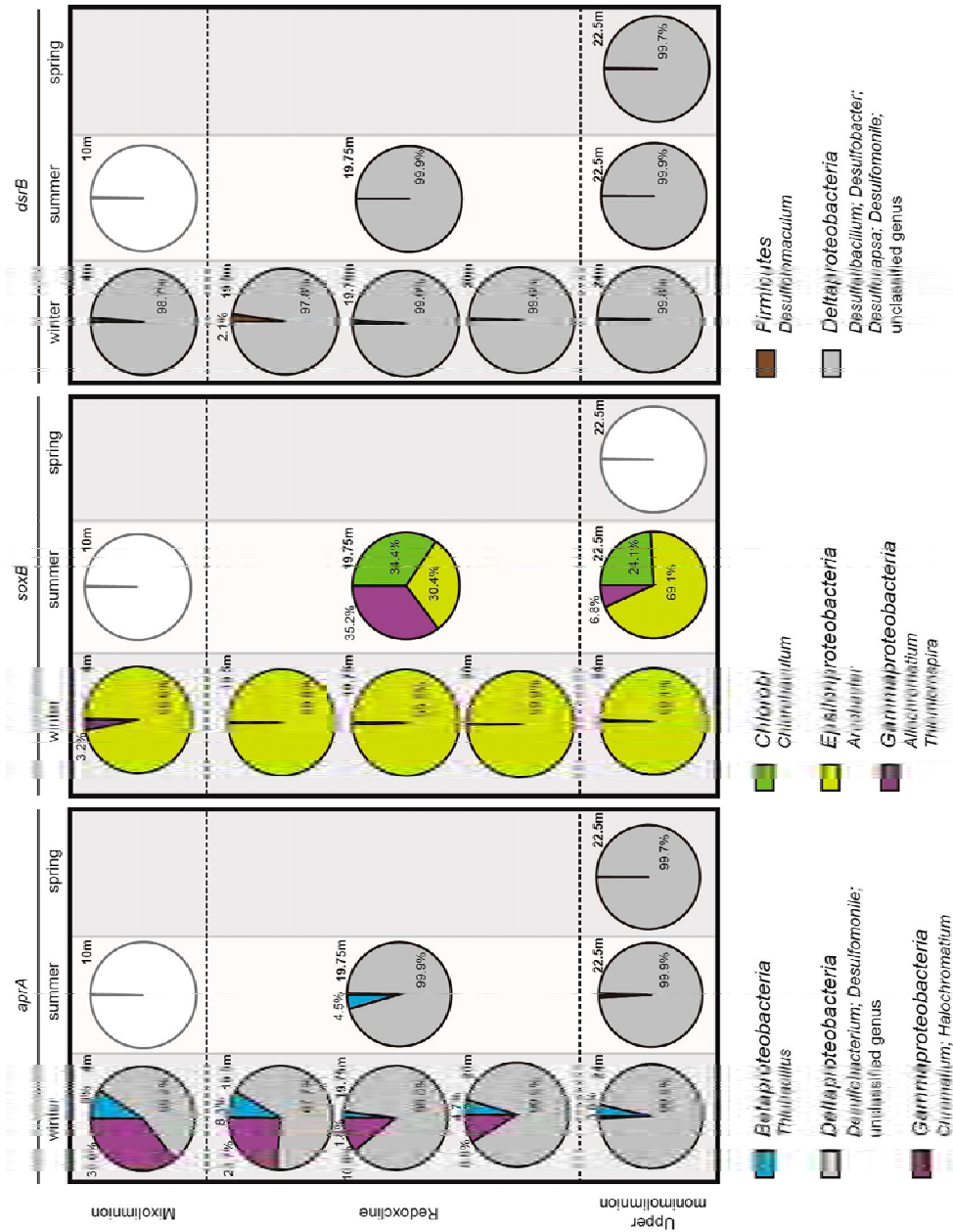
Figure 6.1. Distribution, diversity and relative abundance of *aprA*, *soxB* and *dsrB* functional gene markers in selected samples from different depths and seasons. Taxonomic assignments were done at phylum level (class for *Proteobacteria*). Main genera detected for each group are also indicated. Uncolored pie charts correspond to samples where amplification failed.

6.1.2. *Phylogenetic composition of the planktonic bacterial assemblage based on 16S rRNA gene marker*

Pyrotag libraries of 16S rRNA gene amplicons from the same sample set was carried out to determine to what extent the phylogenetic composition of the planktonic community of both sulfur-oxidizing and sulfate-reducing bacteria in basin C-III mirrored that obtained using functional gene markers. Besides, this complementary analysis allowed us to evaluate the relative contribution of putative SOB and SRB to total bacterial assemblage (Fig. 6.2).
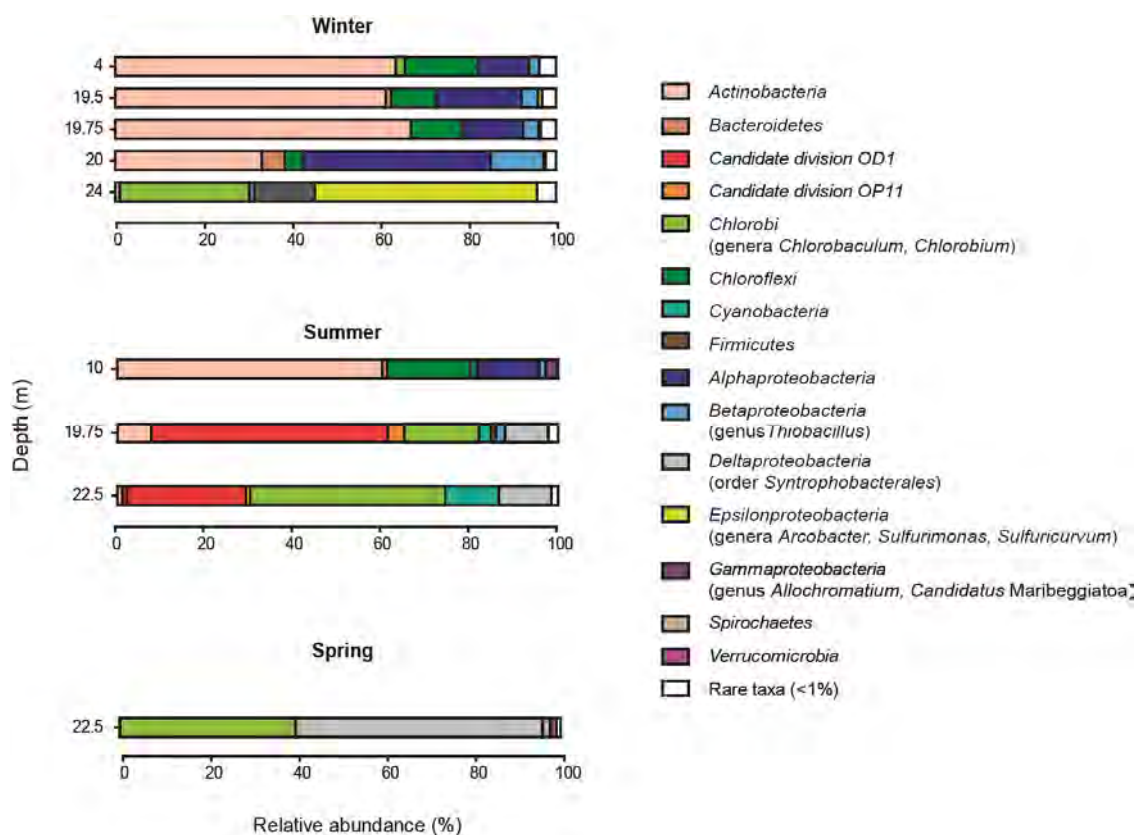
Figure 6.2. Taxonomic breakdown and relative abundance at the phylum level (class for *Proteobacteria*) for all samples analyzed. Taxa with a relative abundance less than 1% were grouped as "Rare taxa".

Pyrotag libraries showed a high variability on the relative abundance of common lineages of freshwater bacteria such as: *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobia*. Sequences affiliated to phylum *Actinobacteria* were predominant in pyrotag libraries from mixolimnetic samples collected in winter and summer (63.6% and 60.1% of total sequences, respectively) and in those from winter redoxcline samples (61.3%, 67.1% and 33.2% of total sequences at 19.5, 19.75 and 20 m depth, respectively). These samples

showed the maximal diversity according to the Shannon index (Table 6.2). In winter, samples from the upper monimolimnion showed an outstanding dominance of class *Epsilonproteobacteria* (50.4% of total sequences) and, to a lesser extent, of the phylum *Chlorobi* (29.4%). Sequences affiliated to *Candidate* division OD1 and *Chlorobi* were prevalent at the redoxcline and the upper monimolimnion in summer. In spring, the sample from the upper monimolimnion showed a clear predominance of sequences affiliated to Green Sulfur Bacteria (*Chlorobi*) and sulfate-reducing bacteria (*Deltaproteobacteria*) (39.9% and 55.9% of total sequences, respectively).

6.1.2.1. Sulfur-Oxidizing Bacteria

Putative SOB recovered in 16S rRNA libraries corresponded to classes *Beta-*, *Gamma-*, and *Epsilonproteobacteria*, and the phylum *Chlorobi*. SOB were mainly represented by Epsilonproteobacteria (*Arcobacter* spp.) in winter samples and Green Sulfur Bacteria (genus *Chlorobaculum* and *Chlorobium*) in summer and spring libraries.

Despite we only detected 16S rRNA gene sequences affiliated to genus *Arcobacter* in winter samples from the monimolimnion, sequences of *soxB* gene that affiliated to this genus were dominant in libraries from all depths in winter. Similarly, 16S rRNA libraries clearly identified sequences affiliated to *Chlorobaculum* and *Chlorobium* (phylum *Chlorobi*), whereas only *soxB* sequences related to *Chlorobaculum* sequences were retrieved from summer *soxB* libraries. In turn, a low representativeness (<1%) of 16S rRNA gene sequences affiliated to genus *Thiobacillus* (*Betaproteobacteria*) were identified from the redoxcline and the upper monimolimnetic winter samples thus mirroring the structure of the community obtained from *aprA* libraries (see section 6.1.1.1.). Finally, few sequences related to class *Gammaproteobacteria* were also recovered in 16S rRNA pyrotag libraries (genera *Allochromatium* and *Candidatus* Maribeggiatoa) although a larger diversity of *aprA* and *soxB* sequences related to gammaproteobacterial SOB (*e.g. Chromatium, Halochromatium, Beggiatoa, Thiothrix, Allochromatium* and *Thiomicrospira*) were identified.

6.1.2.2. Sulfate Reducing Bacteria

Sequences related to putative SRB (order *Syntrophobacterales*, class *Deltaproteobacteria*) were recovered from samples collected in all water compartments and dates being the mixolimnetic summer sample an exception to this rule. Notwithstanding this, deltaproteobacterial sequences represented less than 1% of total sequences in 16S rRNA gene libraries from mixolimnetic and the redoxcline winter samples. In the monimolimnion, however, the relative abundance of deltaproteobacterial sequences was 13.6%, 11.9%, and 55.9% for winter, summer and spring dates, respectively. In the summer redoxcline, 16S rRNA gene sequences affiliated to *Deltaproteobacteria* accounted to 9.8% of total sequences. All these sequences affiliated to genera within the order *Syntrophobacterales* whereas *dsrB* and *aprA* libraries also recovered, although in minor relative abundance, sequences assigned to order

Desulfobacterales (genera *Desulfobacter*, *Desulfocapsa*, *Desulfatibacillum* and *Desulfobacterium*).

6.1.3. Beta-diversity and contribution of environmental variables on community composition

6.1.3.1. Community structure based on 16S rRNA

Hierarchical clustering analysis (Bray-Curtis distance) based on the phylogenetic composition of planktonic communities split samples in two well-defined clusters (Fig. 6.3A). The vertical physicochemical gradient along the water column appears to be the main factor behind this segregation, grouping together samples from the mixolimnion and the upper redoxcline (characterized by a dominance of *Actinobacteria*) and those from the lower redoxcline and the upper monimolimnion (where *Candidate* Division OD1, *Chlorobi* and *Deltaproteobacteria* had an important contribution) (Fig. 6.2). Distance-based redundancy analysis (dbRDA) identified sulfide, total phosphorous and oxygen as the main predictor variables explaining 72.1% of total variation in bacterial community composition (Fig. 6.3B).

6.1.3.2. Community structure based on aprA *gene*

The same hierarchical clustering analysis was performed using the taxonomic composition of samples according to *aprA* gene sequences. In this case, the analysis distributed samples in three groups, differing in their relative contribution of both SOB and SRB (Fig. 6.4A). The first cluster contains shallower winter samples from the mixolimnion (Fig. 6.1). A second cluster grouped samples characterized by a low presence of sequences affiliated to genus *Desulfomonile* (OTU-89) but a high contribution of sequences affiliated to unclassified *Deltaproteobacteria* (Fig. 6.4B). Samples from the upper monimolimnion, all of them dominated by *aprA* sequences from sulfate-reducing bacteria, composed the third cluster (Fig. 6.1). In this case, oxygen, ammonium, nitrate, sulfate, total phosphorous and total organic carbon were identified as the main predictor environmental variables, explaining a 87.4% (axis 1: 64.9%, axis 2: 22.5%) of the total variance (Fig. 6.4C).

6.1.3.3. Community structure based on soxB *gene*

Clustering analysis according to community composition based on *soxB* gene phylogeny grouped all winter samples into a single cluster (Fig. 6.5A). These winter samples showed a high predominance of *soxB* gene sequences related to genus *Arcobacter* (OTU-20) (Fig. 6.5B). A second cluster grouped all summer samples, characterized by *soxB* gene sequences affiliated to *Epsilonproteobacteria* (*Arcobacter*), *Chlorobi* (*Chlorobaculum*) and *Gammaproteobacteria* (*Allochromatium*) (Fig. 6.5A). In this case, dbRDA analysis indicated that ammonium, sulfate, sulfide, total phosphorous and total organic carbon explained 99.5% (axis 1: 96.3%, axis 2: 3.2%) of the total variance in *soxB* community composition (Fig. 6.5C).

6.1.3.4. Community structure based on dsrB gene

Clustering analysis based on *dsrB* gene sequences grouped samples into three well-differentiated clusters (Fig. 6.6B). One cluster is formed by samples with an important contribution of OTU-405 and OTU-577, both affiliated to genus *Desulfomonile* (Fig. 6.6A). Winter redoxcline samples composed a second cluster whereas the third one grouped samples with a high relative abundance of *dsrB* sequences affiliated to genus *Desulfocapsa* (OTU-4). In this case, multivariate analyses identified sulfate, total phosphorous, ammonium and nitrate as predictor variables of *dsrB* community composition explaining the 89.1% of total variance (Fig. 6.6C).
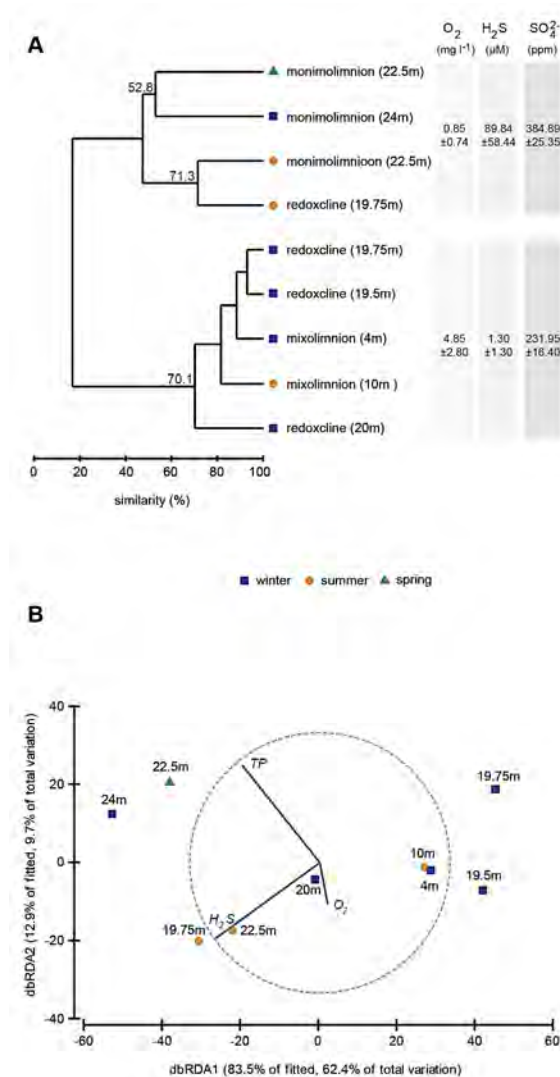
Figure 6.3. (A) Hierarchical clustering analysis (Bray Curtis distance) among samples based on the community composition according to 16S rRNA gene phylogeny. Average values (±SEM) for oxygen, sulfide and sulfate concentrations are indicated for each cluster. (B) Distance-based redundancy analysis (dbRDA) plot showing the result of the distance based linear model (DistLM) based on environmental variables. Significant explanatory variables are indicated by grey vectors, which show direction and strength of multiple partial correlations (>0.3) within a circle of radius 1. Samples correspond to different depths (labeled) of basin C-III of Lake Banyoles in winter (blue squares), summer (orange circles) and spring (green triangles).
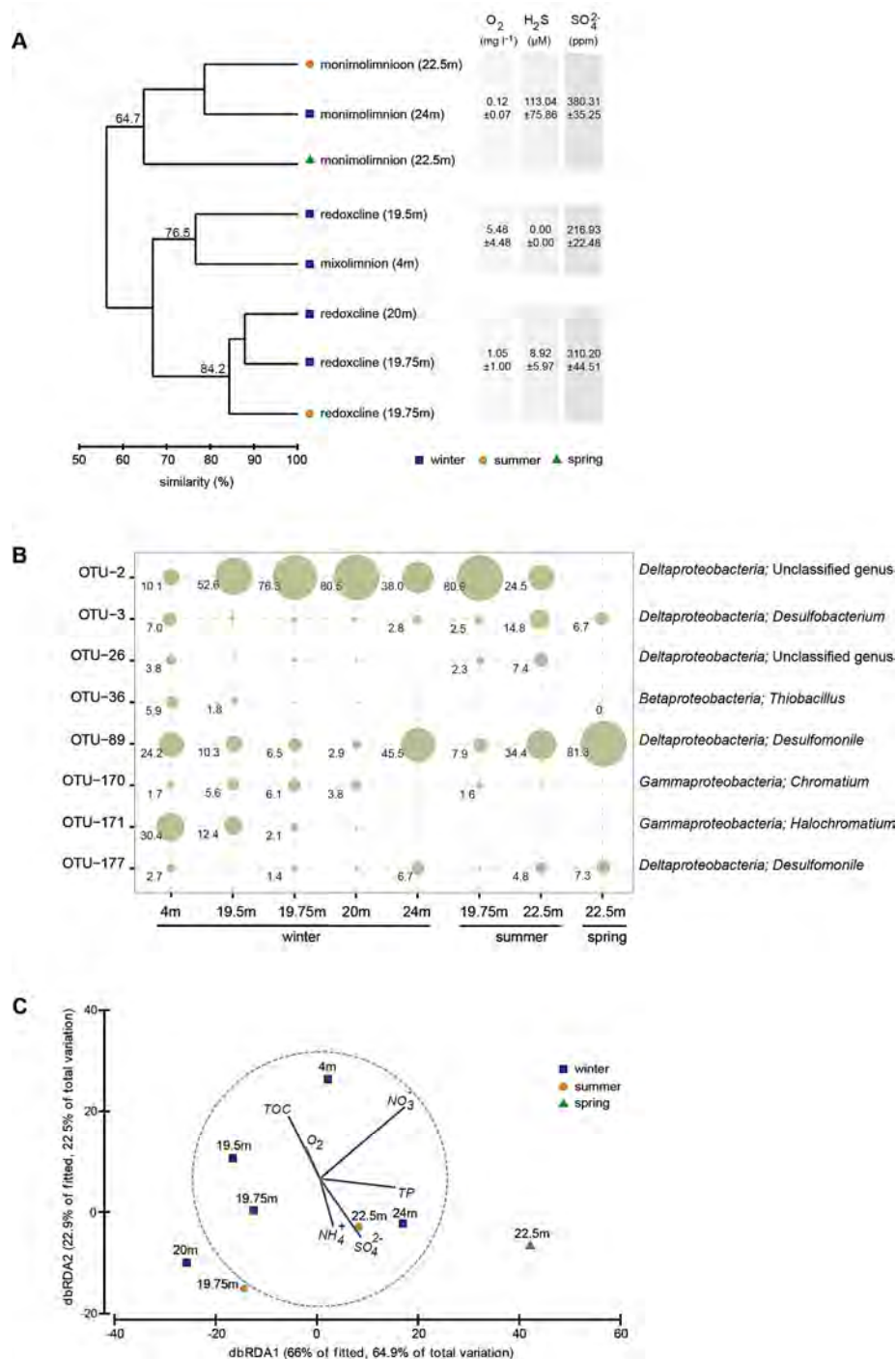
Figure 6.4. (A) Hierarchical clustering analysis (Bray Curtis distance) among samples based on the community composition according to *aprA* gene phylogeny. Average values (±SEM) for oxygen, sulfide and sulfate concentrations are indicated for each cluster. (B) Bubble plot showing the relative abundance of most abundant *aprA* OTUs (>5% of total abundance) and their assigned taxonomy. Values in grey show the relative abundance of each OTU across samples. (C) Distance-based redundancy analysis (dbRDA) plot showing the result of the distance based linear model (DistLM) based on environmental variables. Significant explanatory variables are indicated by grey vectors, which show direction and strength of multiple partial correlations (>0.3) within a circle of radius 1. Samples correspond to different depths (labeled) of basin C-III of Lake Banyoles in winter (blue squares), summer (orange circles) and spring (green triangles).
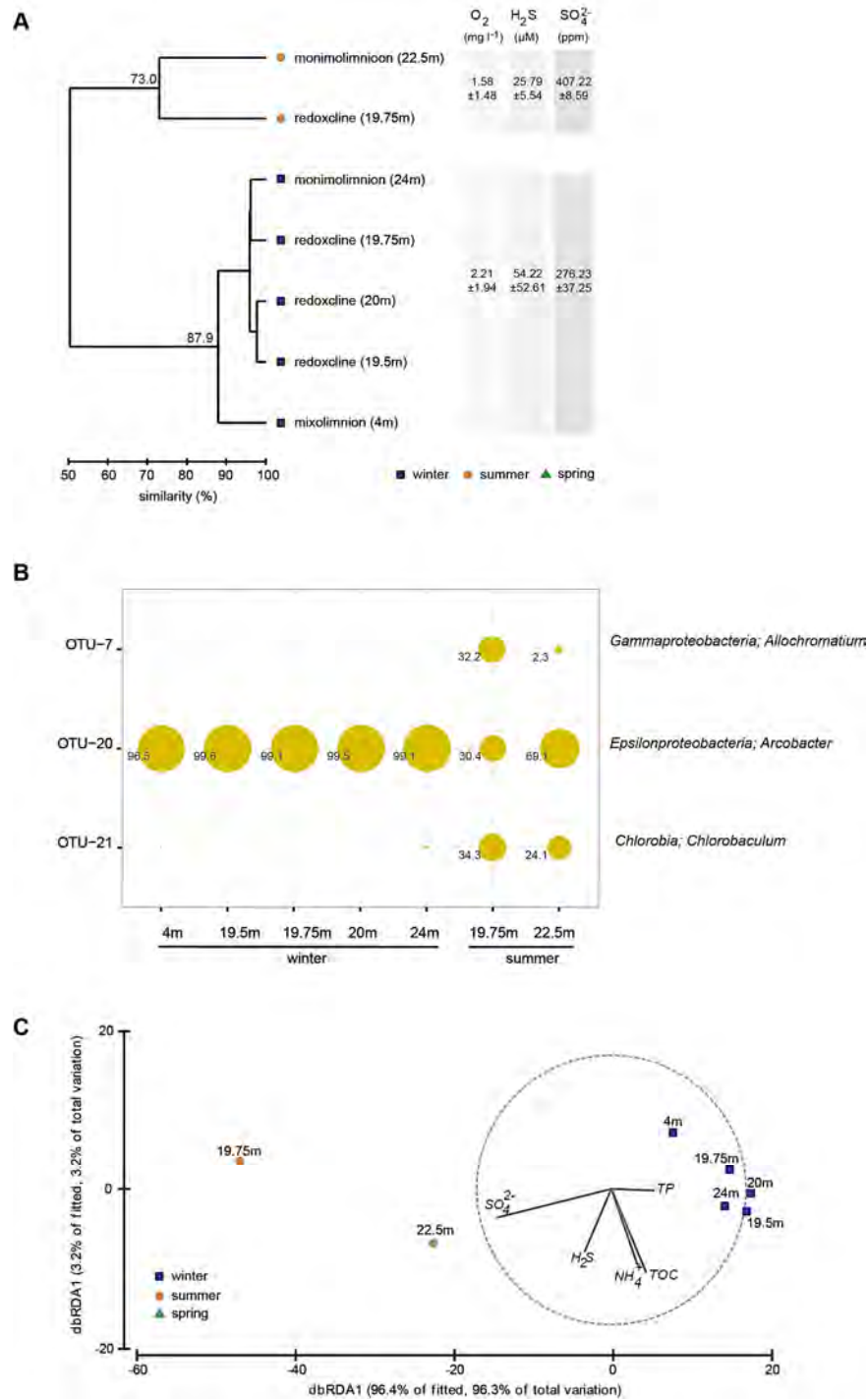
Figure 6.5. (A) Hierarchical clustering analysis (Bray Curtis distance) among samples based on the community composition according to *soxB* gene phylogeny. Average values (±SEM) for oxygen, sulfide and sulfate concentrations are indicated for each cluster. (B) Bubble plot showing the relative abundance of most abundant *soxB* OTUs (>5% of total abundance) and their assigned taxonomy. Values in grey show the relative abundance of each OTU across samples. (C) Distance-based redundancy analysis (dbRDA) plot showing the result of the distance based linear model (DistLM) based on environmental variables. Significant explanatory variables are indicated by grey vectors, which show direction and strength of multiple partial correlations (>0.3) within a circle of radius 1. Samples correspond to different depths (labeled) of basin C-III of Lake Banyoles in winter (blue squares), summer (orange circles) and spring (green triangles).
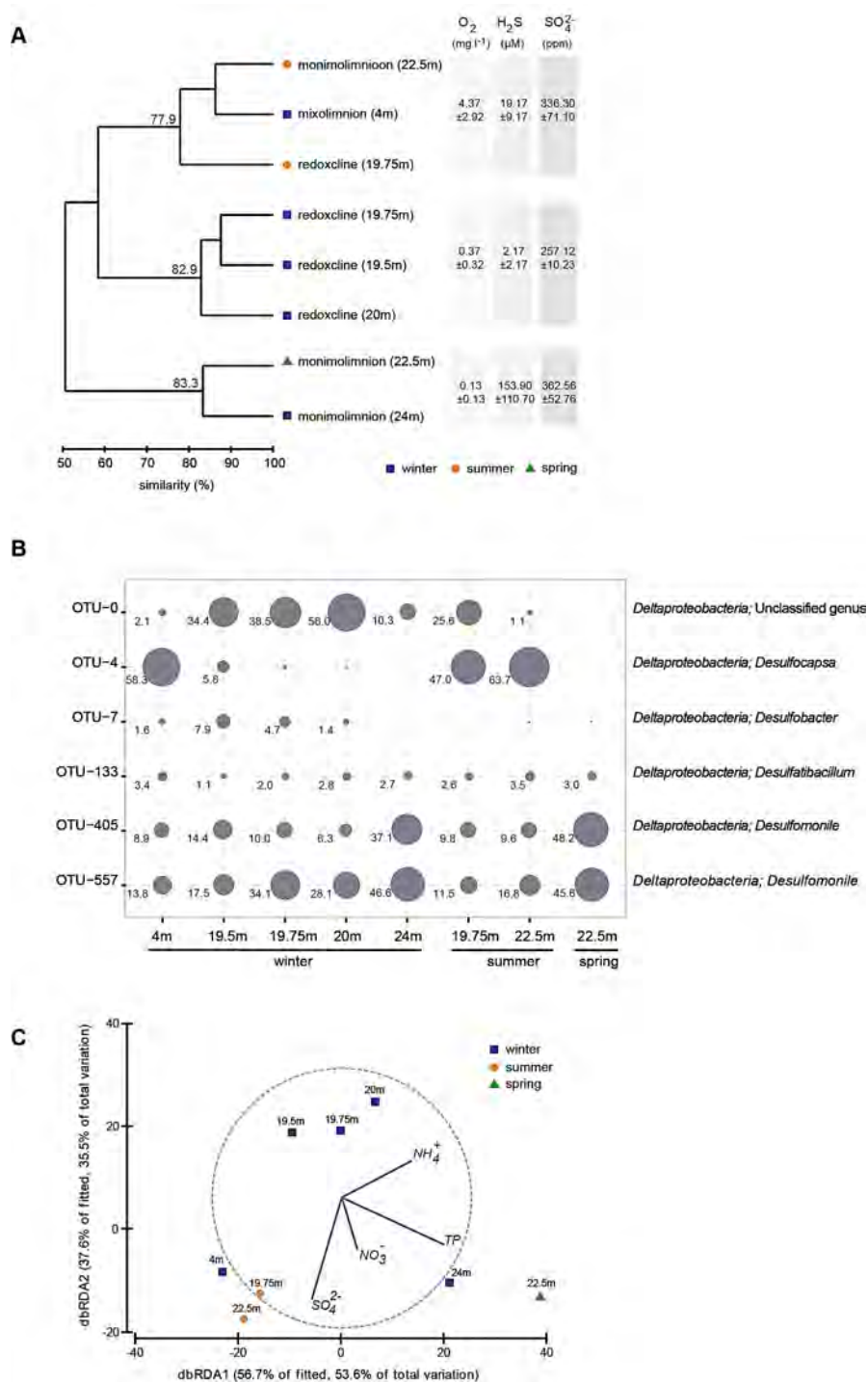
Figure 6.6. (A) Hierarchical clustering analysis (Bray Curtis distance) among samples based on the community composition according to *dsrB* gene phylogeny. Average values (±SEM) for oxygen, sulfide and sulfate concentrations are indicated for each cluster. (B) Bubble plot showing the relative abundance of most abundant *dsrB* OTUs (>5% of total abundance) and their assigned taxonomy. Values in grey show the relative abundance of each OTU across samples. (C) Distance-based redundancy analysis (dbRDA) plot showing the result of the distance based linear model (DistLM) based on environmental variables. Significant explanatory variables are indicated by grey vectors, which show direction and strength of multiple partial correlations (>0.3) within a circle of radius 1. Samples correspond to different depths (labeled) of basin C-III of Lake Banyoles in winter (blue squares), summer (orange circles) and spring (green triangles).

6.2. Discussion

6.2.1. Sulfur-oxidizing and sulfate-reducing bacteria in the planktonic assemblage

Comparison of the composition of the planktonic bacterial assemblage based on the 16S rRNA gene and the selected functional gene markers yielded differences that deserve detailed comments. Some discrepancies might have their origin on methodological issues, for instance: *i)* the known bias of PCR amplification (Kunin et al., 2010; Schirmer et al., 2015); *ii)* the specificity and coverage of the different primers used (Cai et al., 2013); *iii)* the coverage of different bacterial lineages in 16S rRNA and functional gene databases (Penton et al., 2013; Kim and Liesack, 2014; Poretsky et al., 2014); and *iv)* the different sequencing chemistries (pyrosequencing *vs.* Illumina) used for 16S rRNA and functional gene datasets (Luo et al., 2012). Besides, we have to take into account that our approach is probably not complete since genes encoding other key enzymes involved in the oxidation of reduced sulfur compounds such as the sulfide:quinone oxidoreductase (*Sqr* gene) and the flavocytochrom c sulfide dehydrogenase (*Fcc* gene) (Chan et al., 2009; Gregersen et al., 2011; Han and Perner, 2016) have not been included in our study.

Concerning sulfate-reducing bacteria, the use of a primer pair targeting the *dsrB* gene might result in an incomplete coverage for some SRB taxa. Particularly, the forward primer used (DSRp2060F, (Geets et al., 2006)) only covered a 23%, 39% and 8% of *dsrB* sequences within the class *Deltaproteobacteria*, the phylum *Firmicutes* and other environmental unclassified *dsrB* sequences, respectively. In turn, reverse primer DSR4R (Wagner et al., 1998) covered a 31%, 29% and 20% within the class *Deltaproteobacteria*, the phylum *Firmicutes* and archaeal sulfate-reducers (*i.e. Archaeoglobus*), respectively (Müller et al., 2015). Neither forward nor reverse primers targeted members of the phylum *Nitrospirae* (Müller et al., 2015). Although other primers targeting reductive and oxidative *dsrAB* genes are available (Wagner et al., 1998; Geets et al., 2006; Kondo et al., 2006; Lücker et al., 2007; Loy et al., 2009; Lenk et al., 2011; Haeusler et al., 2014; Pelikan et al., 2015) it is unclear how well these primers cover the currently known *dsrAB* diversity (Müller et al., 2015).

Finally, lateral gene transfer (LGT) events occurring during evolution may add confusion when using functional genes to infer phylogenetic relationships for SOB and SRB (Meyer and Kuever, 2007; Meyer et al., 2007; Müller et al., 2015). The use of *aprBA* genes as functional markers to study microbial diversity of sulfur-oxidizing prokaryotes (SOP) is recommended since these genes have been vertically transmitted during evolution among SOP (Meyer and Kuever, 2007). The same authors, however, pointed out that novel LGT events have also been occurred across different SOP divisions (Meyer and Kuever, 2007). On the other hand, reductive-type *dsrAB* and 16S rRNA branching patterns are generally similar although there is evidence for potential LGT of *dsrAB* gene (Müller et al., 2015). Phylogeny of *soxB* gene seems to reflect the major 16S rRNA gene-based phylogenetic lineages, although again some discrepancies indicated several events of lateral *soxB* gene transfer among SOB (Petri et al., 2001; Meyer et al., 2007).

Overall, and despite the potential biases and limitations that might arise from the above-mentioned issues, the overall picture finally obtained is fairly congruent to previous studies on the composition and functional diversity of the planktonic community in basin C-III obtained from metagenomics (Llorens-Marés et al., 2015).

6.2.2. Distribution and diversity of sulfur-oxidizing bacteria in basin C-III

Diversity of sulfur-oxidizing bacteria was assessed by specifically targeting both *aprA* and *soxB* genes, which encodes key enzymes of the reverse sulfate reduction pathway and the putative SOB using multienzyme Sox System, respectively (Petri et al., 2001; Meyer and Kuever, 2007). The diversity of sulfur-oxidizing bacteria in basin C-III is similar to those detected in other environments with a prevalence of sulfur cycle such as caves, hydrothermal vents and soils (Petri et al., 2001; Chen et al., 2009; Hügler et al., 2010; Tourna et al., 2014). However, important differences have been detected between winter and summer due to the mainly prevalence of sequences of *Arcobacter* spp. in winter, in contrast of more diverse community in summer.

The prevalence of *soxB* sequences affiliated to genus *Arcobacter* in winter agrees with a previous metagenomics study of samples collected at the sulfidic redoxcline and the monimolimnion of basin C-III, which reported an important contribution of order *Campylobacterales* in the S cycle (Llorens-Marès et al., 2015). This observation also agrees with results from previous chapters that identify *Arcobacter* spp. as an active chemolithotrophic sulfur-oxidizing member of the planktonic community (see Chapters 4–5). It is somehow surprising, however, that *soxB* sequences assigned to *Arcobacter* spp., were also detected at depths were sulfide was not present (*i.e.* mixolimnion). The detection of sequences affiliated to *Arcobacter* at these well-oxygenated, sulfide-free depths was also confirmed by qPCR analysis (>4.5x10^2 cells ml^{-1}, see Chapter 5). It should be taken into account, however, that the detection of these sequences does not imply activity since the physicochemical conditions at these water layers are incompatible to chemolithotrophy using sulfide as energy source. Accordingly, this ubiquitous distribution point to alternative energy and carbon sources and thus agrees with previous studies that demonstrate that some *Arcobacter* spp. are able to combine strict chemolithotrophy with organotrophy (Campbell et al., 2006; Berg et al., 2013; Carlström et al., 2013; Roalkvam et al., 2015).

A completely different scenario was observed for samples collected in summer, where the predominant *soxB* sequences were mostly related to green, purple and colorless sulfur bacteria. The dominance of anoxygenic photosynthetic bacteria, both purple and green, in basin C-III during the summer season was consistently observed in previous studies (Borrego et al., 1993, 1997, 1999) and during the current investigation (Chapters 4–5). On the other hand, the presence of *aprA* sequences affiliated to colorless sulfur bacteria also agrees with previous studies reporting the occurrence of Thiobacilli at sulfidic redoxcline of lakes and lagoons of the Banyoles Karstic System (Casamayor, 2010). On the other hand, all the sequences recovered from winter and summer samples that affiliated to class *Alphaproteobacteria* grouped with bacterial clades composed of

chemoorganotrophic representatives (*i.e. Candidate* SAR11 (Joint, 2008; Tripp et al., 2008) and *Pseudorhodobacter* (Uchino et al., 2002)) without direct evidences of being capable to oxidize sulfur. Nevertheless, this possibility could not be formally ruled out since class *Alphaproteobacteria* encompasses well-known SOB (Meyer and Kuever, 2007).

Multivariate analyses identified sulfide, total phosphorous and oxygen as most important environmental variables structuring the composition of the bacterial planktonic community based on the 16S rRNA phylogeny. This distribution agrees with the key role of hydrogen sulfide (and sulfate as substrate for sulfate-reducing bacteria) and oxygen in determining the vertical stratification of microbial communities in meromictic lakes (Camacho et al., 2000, 2001; Grote et al., 2007; Lavik et al., 2009). On the other hand, the influence of TP might be probably related to the well-known role of phosphorous as limiting factor in aquatic systems (Krom et al., 1991; Satoh et al., 2006) and, particularly, in other waterbodies of the Banyoles Karstic System (Bañeras et al., 2010). In the distribution of sulfur-oxidizers based on the *soxB* phylogeny, sulfate and partially sulfide were correlated to summer samples, where colorless and anoxygenic sulfur bacteria were present, especially considering the direction and strength of correlation vectors for these variables. In turn, all winter samples clustered together probably because of the large contribution of *soxB* sequences affiliated to genus *Arcobacter* in winter libraries. In this case, TP, total organic carbon and ammonium appear as the main predictor variables. These results differs to those obtained in other meromictic haloalkaline lakes with high sulfide content, where sulfur-oxidizers seems to be highly influenced by environmental variables such as salinity, pH and oxygen concentration (Sorokin et al., 2007).

6.2.3. Distribution and diversity of sulfate-reducing bacteria in basin C-III

Putative sulfate reduction activity was assessed by targeting *dsrB* and *aprA* functional marker genes (Wagner et al., 1998; Geets et al., 2006; Meyer and Kuever, 2007). Both genes are involved in metabolic pathways used by members of the class *Deltaproteobacteria* and phylum *Firmicutes* that anaerobically oxidize a wide range of organic compounds using sulfate as electron acceptor.

Taxonomic assignments of the retrieved sequences identified *Desulfocapsa* and *Desulfomonile* as main members of the sulfate-reducing planktonic community agreeing with results from other freshwater meromictic environments (Tonolla et al., 2005). The high number of unclassified sequences for both *dsrB* and *aprA* genes within the class *Deltaproteobacteria,* especially in libraries constructed from redoxcline samples, agrees with previous studies (Wagner et al., 2005; Frank et al., 2013; Müller et al., 2015) and suggest that a large fraction of the actual diversity of sulfate reducing bacteria remains unknown. The incompleteness of available *dsrAB* sequence databases is a factor that must be considered to partially explain the large number of unclassified sequences thus making the identification and classification of environmental *dsrB* sequences troublesome (Müller et al., 2015). On the other hand, the diversity of the putative sulfate-reducers community according to *aprA* phylogeny was lower compared to that observed

in other meromictic lakes and freshwater environments where the same primer pair was used (Kondo et al., 2006; Meyer and Kuever, 2007).

The spatial distribution of sulfate-reducing bacteria revealed by our study agrees with previous investigations that demonstrate that anaerobic respiration using sulfate is not restricted to anoxic waters (Holmer and Storkholm, 2001) but it could also take place in aerobic compartments with availability of organic carbon (Hastings and Emerson, 1988; Blaabjerg and Finster, 1998; Manz et al., 1998; Brune et al., 2000; Nalven, 2011; Llorens-Marès et al., 2015). Besides, SRB have been the subject of several investigations due to their role in methylmercury production in marine habitats where this activity has been reported in oxic and suboxic compartments (Gentès et al., 2013). Similar results have been reported in oxic compartments of stratified oligotrophic lakes (Achá et al., 2012). Notwithstanding this, the three main OTUs identified in the oxygenated water layers of basin C-III affiliated to different genera (*Desulfocapsa*, *Desulfomonile* and a third, unclassified genus within the *Deltaproteobacteria*) than SRB identified in oxic water layers of marine (*Desulfofustis* sp., *Desulfomicrobium* sp. and *Desulfotomaculum* sp. (Guyoneaud et al., 2014)) and freshwater systems (*Desulfovibrio* sp., (Achá et al., 2012)).

As expected, sulfate appears as the main predictor variable explaining the composition of the bacterial guild according to *dsrB* gene phylogeny. This is especially evident considering the direction and strength of the correlation vector for sulfate which clearly associates to samples collected at the anoxic, sulfate-rich monimolimnetic waters of basin C-III. This results corroborate the hypothesis that functional gene distribution correlate to substrate availability and the presence of intermediates or products of the corresponding metabolic pathways (Tarlton, 2012).

7

GENERAL DISCUSSION

Epsilonproteobacterial sulfur-oxidizers are widespread in both terrestrial and aquatic ecosystems where oxygen and sulfide occur together. Their metabolic versatility allows them to play pivotal roles in biogeochemical cycles of carbon and sulfur, especially in marine sulfidic redoxclines and hydrothermal vent systems (Campbell et al., 2006).

Contrasting with the wealth of information available on the distribution, abundance and activity of Epsilonproteobacteria in the marine biome, these topics are poorly documented in freshwater habitats. Our study contributes to fill this gap by providing data on the distribution, diversity, seasonal dynamics and activity of Epsilon-proteobacteria in a karstic lake and opening several lines of research regarding their ecological role in habitats characterized by sharp physicochemical gradients and euxinic conditions. Results presented and discussed in this thesis demonstrate that a novel clade of freshwater, chemolithotrophic member of the genus *Arcobacter* forms stable planktonic populations at the sulfidic redoxcline of basin C-III in lake Banyoles. Although our data resolve some questions of ecological interest regarding the key role of Epsilon-proteobacteria in cycling C and S in Lake Banyoles, several other issues remain unanswered and deserve additional comments.

7.1. Marine *vs.* freshwater planktonic communities of Epsilonproteobacteria

7.1.1. Epsilonproteobacteria as key players in C and S cycles in basin C-III

Stratified lakes are characterized by well-defined water compartments with contrasting environmental conditions that determine both the diversity and the activity of microbial populations, thus affecting biogeochemical transformations and nutrient cycling. Previous studies have demonstrated that vertical stratification of the water column structures microbial communities (Shade et al., 2008). Besides, a meta-analysis of bacterial diversity conducted on a worldwide set of stratified lakes demonstrate that euxinic (anoxic and sulfurous) waters promote bacterial endemicity (Barberán and Casamayor, 2011). Results of the current study agree with this idea but also reveal a seasonal dynamics of sulfur oxidizing bacteria (SOB) communities, especially at the redoxcline depth. In this regard, Epsilonproteobacteria are prevalent during winter whereas a more diverse community composed of phototrophic and chemolithotrophic sulfur-oxidizers blooms in summer.

In winter, the bacterial assemblage involved in cycling sulfur consist of: *(i)* putative SRB of class *Deltaproteobacteria* (genus *Desulfomonile* and unclassified genera), which are responsible of the anaerobic oxidation of organic matter using sulfate (entering the lake by bottom springs) as electron acceptor; and *(ii)* chemolithotrophic SOB of class *Epsilonproteobacteria* (mainly represented for *Arcobacter* spp. and a minor contribution of other genera such as *Thiomicrospira, Thiobacillus, Allochromatium, Chromatium, Halochromatium, Beggiatoa, Sulfurimonas* and *Sulfuricurvum*) at the redoxcline and the upper monimolimnion (Fig. 7.1A).

In summer, both SRB and SOB communities appear to be more diverse. SRB include sulfate-reducers of genera *Desulfomonile* and *Desulfocapsa* in the upper monimolimnion and diverse unclassified species at the redoxcline depth. The SOB community encompasses *Epsilonproteobacteria* (*Arcobacter* spp.), *Gammaproteobacteria* (members of genus *Allochromatium*, which comprise both anaerobic facultative photoautotrophs and aerobic chemoorganotrophs), and anaerobic Green Sulfur Bacteria (phylum *Chlorobi*, mainly members of genera *Chlorobaculum* and *Chlorobium*) (Fig. 7.1B).

The seasonal dynamics of both Epsilonproteobacteria and GSB communities is fully supported by both the 16S rRNA gene data already presented and by data based on functional genes. Despite the clear dominance of GSB in late spring and summer the co-occurrence of chemolithotrophic, epsilonproteobacterial sulfide-oxidizers may result in a competition for electron donors (*e.g.* sulfide and other reduced sulfur species) although the precise nature of this interaction cannot be elucidated from the available data.
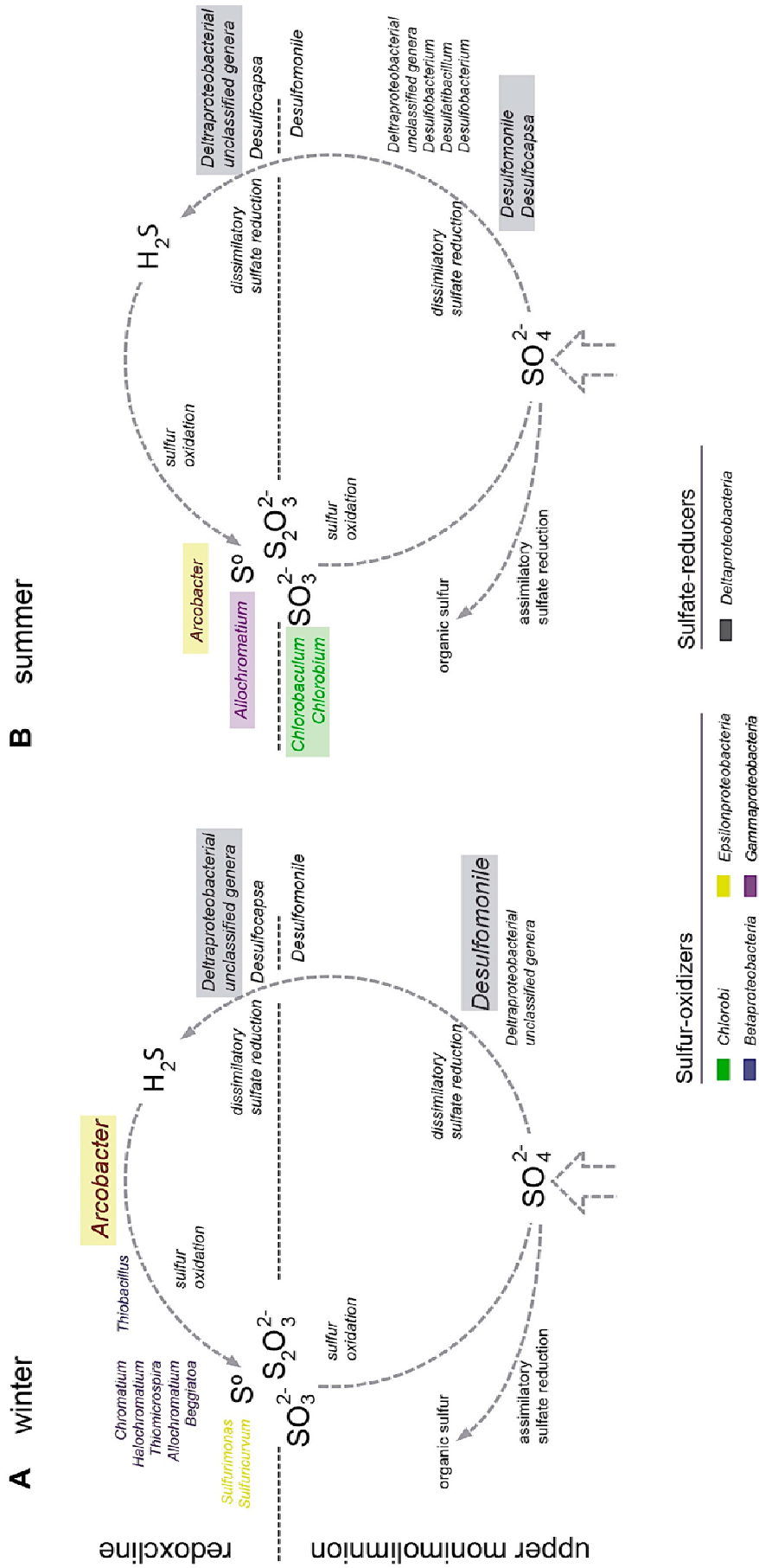
Figure 7.1. Key bacterial agents involved in sulfur transformations at the redoxcline and the upper monimolimnion in basin C-III in (A) winter and (B) summer.

Interestingly, both groups share the same carbon fixation pathway (i.e. the reductive TCA cycle) and the enzymatic pathway for sulfur oxidation (i.e. the Sox system) (Hügler et al., 2005; Evans et al., 1966; Fuchs et al., 1980; Tang and Blankenship, 2010; Gregersen et al., 2011; Yamamoto et al., 2010; Yamamoto and Takai, 2011; Akerman et al., 2013) making it difficult to ascertain why GSB overcompete Epsilonproteobacteria in warm seasons. The high efficiency of brown-colored GSB to thrive under light limitation (Borrego et al., 1997; 1999) may provide a selective advantage over Epsilonproteobacteria when light reaching the sulfide-rich waters progressively increased. The dense plate of GSB at the O_2-H_2S boundary layer then constitutes a biological barrier that prevent sulfide diffusion from the monimolimnion to oxygenated layers. Since Epsilonproteobacteria require the co-occurrence of H_2S and oxygen it is then plausible to assume that their activity is impaired when GSB dominates the sulfidic redoxcline, a situation that agrees with the low rates of dark carbon fixation measured at these depths in summer (see Chapter 4).

To the best of our knowledge, the co-occurrence of both photo- and chemolithotrophic sulfur-oxidizers at the redoxcline of a meromictic lake has only been reported in Mahoney lake (Hamilton et al., 2014). In this case, the SOB community is mainly dominated by Purple Sulfur bacteria (*Gammaproteobacteria*) with lower contribution of *Epsilonproteobacteria* and *Chlorobi*. In Mahoney lake, however, Epsilonproteobacteria was mainly represented by a member closely related to *Sulfurimonas autotrophica* able to carry out both aerobic and anaerobic (*i.e.* using nitrate as electron acceptor) sulfide oxidation (Hamilton et al., 2014). Similarly to Mahoney Lake, the planktonic SOB community in basin C-III is functionally redundant and occupies narrow niches at the redoxcline depth. GSB and Epsilonproteobacteria populations segregate along the vertical profile according to their metabolic and physiological requirements thus interacting at different levels in both spatial and temporal scales. Further research is needed to elucidate the factors that determine their co-existence and interactions regarding the availability of electron donors and acceptors. Data from these studies will also be helpful to decipher the main physic-chemical drivers behind the prevalence of a given species over others.

7.1.2. Differences between epsilonproteobacterial communities from marine and fresh-water environments

In the Central Baltic Sea, planktonic communities of Epsilonproteobacteria are dominated by *Sulfurimonas* sp. (Grote et al., 2007, 2012; Labrenz et al., 2013). Marine pelagic redoxclines of the Black Sea and the Cariaco Basin harbor a more diverse communities, in which *Arcobacter* sp. has also been detected (Madrid et al. 2001; Lin et al. 2006; Grote et al. 2008; Glaubitz et al. 2010). In clear contrast, the Epsilonproteobacteria community in basin C-III is mainly composed of *Arcobacter spp.* whereas *Sulfurimonas* and other genera (*e.g. Sulfuricurvum*) are rare (Chapters 4 and 5) (Table 7.1). The phylogenetic analyses carried out in this work pointed to a chemolithoautotrophic *Arcobacter* spp. closely related to *Candidatus* Arcobacter sulfidicus, a sulfur-oxidizing,

chemolithotrophic marine relative (Wirsen et al., 2002) (Chapter 4). These differences between marine and freshwater epsilonproteobacterial communities raise interesting questions about the influence of environmental drivers on the bacterial communities, and more specifically, on which factors and conditions allow the success of *Arcobacter* spp. in basin C-III.

Table 7.1. Differences in composition and abundance of marine and freshwater epsilonproteobacterial communities in aquatic systems where these SOB have been reported. n.a., not available.

Habitat	Epsilonproteobacteria detected	Maximum abundance[a] (cells mL^{-1}±SEM)	Reference
Central Baltic Sea	*Sulfurimonas gotlandica*	$1.35 \ 10^5 \pm 3.17 \ 10^4$	(Labrenz et al., 2004, 2013; Grote et al., 2007)
Black Sea	*Sulfurimonas* sp.	n.a.	(Grote et al., 2008; Glaubitz et al., 2010)
Cariaco Basin	*Sulfurimonas denitrificans* *Arcobacter nitrofigilis*	n.a.	(Madrid et al., 2001)
Lake Pavin	*Sulfurimonas denitrificans* *Sulfuricurvum* sp.	n.a.	(Biderre-Petit et al., 2011)
Basin C-III (Lake Banyoles)	*Arcobacter* spp.	$9.88 \ 10^6 \pm 6.47 \ 10^4$	This work

[a] The abundance of Epsilonproteobacteria was determined by qPCR.

The high metabolic versatility among Epsilonproteobacteria and, especially, within the genus *Arcobacter* is probably a key factor to explain its ubiquity (Campbell et al., 2006). This genus encompasses animal pathogens as well as chemolithoautotrophic members that thrive in terrestrial, freshwater and marine environments (Gevertz et al., 2000; Wirsen et al., 2002; Vandieken et al., 2012; Roalkvam et al., 2015). Some *Arcobacter* species require higher sulfide concentrations for growth than *Sulfurimonas* spp. (previously known as *Thiomicrospira*, Wirsen et al., 2002). This could be a limiting factor in marine environments compared to some freshwater habitats such as basin C-III, where high sulfide concentrations are usually detected at the redoxcline and the upper monimolimnion especially in winter (Chapter 4). Otherwise, salinity could also appear as a crucial aspect to explain the low representativeness of *Sulfurimonas* spp. in Lake Banyoles assuming its adaptation to brackish or marine waters. In this regard, conductivity values measured in basin C-III are well below values for seawater but higher than standard values for "true" freshwater (Table 7.2). Unfortunately, all attempts to cultivate the dominant *Arcobacter* strain in Lake Banyoles were unsuccessful (see Section 7.3), thus making impossible to determine neither its salt tolerance nor other

physiological requirements that would be useful to understand its ecological success in basin C-III.

Table 7.2. Conductivity values measured in different marine and freshwater habitats.

Habitat	Conductivity ($\mu S\,cm^{-1}$)	Reference
Black Sea (stratified water column, SE Europe-W Asia)	20,000-23,500	(Manske et al., 2005)
Caribbean Sea (stratified water column, Venezuela)	47,500-49,250	(National Centers for Environmental Information, 2010)[a]
Egg Pond (meltwater pond, Antarctica)	8,200-93,600	(Archer et al., 2014)
La Chimba (coastal spring, Chile)	25,300	(Herrera Lameli and Custodio, 2014)
Coffee Pots Hot Spring (thermal spring, Yellowstone, US)	555	(Hall et al., 2008)
Lake Cadagno (meromictic alpine lake, Switzerland)	100-310	(Lüthy et al., 2000)
Lake Pavin (crater meromictic lake, France)	75-475	(Biderre-Petit et al., 2011)
Lake Banyoles (meromictic karstic basin C-III, Spain)	1,500-2,700	This work

[a] Data available on:
ftp://ftp.aoml.noaa.gov/phod/pub/rsmith/VS_VP_Trans_Study/SUMMER_2010/CTD_data

7.2. Epsilonproteobacteria in neighboring lakes of the Banyoles Karstic System

Euxinic conditions and oxic-anoxic interphases (*i.e.* redoxclines) also occurs in other basins of Lake Banyoles (e.g. basin C-IV and basin C-VI in the northern lobe) as well as in neighboring lagoons of the Banyoles Karstic System such as lakes Vilar, Cisó and Coromina (Garcia-Gil et al., 1988; Garcia-Gil and Abella, 1992; Borrego et al., 1997; Llirós et al., 2008; Casamayor, 2010; Casamayor et al., 2012). It is then plausible that Epsilonproteobacteria also thrive in these waterbodies playing a relevant role in cycling C and S.

Lake Banyoles and neighboring lagoons have been studied by different research groups due to their particular limnological and biological features. Most of these studies dealt with the abundance and seasonal dynamics of purple and Green Sulfur Bacteria and

their impact on nutrient cycles and adaptation to environmental conditions (Garcia-Gil et al., 1988; Borrego et al., 1993; E. O. Casamayor, 2010). Main autotrophic bacterial populations inhabiting the sulfidic redoxclines and anoxic, sulfide-rich water layers of these systems consist of both oxic and anoxic phototrophs including algae (e.g. *Cryptomonas* spp.), purple sulfur bacteria (*Amoebobacter* sp. and *Chromatium* spp.), Green Sulfur Bacteria (*Chlorobaculum limnaeum* (formerly known as *Chlorobium phaeobacteroides*) and *Chlorobium clathratiforme* (formerly known as *Pelodictyon clathratiforme*)), and chemolithoautotrophic bacteria (mainly members of class *Gammaproteobacteria* such as sulfur-oxidizing *Thiobacillus* spp. (Borrego et al., 1993; Garcia-Gil et al., 1993; Casamayor, 2010).

Several studies have also been carried out in these habitats to investigate the contribution of planktonic microbial communities to C cycle, and particularly, to dark C fixation (Pedrós-Alió and Guerrero, 1991; García-Cantizano et al., 2005; Casamayor et al., 2008, 2012; E. O. Casamayor, 2010). These studies demonstrate that the contribution of dark carbon fixation to total autotrophic activity is comparable or even higher to that reported for photoassimilation at the redoxcline. In different lagoons, such as lake Cisó most of the fixed CO_2 was due to chemolithoautotrophs populations, followed by the anoxygenic phototrophs (García-Cantizano et al., 2005). More detailed studies carried out in lake Cisó revealed an active contribution of photosynthetic purple sulfur bacteria to dark carbon fixation processes (Casamayor et al., 2008). In basin C-III, vertical distribution of aerobic, chemoautotrophic *Thiobacilli*-like sulfur-oxidizing bacteria matched vertical profiles of dark CO_2 fixation rates thus suggesting a putative role of these microorganisms on C cycle (Casamayor, 2010). In the same study, the potential presence of other chemolithotrophic bacterial populations contributing to dark CO_2 fixation coupled to sulfur oxidation was already suggested.

Results of our investigations demonstrated that Epsilonproteobacteria (*i.e. Arcobacter* spp.) assimilate CO_2 in the dark probably using reduced sulfur compounds as energy source and being the main responsible for the high rates of inorganic carbon fixation in the dark measured in winter at the redoxcline of basin C-III (Chapters 4 and 6). These data suggest that a similar contribution might be expected in other lakes and lagoons of the Banyoles Karstic System that share similar physicochemical conditions. Accordingly, studies addressing the presence, diversity and activity of Epsilonproteobacteria in these waterbodies are encouraged in order to have a better picture of the distribution and ecological role of Epsilonproteobacteria in the Banyoles Karstic System. It would be also of great interest to evaluate if *Arcobacter* spp. also succeeds in neighboring lakes differing from basin C-III in their trophic status and limnological features (e.g. lakes Vilar and Cisó, or hypereutrophic lake Coromina).

Similar surveys in other stratified freshwater lakes such as La Cruz, El Tejo, El Tobar or Arcas (Cuenca), lake Estanya (Huesca) and lake Cadagno (Switzerland) where high dark carbon fixation rates have been measured at the oxic-anoxic interphase (Camacho et al., 2001, 2003; Ferrera et al., 2004; Casamayor et al., 2012) would be also of interest to assess if planktonic Epsilonproteobacteria play a role. Interestingly, sequences affiliated to

Thiovulum, a sulfur-oxidizing Epsilonproteobacteria, were already detected at the redoxcline of lake Cadagno, thus suggesting that members of this class are ubiquitous in O_2/H_2S interphases of stratified lakes (Demarta et al., 1998). Similarly, it would be of interest to investigate the presence of epsilonproteobacterial sulfur-oxidizers in other aquatic environments where high rates of dark CO_2 fixation were also measured, such as the Ebro River Salt Wedge Estuary (Casamayor et al., 2001).

7.3. Cultivability and enrichment strategies

The characterization and description of new species can only be properly accomplished after time-consuming enrichment procedures and further isolation of target microorganism as pure culture. This experimental pipeline is, however, especially tricky taking into account the low cultivability of most microorganisms (Amann et al., 1995). To tackle the problem microbial ecologists have designed innovative enrichment strategies, from dilution to extinction techniques to more sophisticated cultivation systems using state-of-the-art technologies (Connon and Giovannoni, 2002; Bruns et al., 2003; Hahn et al., 2004; Stevenson et al., 2004; Ferrari et al., 2005; Nichols et al., 2010). Although some of these strategies have provided promising results, uncultivable microorganisms outnumber "domestic" bacteria by several orders of magnitude. This issue poses a "Gordian knot" that genomic approaches try to overcome (Handelsman, 2004; Schloss and Handelsman, 2005; Tyson and Banfield, 2005; Kvist et al., 2007). Despite the advantage of culture-independent procedures to unveil the hidden diversity of non-cultivable prokaryotes, pure cultures are still necessary to have a better knowledge of environmental relevant microbes and to properly interpret molecular data in an ecological context (Rothschild, 2006; Nichols, 2007).

As expected, low cultivability indices are also common for planktonic bacteria in lakes, including heterotrophic bacteria (Bussmann et al., 2001). Within class *Epsilonproteobacteria*, some species have been already isolated as pure cultures although most of them correspond to known human pathogens (e.g. members of genus *Campylobacter, Helicobacter and Arcobacter*). In clear contrast, isolates of sulfide-oxidizing Epsilonproteobacteria are less represented in public culture collections although 16S rRNA phylogenetic studies reveal a large diversity within the class. According to DSMZ collection database, there are 175 members of the order *Campylobacterales* cultured but only 15.4% have been isolated from natural habitats, and from these, only a 3.4% have been isolated from marine and freshwater environments. Otherwise, cultured members of the *Nautiliales* account for only 8 species (deposited in the DSMZ) that have been isolated from hydrothermal vents, including symbionts of the *Alvinella pompejana* polychaete (Fig. 7.2).

Recently, the isolation of the dominant member of the epsilonproteobacterial community at sulfidic redoxclines in the Baltic Sea, *Sulfurimonas gotlandica* (formerly known as *Sulfurimonas* strain GD1), allowed its complete physiological and genomic characterization thus providing the necessary clues to understand its success in the

system (Grote et al., 2012). Regarding *Arcobacter* spp., only one free-living chemolithoautotrophic, sulfur-oxidizing species has been described so far, *Candidatus* Arcobacter sulfidicus (Wirsen et al., 2002). This species was enriched using microaerophilic conditions and O_2/H_2S gradients in semi-solid media. This strategy permitted a general description of its main physiological requirements and optimal growth conditions but no pure cultures were finally obtained (Wirsen et al., 2002). After 14 years since its description this species has not been deposited yet in public collections (*e.g.* the German Collection of Microorganisms and Cell Cultures (DSMZ) and the American Type Culture Collection (ATCC)) and it remains as *Candidatus* species. In fact, catalogues of DMSZ and ATCC have only 13 and 11 *Arcobacter* strains currently available, being most of them known pathogenic strains with the exception of *Arcobacter marinus*, *A. nitrofigilis, A. anaerophilus* and *A. halophilus.*
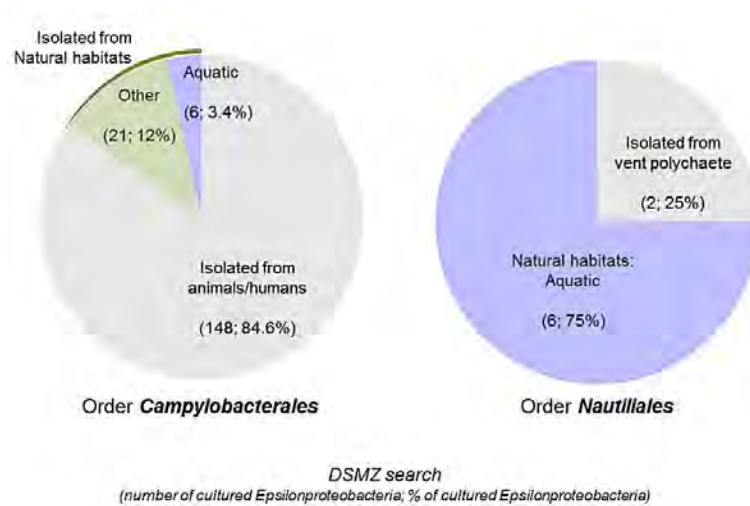
Figure 7.2. Relative contribution of cultured microorganisms within class *Epsilonproteobacteria* deposited in the DSMZ Collection according to the habitat from which they were isolated. Source: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (search carried out in February 2016).

Assuming that cultivability is a key factor to fully understand the potential capabilities of relevant microorganisms in the environment, we specifically tried to isolate the dominant *Arcobacter* strain in basin C-III to have a better understanding of their ecological role in the system. According to our results this strain was capable of using sulfur reduced compounds as energy source to aerobically fix inorganic carbon. On the other hand, the potential use of nitrate as alternative electron acceptor under anoxic conditions (autotrophic denitrification) was also taken into account when designing enrichment conditions. We then tried to enrich the target strain from two water samples collected in winter 2014 at the redoxcline depth using both a basal medium consisting in water from the same basin sterilized by filtration and supplemented with vitamins and a synthetic mineral medium. Both media were amended with different reduced sulfur compounds according to previous studies (Wirsen et al., 2002; Pati et al., 2010; Carlström

et al., 2013; Roalkvam et al., 2015) (*i.e.* thiosulfate (10 mM final concentration); sulfur (13 mM). Enrichment cultures were then incubated under fully oxic or anoxic conditions (Table 7.3, Fig. 7.3). Anoxic conditions were achieved flushing nitrogen gas and adding nitrate (10mM) as a putative electron acceptor.

Table 7.3. Culture conditions used and cultivability strategies tested to achieve the isolation of *Arcobacter spp.* from basin C-III.

Aerobic conditions[a,b]			
e- donor	e- acceptor	C source	Incubation support
$S_2O_3^{2-}$	O_2	CO_2	96-well plates
$S_2O_3^{2-}$	O_2	$CH_3CO_2^-$	

Anaerobic conditions[a,b]			
e- donor	e- acceptor	C source	Incubation support
$S_2O_3^{2-}$	NO_3^-	HCO_3^-	96-well plates
$S_2O_3^{2-}$	NO_3^-	$CH_3CO_2^-$	
S^{2-}	NO_3^-	HCO_3^-	96-well plates and tubes
S^{2-}	NO_3^-	$CH_3CO_2^-$	

[a] All cultures were adjusted at pH: 7.1-7.3, incubated at 18°C
[b] All culture conditions were carried out with and without bromothymol blue as pH indicator.

All enrichments were monitored by microscopic observation. Isolates obtained were subjected to DNA extraction and sequencing of their 16S rRNA genes that were then used for their taxonomic assignment using public 16S RNA gene databases. Unfortunately, none of the isolates affiliated to either *Arcobacter* spp. or Epsilonproteobacteria. According to these results, further efforts must be addressed to design a better enrichment and cultivation strategy. Besides, the abundance of the target microorganism in the source sample is of great concern. Needless to mention that the probability of success in downstream enrichment and isolation procedures will be higher as more target cells will be in the natural sample used as inoculum.

Figure 7.3. Different strategies carried out to isolate the target *Arcobacter* strain: (A) Positive wells were identified by their yellow color that indicated acidification of the growth medium caused by the utilization of thiosulfate. Ten-fold serial dilutions of cell suspensions from positive wells were carried out to isolate the target microorganism. (B) Cultures were then scaled-up to larger volumes. (C) Some tubes were prepared using a bottom agar layer containing sodium sulfide as a source of sulfide that diffuse upwards and creates a vertical gradient that mimics the conditions occurring in oxic-anoxic interphases of stratified lakes.

Despite these setbacks, in our opinion the isolation of this freshwater, chemolithotrophic, sulfur-oxidizing member of the genus *Arcobacter* is of primary significance. Only after its isolation and complete characterization we would be able to properly interpret our results and provide answers to all the microbiological and ecological questions that remain unanswered such as the putative metabolic versatility of this species, which would be of great interest not only to decipher the ecological role of this Arcobacter strain in Lake Banyoles but also to compare its physiologic and genomic traits to those obtained from their close phylogenetic relatives, either *Arcobacter* spp. (Wirsen et al., 2002; Roalkvam et al., 2015) or *Sulfurimonas gotlandica* (Glaubitz et al., 2014). The availability of a pure culture of the *Arcobacter* spp. will also open new lines of research to evaluate its potential affinity for sulfide and sulfur in comparison to other sulfide-oxidizing prokaryotes occurring in the same niche (*e.g.* Green Sulfur Bacteria).

8

CONCLUDING REMARKS

I) Epsilonproteobacteria constitute stable populations at the O_2/H_2S interface of the studied basin of Lake Banyoles, showing maximal abundances in winter.

II) Planktonic populations of Epsilonproteobacteria are mainly composed of members of the genus *Arcobacter*. Particularly, the community is dominated by a phylotype closely related to *Candidatus* Arcobacter sulfidicus, an autotrophic, marine sulfide-oxidizing Epsilonproteobacteria.

III) The probe EPSY914, which has traditionally been considered specific to Epsilonproteobacteria, shows unspecific hybridization with members of the phylum *Chlorobi*. This is of special concern since Green Sulfur Bacteria usually bloom when sulfidic redoxclines are located at photic depths. Under these conditions, the use of probe EPSY914 is not recommended since the presence of Green Sulfur Bacteria may cause an overestimation on the abundance of Epsilonproteobacteria.

IV) To overcome the problems caused by unspecificities of probe EPSY914 we designed primer pairs and a TaqMan probe to specifically quantify members of the dominant *Arcobacter* strain thriving in basin C-III. Despite some technical constraints and limitations, the qPCR-TaqMan protocol allowed the proper quantification of *Arcobacter* spp. with no cross-reaction with other planktonic species inhabiting the studied system.

V) In winter, a third of total carbon fixation corresponds to dark fixation processes at the redoxcline of basin C-III. In turn, photoassimilation is the dominant processes in the water column of basin C-III in summer.

VI) *In situ* incubations using radiolabeled bicarbonate and measurements using *MicroAutoRadiography Catalyzed Reported Deposition-Fluorescence In Situ Hybridization* indicated that Epsilonproteobacteria actively assimilates CO_2 in the dark, likely being mainly responsible for the autotrophic activity at the redoxcline in winter. Additional data suggest that the *Arcobacter* spp. identified in basin C-III is able to assimilate inorganic carbon using the reductive Tricarboxylic Acid Cycle.

VII) Phylogenetic analyses of *soxB* gene confirmed the presence of *soxB* sequences affiliated to genus *Arcobacter* throughout the water column of basin C-III. These data suggest that *Arcobacter* spp. may be able to oxidize reduced sulfur compounds via multienzyme Sox System.

VIII) This work support the key role of Epsilonproteobacteria in linking C and S cycles and extend their influence to freshwater systems characterized by sharp oxic-anoxic interfaces and euxinic waters.

9

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