Universitat de Girona

DIVERSITY, DYNAMICS AND ACTIVITY OF MESOPHILIC ARCHAEA IN STRATIFIED FRESHWATER LAKES. IMPLICATIONS IN BIOGEOCHEMICAL CYCLES

Marc LLIRÓS DUPRÉ

ISBN: 978-84-693-3376-1
Dipòsit legal: GI-579-2010
http://www.tdx.cat/TDX-0422110-114614

Universitat de Girona

Diversitat, dinàmica i activitat d'*Archaea* mesòfiles en llacunes estratificades. Implicacions en els cicles biogeoquímics

Programa de Doctorat en Ciències: Química i Física de les molècules i els materials, Biotecnologia i Ciències de la Salut

Marc LLIRÓS DUPRÉ

Universitat de Girona

PhD thesis

Diversity, dynamics and activity of mesophilic *Archaea* in stratified freshwater lagoons. Implications in biogeochemical cycles

Marc Llirós Dupré

2009

Universitat de Girona

gEMM Group of Molecular Microbial Ecology
Institute of Aquatic Ecology, University of Girona

Diversitat, dinàmica i activitat d'*Archaea* mesòfils en llacunes estratificades. Implicacions en els cicles biogeoquímics

Memòria redactada per Marc Llirós Dupré i inscrita al programa de doctorat en Ciències: Química i Física de les molècules i els materials, Biotecnologia i Ciències de la Salut de la Universitat de Girona per optar al grau de Doctor en Biologia per la Universitat de Girona.

El treball recollit en la present memòria s'ha realitzat tant als laboratoris del grup de recerca en Ecologia Microbiana Molecular (gEMM) de la Universitat de Girona sota la direcció del Dr. Carles M Borrego Moré així com del grup de Limnologia del Departament d'Ecologia Continental del Centre d'Estudis Avançats de Blanes-CSIC (CEAB-CSIC) sota la direcció del Dr. Emilio O Casamayor

Vist-i-plau directors

Carles M. Borrego

Emilio O. Casamayor

Autor

Marc Llirós

Girona 2009

Contents/Continguts

Summary/Resum/Resumen

Summary

According to the goals set out for this PhD thesis, we have been studying the diversity (richness and evenness), distribution and dynamics of planktonic Archaea in several temperate stratified freshwater lakes to shed some light on their distribution and potential activity in these ecosystems in relation to prevalent biogeochemical cycles.

A five consecutive years (2001-2005) survey was conducted in Lake Vilar to study the distribution and dynamics of the archaeoplankton community using a molecular approach. Results clearly showed the presence of a low abundant but very rich archaeal assemblage mainly composed by MCG (*Miscellaneous Crenarchaeotic Group*) and DHVE (*Deep Hydrothermal Vent Euryarchaoeta*). Both lineages were mainly retrieved from the anoxic hypolimnion and shared persistence through seasons. Additionally, other rare archaeal taxa appeared occasionally. However, no significant correlations could be found between the archaeal community composition and the prevalent physico-chemical conditions although differences in richness and seasonality were observed. In turn, the archaeoplankton community of a neighbouring hypereutrophic holomictic freshwater lagoon was mainly dominated by MCG- and *Methanosaeta*-related phylotypes. Both groups were also mostly found in the anoxic sulfide-rich hypolimnion. Although the archaeal community studied were unexpectedly rich in both environments, their low abundance suggest that the prevalent conditions were not optimal for their growth and that the planktonic Archaea constitute a sort of *seed-bank* waiting for better conditions to bloom. A completely different picture was found in Lake Kivu, an oligotrophic and meromictic freshwater lake located in a volcanic region between Rwanda and the Democratic Republic of Congo. In this African lake, the archaeal planktonic assemblage was mainly composed by members of ammonia oxidising archaeal lineages (AOA; belonging to *Marine I.1a* and *Soil I.1b Crenarchaeota* groups). Below the redoxcline, the structure of the assemblage clearly changed and members of the MCG and methanogenic lineages were clearly prevalent.

To determine the potential activity of the archaeal communities studied, we applied two different approaches. Firstly, we focused on the detection of a signature gene for archaeal nitrification (the archaeal *amoA* gene, codifying for the α-subunit of the ammonia monooxygenase enzyme). This approach was succesfully applied on water samples from Lake Kivu. The detection of intense signals of archaeal *amoA* gene coinciding with nitrate and nitrite maxima at the redoxcline give additional evidence that AOA might be actively

involved in nitification processes in this lake. Moreover, the results evidenced archaeal *amoA* gene segregation according to habitat and related to low or very low organic matter content. Secondly, a single-cell approach combining the use of a radiolabeled tracer ($NaH^{14}CO_3$) and fluorescence *in situ* hybridisation was used to detect those archaeal cells actively uptaking bicarbonate as carbon source. This approach was performed in the hypereutrophic Coromina lagoon. *In situ* incubation experiments using $NaH^{14}CO_3$ revealed a predominance of photo- rather than chemotrophy all over the epi- and metalimnetic waters of the lagoon where oxygenic and anoxygenic phototrophs dominated the planktonic assemblage. Moreover, MICRO-CARD-FISH analyses revealed a low contribution (2 to 10%) of the archaeal planktonic fraction to dark carbon fixation processes in the lagoon. These results together with the accumulation of reduced compounds in the water columm of the lagoon suggested that these mesophilic Archaea might perform hetero- or mixotrophic processes. Additionally, MICRO-CARD-FISH analyses also revealed active cells positively hybridised with the *Cytophaga-Flavobacteria-Bacteroidetes* probe which represent a broader metabolic capability for this large bacterial group.

Finally, a phylogenetic lineage-based analysis on the global distribution of lacustrine Archaea was conducted using 16S rRNA gene sequences in combination with statistical and general ecology tools. In this regard, oxygen and salinity appeared as the main environmental drivers affecting the global distributional patterns of mesophilic lacustrine Archaea. MCG cluster appeared as indicator taxa for small stratified karstic lakes with active sulfureta, e.g., Lake Vilar and Coromina lagoon. In turn, the Marine Crenarchaeota Group I.1a appeared as the indicator taxa for large oligotrophic stratified lakes, e.g., Lake Kivu.

Resum

D'acord amb els objectius plantejats en aquesta tesi doctoral, es va estudiar la diversitat (riquesa i abundància), la distribució i la dinàmica de les comunitats d'Archaea planctòniques presents a diferents llacs estratificats temperats d'aigua dolça per tal d'aportar evidencies de la seva distribució i la seva possible activitat en aquests ecosistemes en relació als cicles biogeoquímics presents en aquests ecosistemes.

Per tal d'estudiar la distribució i la dinàmica de la comunitat d'Archaea planctòniques presents a l'estanyol del Vilar es va utilitzar una aproximació molecular basada en el gen 16S rRNA durant cinc anys consecutius (2001-2005). Els resultats clarament varen mostrar que l'estanyol presentava una comunitat d'Archaea poc abundant, però molt rica formada principalment pels grups MCG (*Miscellaneous Crenarchaotic Group*) i DHVE (*Deep Hydrothermal Vent Euryarchaeota*). Ambdós llinatges eren recuperats habitualment a l'hipolinion anòxic de l'estanyol i es mantenien al llarg del temps. A més a més, altres grups menys habituals apareixien ocasionalment. Malgrat això, no es varen trobar correlacions significatives entre la composició de la comunitat d'Archaea i les condicions fisicoquímiques predominants tot i les diferencies observades en la distribució de la riquesa de filotips i en la estacionalitat dels mateixos. Per altra banda, la comunitat d'Archaea planctòniques d'una llacuna estratificada hipereutròfica propera estava dominada per filotips relacionats amb el grup MCG i *Methanosaeta*. Ambdós grups van ser majoritàriament recuperats a l'hipolimnion anòxic i ric en sulfhídric de la llacuna. Malgrat que la comunitat d'Archaea estudiada va ser inesperadament rica en ambdós ambients, la seva baixa abundància indicaria que les condicions presents en aquests ambients no serien les optimes pel seu creixement i que la comunitat d'Archaea constituiria un rebost de filotips (*seed-bank*) esperant unes condicions més propícies pel seu creixement. En canvi la imatge obtinguda del Llac Kivu, un llac oligotròfic i meromictic africà d'origen volcànic situat entre Rwanda i la República Democràtica del Congo, va ser totalment diferent. En aquest llac, la comunitat planctònica d'Archaea estava formada principalment per llinatges d'Archaea oxidadors d'amoni (AOA; relacionats amb els Crenarchaeota dels *Marine Group I.1a* i *Soil Group I.1b*). La estructura de la comunitat era clarament diferent per sota de la redoxclina, amb predomini dels llinatges MCG i metanogens.

Per tal de determinar l'activitat potencial de les comunitats d'Archaea estudiades, es varen aplicar dues aproximacions diferents. En primer lloc, ens varem centrar en la detecció d'un gen clau en la nitrificació realitzada per Archaea (el gen *amoA* d'Archaea que

codifica per la subunitat α de l'enzim amoni monoxigenasa). Aquesta aproximació es va dur a terme amb èxit en mostres d'aigua del Llac Kivu. La coincidència de senyals moleculars més intenses per a aquest gen amb màxims de nitrit i nitrat justament a la redoxclina varen evidenciar el fet que les AOA podrien estar activament implicades en els processos de nitrificació del llac. A més a més, els resultats varen mostrar una segregació d'acord amb l'hàbitat de detecció del gen *amoA* d'Archaea i amb el fet de trobar quantitats baixes o molt baixes de matèria orgànica. En segon lloc i per tal de detectar aquelles Archaea que incorporaven activament bicarbonat com a font de carboni, es va utilitzar una aproximació a nivell de cèl·lula individual combinant l'ús d'un traçador radioactiu ($NaH^{14}CO_3$) amb hibridació *in situ* fluorescent. Aquesta aproximació es va realitzar a la llacuna hipereutròfica de Can Coromina. Els experiments d'incubació *in situ* utilitzant $NaH^{14}CO_3$ varen mostrar un predomini dels processos fòtics per damunt dels afòtics o quimiotròfics tant a l'epi- com al metalimnion de la llacuna on els microorganismes fototròfics, tant oxigènics com anoxigènics, dominaven la comunitat planctònica. A més a més, els resultats dels anàlisis amb MICRO-CARD-FISH varen mostrar una pobre (entre un 2 i un 10%) contribució de les Archaea planctòniques a la fixació fosca de carboni. En aquest context, els resultats anteriors juntament amb l'acumulació de compostos reduïts a la columna d'aigua de la llacuna suggereixen que la comunitat planctònica d'Archaea mesòfiles durien a terme metabolismes hetero- o mixotròfics. Els resultats de MICRO-CARD-FISH també varen mostrar la presencia de cèl·lules hibridades amb la sonda pel grup *Cytophaga-Flavobacteria-Bacteroidetes*, el que representa ampliar les capacitats metabòliques descrites fins ara per aquest ampli grup bacterià.

Finalment, un altre aspecte tractat en aquesta tesi doctoral va ser l'anàlisi de patrons de distribució global de les Archaea mesòfiles. En aquest sentit es va realitzar un anàlisi a nivell de llinatge de la distribució global de les Archaea lacustres combinant seqüències del gen 16S rRNA i diferents eines estadístiques i d'ecologia general. Els resultats varen determinar que l'oxigen i la salinitat eren les principals forces ambientals que controlarien la distribució global de les Archaea mesòfiles d'ambients d'aigua dolça. D'altra banda, el grup MCG va mostrar-se com a grup indicador pels ambients lacustres estratificats d'origen càrstic amb una sulfureta activa, com p.e. l'estanyol del Vilar o de Can Coromina. Per altra banda, el grup Marine Crenarchaeota Group I.1a va definir-se com a grup indicador per aquells ambients lacustres oligotròfics, com ara el Llac Kivu.

Resumen

De acuerdo con los objetivos planteados en la presente tesis doctoral, se estudió la diversidad (riqueza y abundancia), la distribución y la dinámica de las Archaea planctónicas presentes en distintos ambientes lacustres estratificados templados para poder aportar evidencias de su distribución y su posible actividad en dichos ecosistemas en relación a los ciclos biogeoquímicos imperantes en estos ambientes.

Con el fin de estudiar la distribución y la dinámica de la comunidad planctónica de Archaea presentes en la laguna del Vilar se utilizó una aproximación molecular basada en el gen 16S rRNA durante cinco años consecutivos (2001-2005). Los resultados pusieron de manifiesto que la laguna presentaba una comunidad de Archaea poco abundante aunque muy rica, principalmente formada por los grupos MCG (*Miscellaneous Crenarchaotic Group*) y DHVE (*Deep Hydrothermal Vent Euryarchaeota*). Ambos linajes eran principalmente recuperados en el hipolimnion anóxico de la laguna y se mantuvieron a lo largo del periodo de estudio. A su vez, otros grupos menos habituales aparecían ocasionalmente. A pesar de esto, no se pudieron establecer correlaciones significativas entre la composición de la comunidad de Archaea y las condiciones fisicoquímicas imperantes en el periodo estudiado a pesar de las diferencias detectadas en relación a la distribución de la riqueza y la estacionalidad de los filotipos recuperados. Por otro lado, la comunidad de Archaea planctónica de una laguna hipereutrófica de la misma región mostró un claro predominio de los filotipos relacionados con el grupo MCG y *Methanosaeta*. Ambos grupos fueron mayormente recuperados en el hipolimnion anóxico y rico en sulfhídrico de la laguna. A pesar de la inesperada gran riqueza de la comunidad planctónica de Archaea en las dos lagunas estratificadas, su baja abundancia indicaría que las condiciones presentes no serian las óptimas para su crecimiento. Así pues, la comunidad de Archaea constituiría un sumidero de filotipos (*seed-bank*) esperando unas condiciones más propicias para su desarrollo. En cambio, la imagen que se obtuvo del Lago Kivu, un lago africano oligotrófico y meromíctico de origen volcánico situado entre Rwanda y la República Democrática del Congo, fue totalmente diferente. En este lago, la comunidad de Archaea estaba formada principalmente por linajes de Archaea oxidadoras de amonio (AOA; relacionados con los Crenarchaeota *Marine Group I.1a* y *Soil Group I.1b*).A su vez, la estructura de la comunidad era claramente distinta por debajo de la redoxclina, con un predominio de los linajes MCG y metanogenos.

Con el fin de determinar la actividad potencial de las comunidades de Archaea estudiadas, se aplicaron dos aproximaciones distintas. En primer lugar, nos centramos en la detección

de un gen clave en la nitrificación realizada por Archaea (el gen *amoA* de Archaea, que codifica para la subunidad α de la enzima amonio monooxigenasa). Esta aproximación se realizó con éxito en muestras del Lago Kivu. La coincidencia de señales moleculares más intensas para este gen con máximos de nitrito y nitrato en la redoxclina evidenciaron que las AOA podrían estar activamente implicadas en los procesos de nitrificación del lago. Además, los resultados mostraron una segregación de acuerdo con el ambiente de detección del gen *amoA* de Archaea y con el hecho de encontrar cantidades bajas o muy bajas de materia orgánica. En segundo lugar y con el fin de detectar aquellas Archaea que incorporaban activamente bicarbonato como fuente de carbono, se utilizó una aproximación a nivel de célula individual combinando el uso de un trazador radioactivo ($NaH^{14}CO_3$) con hibridación *in situ* fluorescente. Esta aproximación se realizó en la laguna hipereutrófica de Coromina. Los experimentos de incubación *in situ* utilizando $NaH^{14}CO_3$ mostraron un predominio de los procesos fóticos en relación a los afóticos o quimiotróficos tanto en el epi- como en el metalimnion de la laguna donde los microorganismos fototróficos, tanto oxigénicos como anoxigénicos, dominaban la comunidad planctónica. Además, los resultados de los análisis de MICRO-CARD-FISH revelaron una pobre contribución (entre un 2 y un 10%) de las Archaea planctónicas a los procesos de fijación oscura de carbono. En este sentido, los resultados anteriores junto con la acumulación de compuestos reducidos en la columna de agua de la laguna sugieren que estas Archaea realizarían un metabolismo hetero- o mixotrófico. Por otro lado, los resultados de MICRO-CARD-FISH revelaron también la presencia de células hibridadas con la sonda para el grupo *Cytophaga-Flavobacteria-Bacteroidetes*, lo que supone ampliar las capacidades metabólicas descritas hasta la fecha para este amplio grupo bacteriano.

Finalmente, otro aspecto tratado en la presente tesis doctoral fue analizar los patrones globales de distribución de las Archaea mesófilas. En este sentido se realizó un análisis a nivel de linaje de la distribución global de las Archaea lacustres combinando secuencias del gen 16S rRNA y distintas herramientas estadísticas y de ecología general. En este sentido, se determinó que el oxígeno i la salinidad eran las principales fuerzas ambientales que determinarían la distribución global de las Archaea mesófilas en ambientes lacustres. Por otro lado, el grupo MCG se mostró como el grupo indicador para los ambientes lacustres estratificados de agua dulce con una sulfureta activa, como por ejemplo la laguna del Vilar y Coromina. Por otro lado, el grupo Marine Crenarchaeota Group I.1a fue definido como indicador para los ambientes lacustres oligotróficos, como el lago Kivu.

1. Introduction

After the pioneering work of Woese and coworkers dealing with the molecular analyses of ribosomes (at protein and RNA level) the phylogenetic classification of living organisms suffered a drastic change with the proposal and further acceptance of a third Domain, the *Archaea* (Balch et al 1977; Fox et al 1977; Woese & Fox 1977; Woese 1987; Woese et al 1990). In fact, the discovery and furhter characterisation of the *Archaea* (Woese 2000) is one of the best examples to illustrate how our concept of the diversity of (micro)-organisms has changed. The Domain *Archaea* was initially composed by three distinct kingdoms, the so-called *Euryarchaeota*, *Crenarchaeota* and *Korarchaeota* (Woese et al 1990, Woese 2000). In those days, all *Archaea* consisted of extremophiles that somehow reflected our early perception regarding their ancient and primitive origin (hotsprings, deep-sea hydrothermal vents, anoxic environments, among others; Brown 1999). In fact, archaeons have been found in all extreme environments with abundances clearly above those of Bacteria (Huber et al 2000) although under other extreme conditions (high salt, high or low pH, low temperature and high preassure) members of the Domain *Bacteria* can be found in close viccinity with *Archaea* and/or *Eukarya* (Rothschild & Mancinelli 2001). In the last two decades the perception of *Archaea* as extremist has dramatically changed (DeLong 1998). The application of molecular techniques to microbial ecology has revealed that *Archaea* are diverse and widespread microorganisms. In this regard, some useful reviews on Archaea have been recently published (Forterre et al 2002; Schleper et al 2005; Cavicchioli 2007; Garret & Klenk 2007).

1.1. General concepts on *Archaea*

Archaea are microbes that, in some molecular features, resemble eukaryotes more than bacteria. Archaea are anuclear cells (the so-called prokaryotes; but see Pace 2006, 2009 for current controversies about this concept) with an overall cellular architecture similar to that of Bacteria. Some characteristic features of Archaea are depicted in Table 1.1. Archaea are not easily distinguished from Bacteria by their size, shape, or metabolic requirements. Moreover, the number of archaeal morphotypes is less than that for Bacteria. However, some Archaea posses morphologies not found in Bacteria to date such as polygonal cells (halophilic archaea), irregular cocci (some hyperthermophiles) (Woese & Fox 1977) or amoeba-like forms (*Thermoplasma* and *Ferroplasma*; Searcy & Hixon 1991; Hixon & Searcy 1993; Golyshina et al 2000). In turn, complex cell stages with cellular differentiation such as those in *Streptomyces* and *Spirochaetes,* are actually unknown in Archaea (Kletzin 2007). This fact, however, might be a consecuence of either an incomplete sampling or the incapacity to culture them (Stanley & Konopka 1985; Pedrós-Alió 2006, 2007). In relation to their metabolism, the Archaea appears to be fairly similar to that of Bacteria although some bias may be introduced due to the few archaeal culture representatives available. With the remarkable exception of methanogenesis (which it has been not described yet in Bacteria), almost all metabolic pathways observed to date in Archaea also exist in Bacteria. On the other hand, the core components of archaeal information processing machinery (replication, transcription, translation and DNA repair) show striking similarities to those in eukaryotes (Huet et al 1983; Reiter et al 1988; Forterre et al 2002; Makarova & Koonin 2003; Allers & Mevarech 2005) but, in turn, archaeal genomes are typically prokaryotic in terms of genome size (small) and organization. Based on the unique archaeal information-processing machineries, conserved archaeal “core” genes and archaeal transcriptional signals, it is possible to differentiate archaeal and bacterial genomes, and even between euryarchaeotes and crenarchaeotes (Makarova & Koonin 2003). Altogether, Archaea are organisms that use eukaryote-like proteins in a bacterium-like context.

Table 1.1. Distinctive features among the Three Domains of Life.

Property	***Archaea***	***Bacteria***	***Eukarya***
Cellular features			
Cell organization	Unicellular	Unicellular	Uni/Multicellular
Cell wall	No Murein	Murein	No Murein
Membrane lipids	Ether linked	Ester linked	Ester linked
Organelles	-	-	+
Cytoskeleton	-	-	+
Growth above 80ºC	+	+	-
Molecular features			
Nucleus	-	-	+
Genetic material	Small circular	Small circular	Linear and complex
	Relaxed or positively supercoiled	Negatively supercoiled	Negatively supercoiled
	Genome associated with histones	Genome no associated with histones	Genome associated with histones
RNA polymerase	1 type, complex structure	1 type, simple structure	3 types, complex structure
Ribosome	70s	70s	80s
Operons	+	+	Rare
Introns	Rare	-	+

1.1.1. Cell features

One of the traits that radically distinguish Archaea from Bacteria and Eukarya is the structure and lipidic composition of the cellular cytoplasmatic membrane. Bacteria generally possess bi-layered cell membranes whereas in Archaea the membrane is composed by lipid mono-layers with ether-linkages instead of ester ones (Figure 1.1). Furthermore, the absence of murein in the cell walls and the absence of a periplasmatic space are other features that distinguish Archaea from Bacteria. A single S-layer of glycoproteins can represent the unique cell wall component in many Archaea which provides a high level of envelope rigidity. Nevertheless, some exceptions are described among Archaea, as *Thermoplasma* and *Ferroplasma* lacking cell walls or *Halococcus* and *Methanobacteriales* which have rigid cell walls based on heteropolysaccharides or pseudomurein, respectively (Kleitzin 2007).

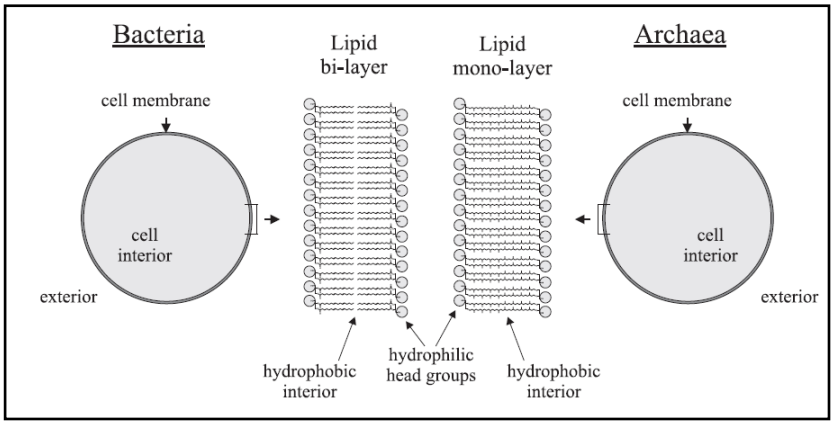


Figure 1.1. Bacteria (left) and Archaea (right) lipidic membranes.

Archaea synthesise unique membrane lipids compared to Bacteria and Eukarya. This feature has been used as one of the arguments to propose Archaea as a third Domain of Life (Woese et al 1990). While membrane lipids of Bacteria and Eukarya consist predominantly on fatty acids esterified to glycerol, archaeal membrane lipids are ether-linked which consist on long-chain of isoprenoid diphytanyl glycerol diethers (DGDs) and of glycerol dibiphytanyl glycerol tetraethers (GDGTs) (Fig. 1.2). Furthermore, the archaeal stereochemistry of glycerol backbone phospholipids is the enantiometric image and clearly contrasting to that of the other two domains (Kates 1993; Wuchter et al 2003; Fig. 1.2). In fact, the capacity to resist extreme physico-chemical conditions such as high salinity, high or low pH, high or low temperature, high pressure may be attributed to the unique propierties of archaeal cell membranes since ether lipids provide higher membrane stability than ester lipids present in Bacteria (Elferinck et al 1994; Patel & Sprott 1999).

The most widespread GDGT among hyperthermophilic Archaea is Caldarchaeol (GDGT-0; de Rosa et al 1987; Lanzotti et al 1989; Sugai et al 2000, 2004; Boucher 2007). In turn, non-thermophilic Crenarchaeota contain Crenarchaeol (GDGT-4) as unique membrane lipid. This molecule contains one hexacyclic ring and four pentacyclic moieties (Sinninghe-Damsté et al 2002a; Fig. 1.2).

Furthermore, the membrane lipidic composition of Archaea could be used not only as a biomarker for their presence in ancient environments but also as a proxy for paleoclimate analyses. GDGT were initially detected in hyperthermophilic habitats (de Rosa & Gambacorta 1988; Uda et al 2001) but their further detection in non-extreme environments (such oceans and soils) suggested a direct correlation with the presence of non-thermophilic Crenarchaeota (Hoëfs et al 1997; Schouten et al 2000; Sinninghe-Damsté et al 2002a, 2002b). Moreover, the number of cyclopentane ring-containing GDGTs has been correlated with increases in the temperature at which the microorganism has grown (de Rosa & Gambacorta 1988; Uda et al 2001). Accordingly, the number of cyclic moieties in GDGTs is currently used as a proxy (TEX_{86}; TetraEther indeX of lipids with 86 carbon atoms) for paleoclimate reconstructions (Schouten et al 2002; Hopmans et al 2004; Powers et al 2004; Wuchter et al 2004, 2005).

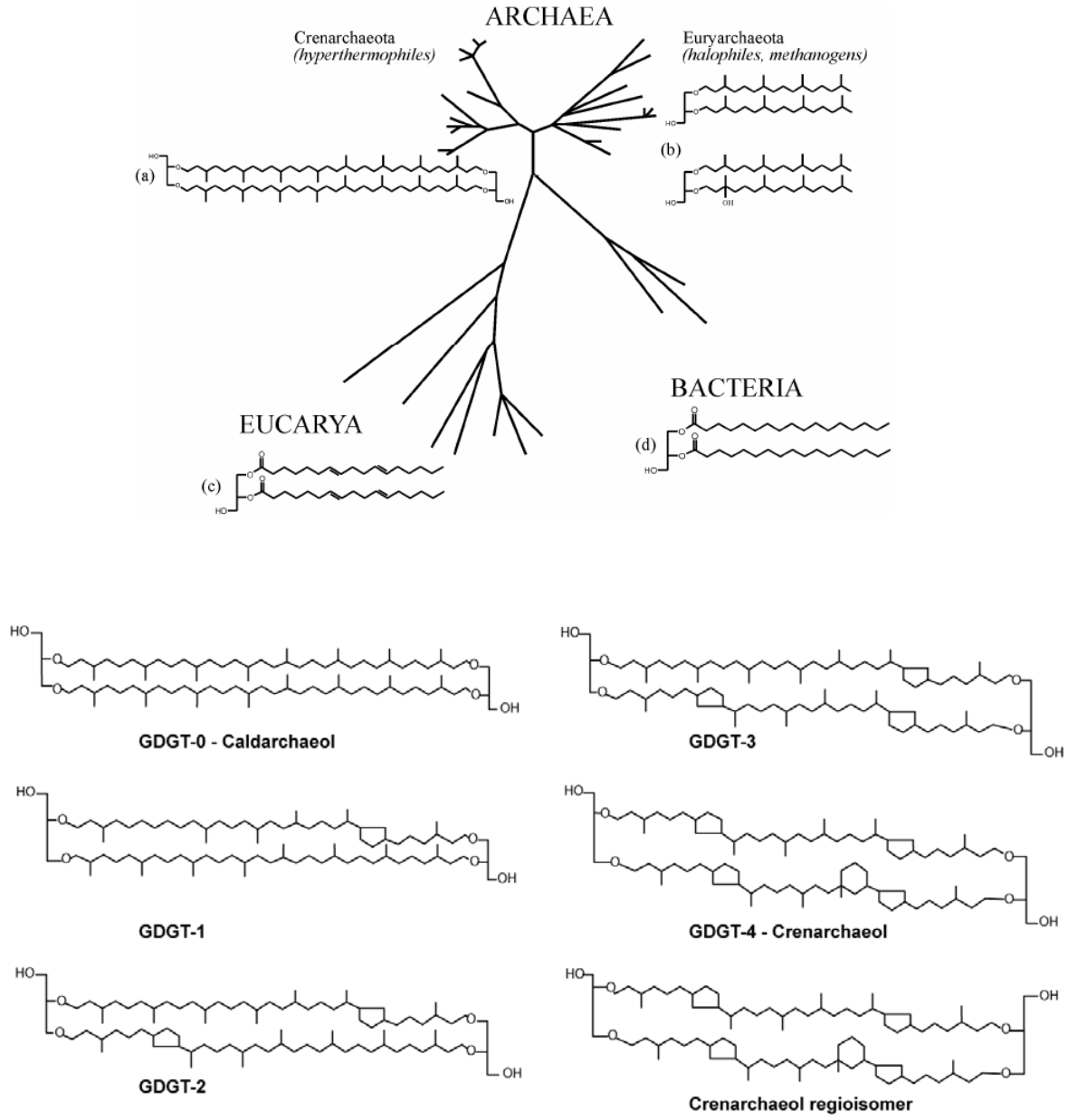


Figure 1.2. The different membrane lipid composition in the three Domains (up): Archaea contain (a) glycerol dibiphytanyl glycerol tetraethers (GDGTs) for hyperthermophilic Crenarchaeota and (b) diphytanyl glycerol diethers for Euryarchaeota (halophiles and methanogens); while Eukarya (c) and Bacteria (d) posses fatty acids with glycerol diesters, respectively. The lower image highlight core tetraether membrane lipid structures of marine Crenarchaeota with: none (GDGT-0 or Caldarchaeol), one (GDGT-1), two (GDGT-2), three (GDGT-3) and four (GDGT-4 or Crenarchaeol and its regioisomer) cyclo-pentane moieties (extracted and modified from Wuchter 2006a).

Archaea posses distinct types of surface structures (Ng et al 2008). Some of them (flagella and pilli) resemble their bacterial counterparts with an archaeal "twist"; while some others (cannulae and hami) are unique to the Domain (Fig. 1.3). Whereas the archaeal flagella, pilli and cannulae are cell strutures widely distributed throughout all cultured representatives (Nickell et al 2003; Ng et al 2006; Jarrell et al 2007; Zolghadr et al 2007); the hamus is a novel cell surface appendage recently described in the unique biofilm exclusively composed by Euryarchaeota cells (Rudolph et al 2001; Moissl et al 2005; Henneberger et al 2006).

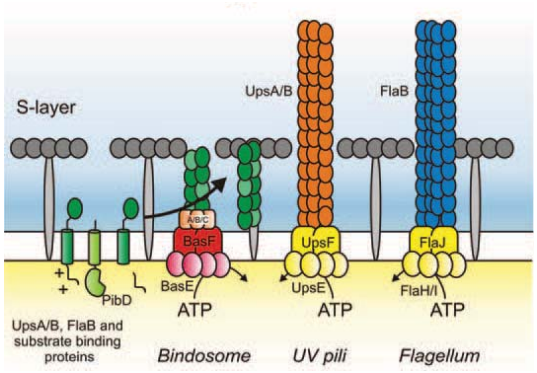


Figure 1.3. Hipotetical scheme of some cell surface structures currently described in *Archaea* (Ng et al 2008).

1.1.2. Archaeal proteins

At the molecular level, the definition of Archaea as a group has been certified by different comparative genomic analyses (Forterre 1997; Olsen & Woese 1997; Fitz-Gibbon & House 1999; Forterre & Philippe 1999; Makarova et al 1999; Snel et al 1999; Tekaia et al 1999; Wolf et al 2001). In Archaea, informational proteins (those involved in DNA replication, transcription and translation) are usually more similar to those of Eukarya than to those of Bacteria. Archaeal genomes encode homologues of nearly all eukaryal DNA replication proteins but only one homologue of a bacterial DNA replication protein (Edgell & Doolittle 1997; Forterre 1999; Leipe et al 1999). Interestingly and in contrast to informational proteins, most operational proteins (metabolic enzymes, membrane proteins and some cell division proteins) of Archaea are bacterial-like (Koonin et al 1997; Jain et al 1999; Fig. 1.4). This could be interpreted as a result of Lateral Gene Transfer (LGT) processes of operational proteins between the two prokaryotic domains (Faguy & Doolittle 1999; Gribaldo et al 1999; Makarova et al 1999; Forterre et al 2000; Ruepp et al 2000; Daubin et al 2001; Nesbo et al 2001). Furthermore, some data of comparative genomics support the division of Archaea in two distinct Kingdoms, since many proteins present in all Euryarchaeota genomes are missing in Crenarchaeota and vice versa (Bernander 2000; Myllykallio & Forterre 2000). However, it still unknown if

these differences are specific to cultured archaeal representatives or if they are a general characteristic for this Domain.

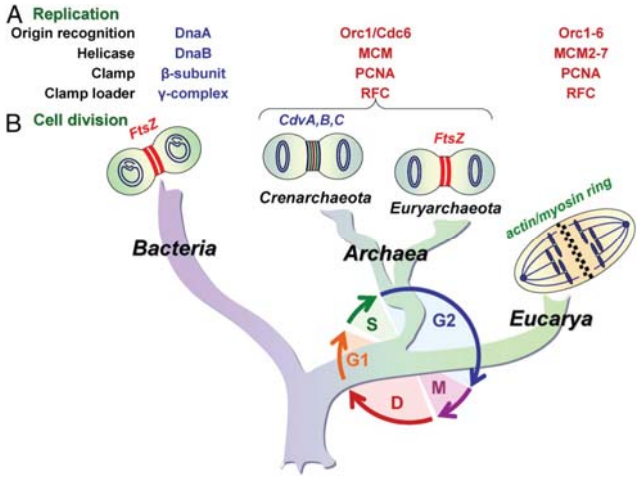


Figure 1.4. Schematic representation of a life phylogenetic tree showing that *Archaea* and *Eukarya* share similar replication machineries (A) and that *Archaea* has different mechanisms for cell division (B). A cell cycle timming for archaeal and eukaryal cells is shown at the corresponding radiation point (from Cann 2008).

1.1.3. Antibiotic Resistance

The difference between Archaea and Bacteria at the molecular level is also exemplified by the resistance of Archaea to most antibiotics that are active against Bacteria. Early studies on the molecular biology of Archaea (e.g., Zillig 1991) have shown that this resistance was mainly due to critical differences in the antibiotic targets (e.g., RNA polymerase or ribosomal proteins) but also due to their extreme growth conditions (Böck & Kandler 1985). As an example, almost all antibiotic substances inhibiting bacterial translation are ineffective against Archaea (and Eukarya) and viceversa, regardless of the targeted ribosomal subunit (30S or 50S; Kletzin 2007).

1.2. Metabolism

In genereal, Archaea posses nearly the same metabolic pathways than Bacteria (Table 1.2 and 1.3), being the methanogenesis an exception to this trait. Archaea could be either hetero- or autotrophs using a large variety of electron donors or acceptors (Huber et al 2000). Photosynthesis based on chlorophyll has not been still found in Archaea, whereas bacteriorhodopsin-based photosynthesis, once believed unique to halophilic Archaea, has been recently described in planktonic Bacteria (Béjà et al 2000, 2001).

Until recently, Archaea were characterised as obligate extremophiles thriving in environments with harsh physico-chemical conditions for other organisms. In this sense, the limited physiological diversity among archaeal cultivated representatives (e.g., most Crenarchaeota cultures are sulfur-metabolizing thermophiles; Burggraf et al 1997) suggested a limited niche distribution of Archaea in nature. Accordingly, the archaeal contribution to global biogeochemical cycles was neglected due to this limited distribution. The recent application of molecular tools to microbial ecology revealed a widespread diversity and ubiquity of Archaea in nature (e.g., Schleper et al 2005; Chaban et al 2006 and references therein). Furthermore, metagenomic analyses had largely increased available information on archaeal functional genes. In this sense, functional genes involved in different steps of the N cycle such as the nitrite reductase (*nir*), the assimilatory nitrate and nitrite reductases (*narB* and *nirA*) and the ammonium monooxygenases (*amoA, B,* and *C*) have been recently identified in archaeal metagenomes from different environments (Treusch et al 2005; Francis et al 2005, 2007; Lin et al 2006; Coolen et al 2007; Alcántara-Hernández et al 2009; Pouliot et al 2009; for a recent review on *amoA* gene see Erguder et al 2009). The two main archaeal groups related with the N cycle, *Marine Crenarchaoeta Group I.1a* and *Soil Crenarchaoeta Group I.1b*, are also the most widespread and abundant archaeal groups in nature (DeLong 1998; Buckley et al 1998). According to recent data, archaeal metabolism may range from strict chemoautotrophy to heterotrophy in both marine and terrestrial habitats (Ouverney & Furhman 1999, 2000; Quaiser et al 2002; Hügler et al 2003; Ochsenreiter et al 2003; Wuchter et al 2003, 2006b; Nicol et al 2005; Francis et al 2005; Treusch et al 2005; Biddle et al 2006; Hallam et al 2006; Nicol & Schleper 2006; Beman et al 2007; He et al 2007). This metabolic plasticity suggests a direct and important role of Archaea in global biogeochemical cycles and energy fluxes, especially for C and N. In this regard, a link between these cycles has been recently established after the isolation of a mesophilic *Crenarchaeota* able to fix carbon in the dark coupled to the oxidation of ammonia (Könneke et al 2005).

1.3. Cultivation

The improvement of cultivation techniques is necessary to increase the low cultivability values for most microorganisms, being the non-extremophilic Archaea one of the best examples. This necessarily is still more urgent for those microbes playing relevant functions in the ecosystem. The availability of well-characterised isolates allows experimentation under controlled conditions and proper characterisation of microorgasnisms' function and regulation. Both aspects are necessary to improve our knowledge on their metabolic capabilities, community interactions and impact on element cycling and nutrient fluxes of Archaea. In this sense, new and imaginative culture techniques and strategies are challenging topics to be addressed by microbial ecologists. Among these new strategies some include the use of relatively low amounts of nutrients (e.g., Connon & Giovannoni 2002; Rappé et al 2002); inocula dilution to extinction (e.g., Baroos 1995; Burns et al 2004; Davis et al 2005; Braüer et al 2006); and the addition of selective inhibitors against Bacteria or Archaea (e.g., Braüer et al 2004, 2006; Könneke et al 2005). In this sense, recent pure and enrichment cultures of mesophilic Archaea have been obtained after the combination of different antibiotics and energy sources as ammonia or reduced iron (Könneke et al 2005; Simon et al 2005; de la Torre et al 2008; Hatzenpichler et al 2008; Kozubal et al 2008). Despite these efforts, only one third of the 49 currently accepted archaeal lineages contain one or more cultured representative (Fig. 1.5; Schleper 2007). In culture collections, no cultures of moderate Archaea are available and, for instance, no isolates from freshwater Crenarchaeota have been obtained so far.

Table 1.2. Summary of the basic metabolic capabilities, required growth conditions and occurrence of the main groups of *Euryarchaeota* (modified from Casamayor & Borrego 2009).

Group	Metabolic process	Energy source	Carbon source	Environmental conditions	Environment location	Culture
Methanogens	Methanogenesis	OM[a] (simple)	OM	Extreme anaerobiosis and Low RedOx potential	Anoxic water compartment of stratified lakes; sediments; rice fields	*Methanococcus; Methanobacterium; Methanosphaera*
		H_2	CO_2		Anoxic microhabitats in saline environments; sewage digestors	Most methanogens
Methanotrophs	Anaerobic Oxidation of Methane	CH_4	OM	Extreme anaerobiosis and active sulphur cycle; Low RedOx potential; Sulfate Reducing Bacteria[b]	Anoxic sediments	None
Halophiles	Aerobic Respiration	OM (complex)	OM	Saline (7%-37% NaCl)	Salt lakes	*Haloarcula, Haloferax, Halorubrum, Natrinena*
	Anaerobic Respiration	OM (complex)	OM	high pH	Soda lakes	*Halobacterium, Haloarcula, Haloferax*
	Fermentation	Sugars, aminoacids	OM	Oxic and/or Anoxic	Hypersaline Antarctic lakes	*Halobacterium*
	Non-photosynthetic photoheterotrophy[c]	Light	OM		Solar salterns	*Halobacterium*

[a]: Organic matter.
[b]: Anaerobic methane oxidation implies a symbiosis between archaeal cells and sulfate-reducing bacteria; a syntrophic consortia is formed.
[c]: Under suboxic or anoxic conditions, light-induced proton translocation within Bacteriorhodopsins which generates ATP.

Table 1.3. Summary of the putative metabolic capabilities, required growth conditions, and occurrence of the main groups of nonthermophilic *Crenarchaeota* (modified from Casamayor & Borrego 2009).

Group	Putative Metabolic process	Energy source	Carbon source	Environmental conditions	Environment location	Culture
Non-thermophiles	Aerobic chemolitotrophy	NH_4^+	CO_2	O_2 and reduced nitrogen compounds (NH_4^+); oligotrophy	Close relatives detected in great lakes and alpine lakes	N. maritimus[a]
	Aerobic chemoorganotrophy Mixotrophy	OM[b] (simple)	OM	Oxic conditions; oligo- to mesotrophy		None
	Anaerobic chemolitotrophy	H_2; H_2S; NO_3^-	CO_2	Suboxic to anoxic conditions; reduced sulfur compounds; meso- to eutrophy	Suboxic-anoxic zones in stratified lakes (meta- and hypolimnia), sediments, saline alkaline soils	None
	Amaerobic chemoorganotrophy	OM (complex)	OM	Suboxic to anoxic conditions	Suboxic-anoxic zones in stratified lakes (meta- and hypolimnia)	None
				meso- to eutrophy	Sediments	

[a]: Isolated from marine environment.
[b]: Organic matter.

1.4. Phylogeny

One of the best tools to establish phylogenetic relationships among organisms is the analysis of the nucleic acids involved in the translation of the molecular information. The processes involved in protein synthesis are ancient biochemical reactions due to similarity of ribosome and rRNA structures among the three domains. Therefore, the translation machinery must appear before the radiation of the three Domains of Life (but see Roberts et al 2008). Ribosomes contain three different RNA molecules: 5S, 16S and 23S. The 5S rRNA is too small (120 nucleotides) for accurate phylogenetic inferences. 23S rRNA is *ca.* 2900 nucleotides long and would provide twice phylogenetic information compared to 16S rRNA molecule (which contains 1500 nucleotides), but the average rate of nucleotide changes of the 23S molecule is significantly higher than that of 16S rRNA. Moreover, both molecules are not functionally independent and they give similar phylogenies (Ludwig et al 1995). Accordingly, SSU rRNA was proposed as "gold standard" for prokaryotic phylogeny. Briefly, SSU rRNA sequences are aligned and pairwise comparisons are made between aligned sequences. The differences found in the data set are considered to be a measure of evolutionary distance between the compared SSU rRNA sequences. No consideration is given to the pass of time, but to nucleotide changes. Therefore, and theoretically, phylogenetic analyses of SSU rRNA could help to trace back the phylogenetic relationships among organisms until the origin of life and to establish their evolutionary history.

To date the most suitable way to distinguish between *Archaea* and *Bacteria* is by looking at their 16S rRNA molecule. Phylogenetic relationships of the archaeal domain based on 16S rRNA had split this domain into two kingdoms, the *Euryarchaeota* and the *Crenarchaeota* (Woese et al 1990; Fig. 1.5). The *Euryarchaeota* have been named using the Greek word for "wide" (or "diversity") because they encompass the greatest phenotypic diversity among known cultivable species (halophiles, methanogens, thermoacidophiles and hyperthermophiles). In contrast, the phenotypic diversity of cultivable *Crenarchaeota* is much more limited to date (hyperthermophilic species and some mesophilic ones) and thus has been named using the Greek word for "spring" (or "origin") from the popular hypothesis that hyperthermophiles are at the origin of all present-day organisms. Nowadays, the division of *Archaea* into *Crenarchaeota* and *Euryarchaeota* is supported by phylogenetic analyses, at least for cultured species. Indeed, the analysis of environmental archaeal sequences has somehow complicated the picture of archaeal phylogeny. Although many of the new archaeal lineages that have been detected by molecular techniques in environmental surveys branch among *Crenarchaeota* and

Euryarchaeota, some of them branch in between. A third Kingdom, the *Thaumarchaeota* (using the Greek word for "wonder") has been recently suggested (Brochier-Armanet et al 2008). Another tentative Kingdom, the *Korarchaeota* (using the Greek word for "young man"), was suggested exclusively from environmental sequences (Barns et al 1996; Fig. 1.5). Sequences that branch even deeper than *Korarchaeota* in the archaeal 16S rRNA tree were also reported (Takai et al 2001a, b). Nevertheless, scientists must be careful in interpreting the diversity and phylogeny of environmental sequences, since divergent copies of rRNA can exist within a single organism (Mylvaganam & Dennis 1992; Amann & Ludwig 2000). However, a consistent archaeal phylogeny must also rely on complementary information. In this regard, comprehensive environmental data must be provided and analysed together with 16S rRNA molecular phylogenies to increase the robustness of tree branching, clustering and naming of archaeal phylogeny.

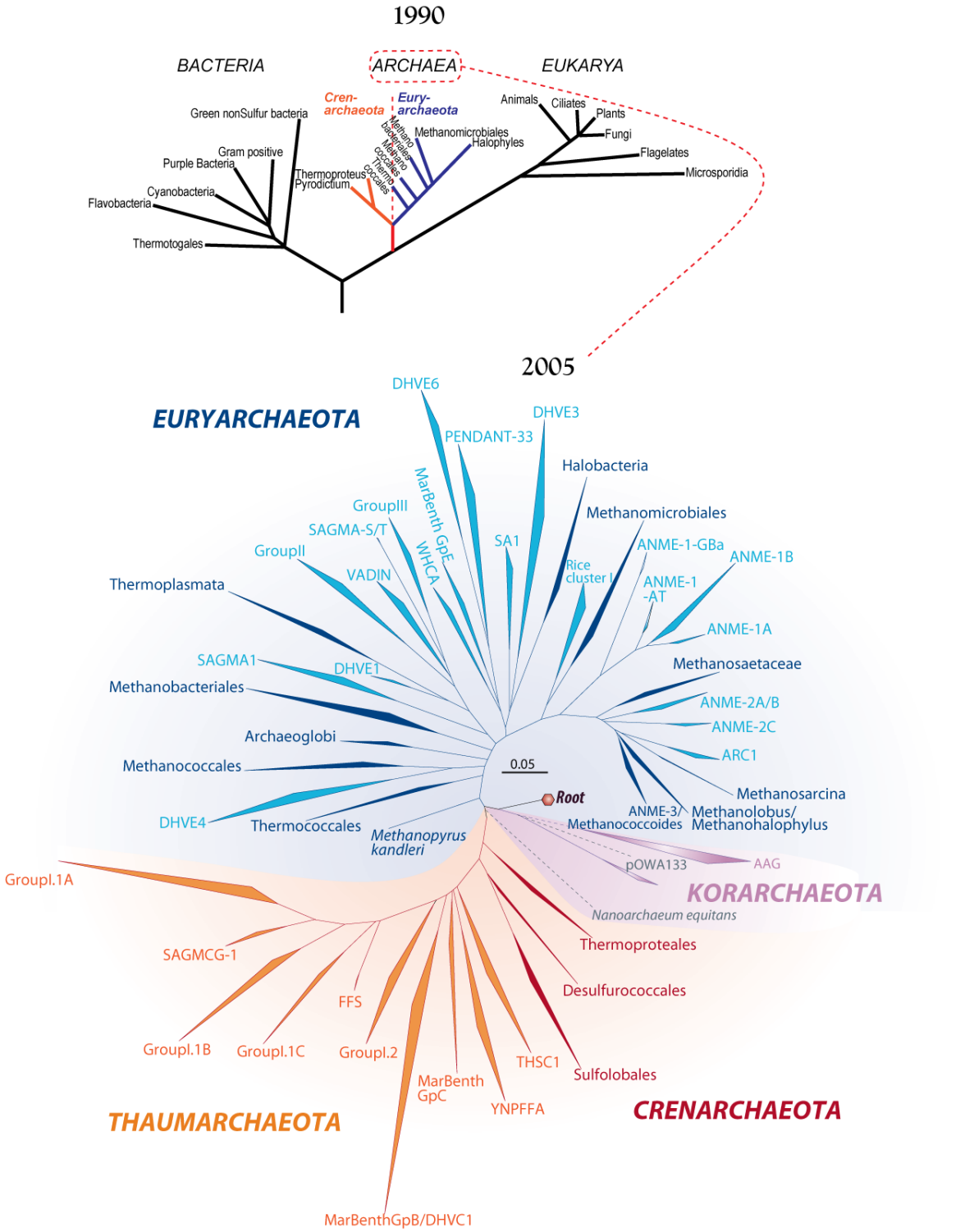


Figure 1.5. Composition of the "evolution" of the Tree of Life. The upper tree corresponds to one of the first tree reconstructions of organisms' phylogeny, while the lower one corresponds to one of the latest archaeal phylogenetical tree reconstructions based on 16S rRNA gene. In the lower tree; dark wedges highlight branches with cultivated species, while light coloured wedges highlight those with exclusively uncultivated species. The size of the wedge is proportional to the number of sequences included (modified from Woese et al 1990; Schleper et al 2005; Brochier-Armanet et al 2008).

1.5. Ecology

The wide use of molecular tools applied to microbial ecology has allowed the study of microbes without the need of culturing them. These new approaches have dramatically changed our perception of microbial diversity. Concerning Archaea, 16S rRNA-based molecular tools revealed great abundances and unexpected diversity. For the last two decades Archaea have been the focus of intensive research due to their ubiquity and abundance in almost every ecosystem on Earth and not only in those classified as extreme (Chaban et al 2006 and references therein). Particularly intense has been the research on marine and soil environments showing that Archaea are one of the main components in this environments, suggesting a more complex ecological role than previously expected. Mesophilic planktonic Archaea have been revealed as ubiquitous and abundant prokaryotes in a wide range of environments from soils (Bintrim et al 1997; Buckley et al 1998; Sandaa et al 1999; Jurgens et al 1997, 2000; Ochsenreiter et al 2003; Simon et al 2005); sediments (MacGregor et al 1997; Boetius et al 2000; Orphan et al 2001); hypersaline environments (van der Wielen et al 2005); marine (DeLong 1992, 2003; DeLong et al 1999; Fuhrman et al 1992; Masana et al 1997, 2000; Karner et al 2001; Varela et al 2008); and inland waters (see Casamayor & Borrego 2009 for a recent review). Overall, current data indicated that Archaea are a common component of freshwater plankton and different uncultured archaeal groups occupy different ecological niches. Accordingly and due to the increasing interest on Archaea, environmental sequences are accumulating at high rates in public databases (e.g. GenBank, http://www.ncbi.nlm.nih.gov/; Fig. 1.6). However, research focused in freshwater environments received less attention considering the low contribution of lacustrine environments to global aquatic environments and the number of freshwater archaeal environmental sequences deposited at public databases (Fig. 1.6).

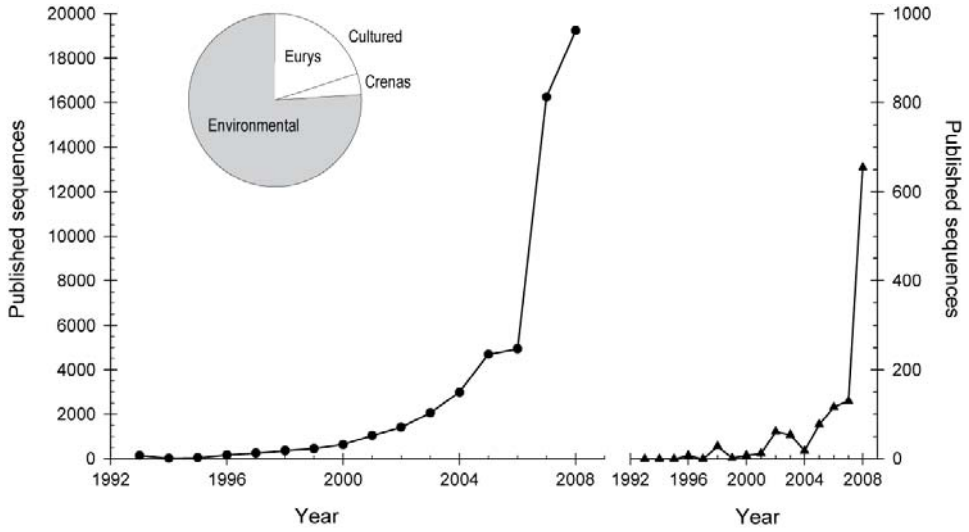


Figure 1.6. Number of archaeal (left; modified from Robertson et al 2005) and freshwater archaeal (right) 16S rRNA sequences submitted to GenBank database since 1993. The inset pie-chart depicts cultured versus environmental origin archaeal 16S rRNA sequences (Cren, Crenarchaeota; Eury, Euryarchaeota).

Nowadays, Archaea are widely seen as ubiquitous microorganisms. Nevertheless, the pool of data available so far from freshwater environments is less comprehensive than that from soil and marine environments mainly due to the physico-chemical heterogeneity of these environments and to the fact that most studies carried out in freshwater ecosystems consisted on snapshots of selected microbial communities in particular habitats (e.g Jurgens et al 1997; Casamayor et al 2000, 2001b; Vetriani et al 2003; Lin et al 2006; Coolen et al 2007; Labrentz et al 2007; Lehours et al 2007; Auguet & Casamayor 2008 among few others). Accordingly, the diversity and function of mesophilic archaeal communities within these mesophilic environments is poorly known. In turn, long-term studies where a freshwater prokaryotic assemblage (e.g., Bacteria) is seasonally monitored for changes in its structure are scarce (but see Lindström 1998; Boucher et al 2006; Koch et al 2006) and only few were focused on Archaea (Ovreas et al 1997; Casamayor et al 2000, 2001b). In this sense, stratified lakes constitute optimal study sites in which investigate links between microbial communities and their metabolic roles under a biogeochemical and ecological framework. Notwithstanding this, their metabolism and physiological roles are still unknown. In this sense, biogeochemical cycles should be carefully revised to incorporate the archaeal contribution (Herndl et al 2005; Schleper et al 2005; Francis et al 2005, 2007; Nicol & Schleper 2006; Erguder et al 2009).

2. Objectives/Objectius/Objetivos

Objectives

The main goals of the present PhD thesis can be summarised as follows:

- To study the diversity, distribution and temporal dynamics of mesophilic planktonic archaeal communities in different temperate stratified freshwater lakes characterised by seasonal anoxia and sulfide-rich hypolimnia.
- To ascertain the internal and external physico-chemical drivers that affect and modulate the distribution and abundance of mesophilic Archaea in stratified freshwater lakes.
- To determine the contribution of the planktonic archaeal assemblage thriving in a stratified freshwater lagoon to inorganic carbon fixation processes and to ascertain their ecological function within this ecosystem.
- To determine global distribution patterns for lacustrine Archaea to identify archaeal indicator taxa in freshwater lacustrine environments.

Objectius

Els principals objectius d'aquesta memòria de tesi doctoral es resumeixen a continuació:

- Estudiar la diversitat, la distribució i la dinàmica temporal de les comunitats d'Archaea planctòniques mesòfiles presents a diferents llacs temperats estratificats d'aigua dolça caracteritzats per una anòxia estacional i un hipolimnion ric en sulfhídric.

- Reconèixer els factors fisicoquímics, tan interns com externs, que afecten i modulen la distribució i abundància de les Archaea mesòfiles en llacs estratificats d'aigua dolça.

- Determinar la contribució de la comunitat planctònica d'Archaea present en un llac estratificat d'aigua dolça al processos inorgànics de fixació de carboni i establir la seva funció ecològica en aquest ecosistema.

- Determinar la distribució global de la comunitat planctònica d'Archaea per a identificar els diferents grups filogenètics d'Archaea indicadors en els ambients lacustres d'aigua dolça.

Objetivos

Los principales objetivos de la presente memoria de tesis doctoral se resumen a continuación:

- Estudiar la diversidad, la distribución y la dinámica temporal de las comunidades de Archaea planctónicas mesófilas presentes en distintos lagos estratificados de agua dulce caracterizados por una anoxia estacional y un hipolimnion rico en sulfhídrico.

- Identificar los factores fisicoquímicos, tanto internos como externos, que afectan y modulan la distribución y abundancia de las Archaea mesófilas en lagos estratificados de agua dulce.

- Determinar la contribución de la comunidad planctónica de Archaea presente en una laguna estratificada de agua dulce a los procesos de fijación de carbono inorgánico y establecer su función ecológica en este ecosistema.

- Determinar la distribución global de las Archaea mesófilas lacustres para identificar grupos filogenéticos indicadores dentro de las Archaea en los ambientes lacustres de agua dulce.

3. Material & Methods

3.1. Study sites

During the present work we have studied different stratified freshwater lakes characterised by distinct physico-chemical gradients that structure their microbial planktonic communites (see Table 3.1).

Lake Vilar is one of the largest water-bodies in the lacustrine system of Banyoles (Spain, Figure 3.1). The lake is composed by two separated circular basins of 10 m depth fed with bottom springs that provide a water inflow with high sulfate concentration (0.8 g l^{-1}). The lake has been traditionally considered as meromictic due to its permanent chemical stratification, which maintains an stable anoxic, sulfide-rich monimolimnion (Guerrero et al 1980, 1985).

Lake Kivu is located between Rwanda and DR Congo (Fig. 3.1) at 1,463 meters above sea level. The lake is a deep (max. 489 m) meromictic lake with step increases in temperature and salinity gradients. The lake has been classified as oligotrophic (Sarmento et al 2006). The basins sampled during the present work are named as: Northern (NB), Southern (SB) and Eastern (EB) basins and Bukavu Bay (BB) according to Sarmento (Sarmento et al 2008). Further details on hydrology, physico-chemistry and the biology of the lake are described elsewhere (Degens et al 1973; Tietze et al 1980; Isumbisho et al 2006; Sarmento et al 2006, 2008).

Coromina lagoon is a round-shaped holomictic and hypereutrophic lagoon located in the Banyoles karstic lacustrine system (Fig. 3.1, Sant Miquel de Campmajor, Girona, Spain). The lagoon is protected against wind due to its shape and sharp shore slope (>50º). Similar to other water bodies of the area, the lagoon develops a thermal stratification during late spring-summer due to the absence of bottom springs (Borrego & García-Gil 1994a). Consequently the water level exclusively depends on the balance between rainfall, evaporation, and water consumption by irrigation (Borrego & Garcia-Gil 1994a). A rich community of anoxygenic photosynthetic bacteria, mainly GSB (Green Sulfur Bacteria), usually prevails in the lagoon as described elsewhere (Borrego & Garcia-Gil 1994a; Abella & Garcia-Gil 1998; Gich et al 2001).

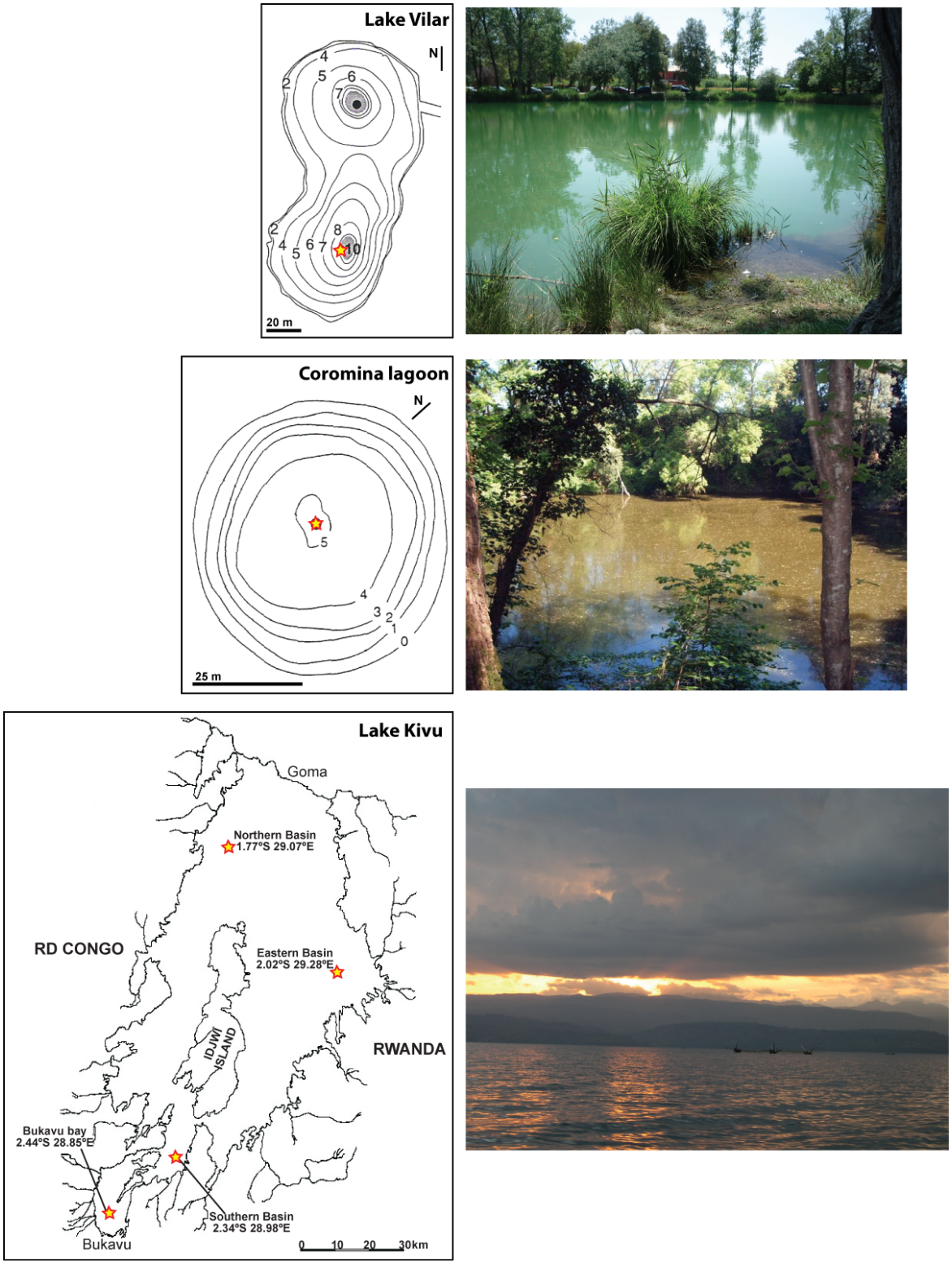


Figure 3.1. Geographical location of the different freshwater environments sampled in the present work. Up, Lake Vilar; middle, Coromina lagoon; down, Lake Kivu. In all ecosystems, water samples were collected at defined sites corresponding to characterised basins (stars in the corresponding figures).

Table 3.1. Freshwater environments sampled in the present PhD thesis.

Lake	Location	Area (km^2)	Volume (km^3)	Altitude (m)	Max. depth (m)	Origin	Type
Vilar	Girona (42º8'N/2º45'E)	0.011	0.00005	172	10	Karstic	Meromictic
Kivu	Congo-Rwanda (2ºS/29ºE)	2,370	580	1,463	489	Volcanic	Meromictic
Coromina	Girona (42º8'N/2º45'E)	0.0065	0.000005	60	4.5	Karstic	Holomictic

3.2. Sampling and analytical procedures

Banyoles Karstic System:

In Lake Vilar, water samples were collected at the southern basin of the lake during 20 sampling cruises at different date intervals for a period of five consecutive years (2001–2005) while two sampling campaigns were conducted on Coromina lagoon (May and October 2007). In both lakes, depth profiles for water temperature, conductivity, pH, redox potential (E_H), and oxygen concentration were determined *in situ* with a multiparametric probe YSI-556MPS (Yellow Spring Instruments, USA). Water samples for biological and chemical analyses were collected from different depths in 1-L sterile glass bottles using a weighted double cone connected to a battery-driven pump allowing laminar water sampling and minimal disruption of the vertical water stratification (Jorgensen et al 1979). Water samples were kept on ice and in the dark until further analysis within 24 h. For sulfide analysis, 10 ml of water were collected in sterile screw-capped glass tubes and fixed by adding zinc acetate (0.1M final concentration) under alkaline conditions (NaOH, 0.1M fin. conc.). Sulfide concentration was measured using the leucomethylene blue method (Brock et al 1971). Figure 3.1 showed the bathymetric profile for each lake.

Lake Kivu:

In this lake, water samples were collected during a sampling campaign conducted on March 2007 (rainy season). The sampling cruise covered four basins: Northern (NB), Eastern (EB) and Southern (SB) basins and Bukavu Bay (BB) (see Fig. 3.1 for exact location of sampling sites). Temperature, conductivity, pH, and oxygen were measured *in situ* at each sampling site with a YSI 6600 V2 multiparametric sonde (Yellow Spring Instruments, USA). Water samples for physico-chemical and biological analyses were collected using a 5-L vertical VanDorn bottle and stored in 4-L plastic bottles until further processing. Ammonia concentrations were determined using the dichloroisocyanurate-salicylate-nitroprussiate colorimetric method (Standing Committee of Analysts 1981). Nitrite concentrations were determined by the sulfanilamide coloration method (APHA 1998). Nitrate concentrations were determined after cadmium reduction to nitrite and quantified under this form following nitrite determination procedure (Jones 1984; APHA 1998). By these methods, detection limits were 0.3, 0.03 and 0.15 μM for NH_4^+, NO_2^- and NO_3^-, respectively.

Table 3.2. PCR conditions, primers and horseradish peroxidase (HRP) labeled oligonucleotide probes used in the present PhD thesis.

Target	Primer pair[a]	sequence (5'-3')	PCR conditions[b]						Ref.[d]
			Cycles	Denaturation °C	Denaturation min	Annealing °C	Annealing min	Elongation °C	Elongation min	
16S rRNA gene										
1st Round										
Universal *Bacteria*	341f	CCTACGGGAGGCAGCAG	30	94	1	55	1	72	1	[1,2]
	907r	CCGTCAATTCMTTTGAGTTT								
Universal *Archaea*	21f	TTCCGGTTGATCCYGCCGGA	30	94	1	56	1	72	2	[3]
	958r	YCCGGCGTTGAMTCCAATT								
General *Archaea*	ARC344f	ACGGGGCGCAGCAGGCGCGA	16+20	94	1	68[c]/60	1	72	1.5	[4]
	ARC915r	GTGCTCCCCCGCCAATTCCT								
2nd Round (nested)										
General *Archaea* PAIR-1	ARC344f	ACGGGGCGCAGCAGGCGCGA	16+10	94	1	68[c]/60	1	72	1.5	[4]
	ARC915r	GTGCTCCCCCGCCAATTCCT								
Marine *Crenarchaeota* PAIR-2	CREN28f	AATCCGGTTGATCCTGCCGGACC	25	94	1	58	1	72	1.5	[5]
	CREN457r	TTGCCCCCCGCTTATTCSCCC								
Freshwater *Crenarchaeota* PAIR-3	ARC337f	ATGGGCACTGAGACAAGG	25	94	1	58	1	72	1.5	[6]
	ARC915r	GTGCTCCCCCGCCAATTCCT								
Functional genes										
Archaeal *amoA*	Arch amoAf	STAATGGTCTGGCTTAGACG	35	94	0.75	55	1	72	1	[7]
	Arch amoAr	GCGGCCATCCATCTGTATGT								

Target	CARD-FISH probe	Final conc.	% FA in HB[e]	[NaCl] in WB[f]	Ref.
Eubacteria	HRP-EUB338I-III	0.28	55	3	[8, 9]
Archaea	HRP-ARC915	0.84	55	3	[10]
Cytophaga	HRP-CF319a	0.28	55	3	[8, 11]

[a]: A GC-rich clamp was attached to the 5' end of each forward primer used in all amplifications reactions used to generate amplicons for DGGE analysis.
[b]: Before each run of cycles, the temperature was held at 94º for 4 min and after all cycles were completed, temperature was kept at 72º during 30 min to allow final template elongation.
[c]: The program consisted in a touch-down protocol where the initial annealing temperature decreased 0.5 ºC each cycle during the first 16 cycles.
[d]: [1] Muyzer et al 1993; [2] Muyzer et al 1995; [3] DeLong 1992; [4] Casamayor et al 2001b; [5] Schleper et al 1997; [6] Llirós et al 2008; [7] Francis et al 2005; [8] Amann et al 1990; [9] Daims et al 1999; [10] Stahl & Amann 1991; [11] Manz et al 1996.
[e]: FA: formamide; HB: hybridisation buffer.
[f]: WB: washing buffer.

3.3. Pigment analyses

Pigment analyses were exclusively carried out in Coromina lagoon. In this sense, water samples for pigment analyses were passed through 0.45 mm pore-size membrane filters (47 mm filter diameter) previously covered with a thin layer of 2.5% $MgCO_3$ (Guerrero et al 1985). Cells retained in the filters were subsequently scrapper from the filters and transferred to light-preserved screw-capped glass tubes containing 3 ml of acetone:methanol (7:2 v/v, HPLC grade, Scharlau, Germany) as extractant. Pigments were extracted after a mild sonication for 30 sec in the dark at 4 ºC using a sonicator (B.Braun-Labsonic 2000, B.Braun, Germany) and stored 24 h at –30 ºC. HPLC analyses were performed as described previously (Borrego & Garcia-Gil 1994b).

3.4. Molecular procedures

3.4.1. DNA extraction

Water samples from different depths along the water column of the different lakes sampled were collected to characterise the prokaryotic planktonic assemblage present on them. Water samples for DNA extraction were first passed through 5.0 µm pore size, 47 mm diameter polycarbonate (ISOPORE^{TM}, Millipore, USA) to remove particulate debris as well as large protozoa, which are potential hosts for endosymbiontic archaea (i.e., methanogens, Casamayor et al 2001b). Eluents were subsequently passed through 0.22 µm pore size, 47 mm diameter polycarbonate filters (ISOPORE^{TM}, Millipore, USA) to retain free-living prokaryotes. Total nucleic acids were extracted from these latter filters using a combination of enzymatic cell lysis followed by a modification of the extraction protocol described by Lodhi and co-workers (1994). The cell lysis was accomplished using a two-step protocol consisting of a first incubation step using lysozyme (fin. conc. 1 mg ml^{-1}) at 37 ºC for 45 min, followed by a second one using proteinase K (fin. conc. 0.2 mg ml^{-1}) at 55 ºC for 1 h. Both steps were performed on a total volume of 300 µl of lysis buffer [40 mM EDTA, 50 mM Tris-HCl (pH 8.3), and 0.75 M sucrose]. DNA extraction was carried out by incubating predigested samples in double-concentrated CTAB extraction buffer (Fluka, Sigma-Aldrich, Switzerland) amended with β-mercaptoethanol (fin. conc. 2% v/v, Sigma-Aldrich, Germany) and polyvinylpyrrolidone (PVP, fin. conc. 2% w/v, Merck, Germany) and incubated at 65 °C for 60 min. After incubation, DNA extracts were purified with chloroform:isoamyl alcohol (24:1; v/v) and precipitated by adding 0.5 vol.

of 7.5 M ammonium acetate (pH 7.5) and 2 vol. of ice-cold absolute ethanol. Extracts were maintained overnight at –30 ºC. Afterwards, DNA was collected by centrifugation, washed with ice-cold 70% ethanol (v/v) and dehydrated in a SpeedVac system (Heto Lab). Dry DNA pellets were finally rehydrated in 50 µl of 10 mM Tris-HCl buffer (pH 7.4) and further purified using Centricon® cleaning columns (Millipore, USA). DNA concentration and purity were further determined by a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop, USA). Clean DNA extracts were stored at –80 °C until use.

3.4.2. PCR amplification of phylogenetic and functional gene markers

Amplification of bacterial and archaeal 16S rRNA gene fragments was carried out using direct and nested-PCR reactions with the primer pairs and PCR conditions summarized in Table 3.2. Amplification of bacterial 16S rRNA gene fragments was performed using 341f/907r primer pair as described in Casamayor (Casamayor et al 2000). In turn, the first archaeal PCR amplification round was performed using the universal primer pair 21f/958r (DeLong 1992). PCR products (*ca*. 900 bp) were later used as templates for nested-PCR reactions using three distinct combinations of internal primers that yielded amplicons suitable for further denaturing gradient gel electrophoresis (DGGE) analysis as follows:

3.4.2.1. Phylogenetic biomarkers

PAIR-1; the ARC344f/ARC915r set, specific for the Domain *Archaea* (Stahl & Amann 1991; Raskin et al 1994) and successfully applied in previous studies in Lake Vilar (Casamayor et al 2000, 2001b);

PAIR-2; the CREN28f/CREN457r set, specific for the Kingdom *Crenarchaeota* (Schleper et al 1997); and finally,

PAIR-3; the ARC337f/ARC915r set, combining the reverse primer of PAIR-1 with a newly designed forward primer biased towards freshwater *Crenarchaeota* (Table 3.2). Primer design was done using PRIMER PREMIER software (http://www.premierbiosoft.com/) based on freshwater *Crenarchaeota* sequences obtained from public database and several 16S rRNA gene clone sequences retrieved from Lake Vilar (AJ937874-AJ937878, and AM076830-AM076837). Primer ARC337f, however, has some flaws that should be taken into consideration. First, it offered reliable results after nested reactions but direct amplification on natural samples yielded unspecified products (e.g.. *Alcaligenes*,

Bacteroidetes; data not shown). ARC337f is very similar to a recently described primer (CREN334f) successfully used in combination with the reporting probe Cren519 for the qPCR quantification of archaea in the Black Sea (Lam et al 2007). Particularly, CREN334f is three and one nucleotide longer than ARC337f primer at the 5'- and 3' ends, respectively. Despite these differences, CREN334f showed the same unspecificities than ARC337f when applied for the direct amplification of archaea using the same PCR conditions (data not shown). A second drawback of ARC337f is its unspecific amplification of *Euryarchaeota* (mainly members of the Deep Hydrothermal Vent *Euryarchaeota* (DHVE) cluster) when combined with ARC915r, probably because the high number of mismatches of this reverse primer (Teske & Sorensen 2008). Considering these limitations, the nested use of ARC337f/ARC915r combination after a previous amplification step using universal archaeal primers (e.g., 21f/958r) may still offer a good approach to enhance the detection of freshwater *Archaea* (see Chapters 1 to 3).

3.4.2.2. Functional biomarkers

Amplification of the functional *amoA* gene coding for the α subunit of the archaeal ammonium monooxygenase was done as described by Francis and co-workers (2005) yielding PCR amplicons suitable for further denaturing gradient gel electrophoresis (DGGE) analysis.

3.4.3. PCR amplification of phylogenetic and functional gene markers

All amplification reactions were carried out in a GeneAmp 2700 thermocycler (Applied Biosystems, Perkin-Elmer, USA). PCR mixtures (50 µl) for first-round amplifications contained 5 µl of 10x PCR buffer containing 15 mM $MgCl_2$ (QIAGEN, GmbH, Germany), 0.8 mM premixed dNTP's (Applied Biosystems, Warrington, UK), 0.5 µL of 50 mM $MgCl_2$ (QIAGEN), 400 ng μl^{-1} of bovine serum albumin (BSA, BioLabs, USA), 0.2 µM of each primer (341f/907r; 21f/958r; ARC344f/ARC915r; or Arch amoAf/amoAr), 0.025 U of QIAGEN-DNA Taq polymerase (QUIAGEN), and 1 µl of template DNA (10-50 ng). The remaining volume was adjusted to 50 µl with sterile, molecular biology-grade water (Eppendorf AG, Germany). Nested reactions were prepared as described above, except for primer pair used (ARC344f/ARC915r; CREN28f/CREN457r and ARC337f/ARC915r), the final $MgCl_2$ concentration (1.5 mM instead of 2 mM), and no BSA addition. Positive PCR products were visualized using electrophoresis on 1.5% (w/v) agarose gels after ethidium bromide staining.

3.4.4. Phylogenetic and functional genes fingerprinting

Fingerprinting of the *Bacteria*, *Archaea* and *Crenarchaeota* planktonic assemblages as well as of the archaeal *amoA* gene fragments were carried out by denaturing gradient gel electrophoresis (DGGE) (Muyzer et al 1993) using an INGENY phorU-2 (Ingeny International BV, the Netherlands) DGGE system. In all cases, between 500 and 1000 ng of PCR product were loaded onto 6.0% polyacrylamide gels and run with 1x TAE buffer using a 30–70% linear gradient of urea and formamide [100% denaturant agent contains 7M urea and 40% deionised formamide (McCaig et al 2001)] for the bacterial 16S rRNA gene fragments, 20–80% linear gradient for the Archaea 16S rRNA gene fragments and a 20–70% vertical denaturing gradient for the functional gene analysis. The archaeal 16S rRNA gradient was broad enough to accommodate most of the archaeal phylotypes (Yu et al 2008). Electrophoreses were run at 60 ºC and at a constant voltage of 120 V for 17 h. After electrophoresis, gels were stained for 30 min with 1x SYBR Gold nucleic acid stain (Molecular Probes Inc.) in 1x TAE buffer, rinsed, and visualised under UV radiation using a GelPrinter system (TDI, Spain). A DGGE ladder composed by a mixture of known SSU rRNA gene fragments (*S. cerevisiae, Mucor sp., Ps. aeruginosa, M. luteus* and *S. solfataricus*; *ca.* 700–800 ng μl^{-1} each) was loaded in all gels to allow inter-gel comparison of band migration. Discrete bands were excised from the DGGE gels and rehydrated in 30 µl of Tris-HCl 10 mM buffer (pH=7.4). DNA was eluted after incubation at 65 ºC for 3 h and amplified using the corresponding primer pairs (without GC clamp) and PCR conditions as showed in Table 2.2, but sizing down the number of PCR cycles up to 20. PCR products were forward and reverse sequenced at Macrogen (Macrogen Inc., Korea).

3.4.5. Gel image analyses

Digital images of polyacrylamide gels were analysed using the GELComparII® software package (Applied Maths BVBA, Belgium). Lanes were manually defined and band positions identified from corrected intensity plots. Comparison between samples loaded on different DGGE gels was performed using normalized values derived from known standards loaded in each DGGE run. A binary matrix showing the presence-absence of identified bands was made for all gel lanes. Further, a similarity matrix based on the Dice coefficient was calculated and samples clustered according to the unweighted pair group average linkage method (UPGMA) algorithm using a tolerance position value of 1.5–2%.

3.4.6. Phylogenetic analyses

All 16S rRNA retrieved sequences were compared for closest relatives to partial 16S rDNA sequences at NCBI sequence database (http://www.ncbi.nlm.nih.gov/blast/) using the BLASTN algorithm tool (Altschul et al 1990). All bacterial 16S rRNA sequences were analysed as previously described (Gich et al 2005) whereas all archaeal 16S rRNA sequences obtained from DGGE bands (*ca.* 400-600 bp), as well as other closely related archaeal sequences (*ca.* 500-1300 bp) from databases were properly aligned using the NAST (DeSantis et al 2006a) aligner web server. The presence of chimera was checked using the Bellerophon (Huber et al 2004) tool implemented at the Greengenes website (http://greengenes.lbl.gov/; DeSantis et al 2006b). An Archaea phylogenetic backbone tree was constructed with ARB software package (http://www.arb-home.de/; Ludwig et al 2004) using a 16S rRNA ARB-compatible database (updated May 2007) available at Greengenes web site. Two base frequency filters ("*Positional Variability by Parsimony*" and "*Archaeal filter by Maximum Frequency*") were applied to exclude highly variable positions before sequences were added to the original Greengenes database using the *parsimony quick add marked* tool implemented in ARB. A ≥98% cut-off was applied when defining phylotypes. Clustering, grouping, and naming used in the entire thesis were based on the cluster redefinition for *Crenarchaeota* and *Euryarchaeota* proposed by Teske & Sorensen (2008).

The phylogenetical analysis of archaeal *amoA* sequences (582 bp) was conducted on DNA rather than predicted aminoacid sequence in order to highlight the genetic (rather than protein) heterogeneity among ammonia-oxidizing *Crenarchaeota* in Lake Kivu. About 900 *amoA* sequences from other environmental studies were analysed using DAMBE software package (Xia & Xie 2001) to retrieve representative haplotypes. A total of 135 haplotypic environmental *amoA* sequences together with *amoA* sequences from this study (16) were aligned and subsequently used for a phylogenetical analyses following Neighbour-Joining algorithm based on Tamura-Nei corrected distances (Saitou & Nei 1987; Tamura & Nei 1993) with 1000 bootstrap replicates. Different phylogenetic reconstructions revealed similar tree topologies. All analyses were conducted using MEGA4.1 (Tamura et al 2007).

All 16S rRNA and *amoA* gene sequences obtained in the present PhD thesis have been deposited in GenBank database (Table 3.3).

Table 3.3. Accession numbers for 16S rRNA and amoA gene sequences according to sampling site.

Study site	Archaeal 16S rRNA	Archaeal *amoA*	Bacterial 16S rRNA
Lake Vilar	AM697959-AM698009 EU683310-EU683429	-	-
Lake Kivu	EU921487 to EU921548 FJ536696 to FJ536719	EU921473 to EU921486 FJ536694 to FJ536695	-
Coromina lagoon	nd[a]	nd	nd

[a]: not yet deposited

3.4.7. Construction of a lacustrine archaeal 16S rRNA gene database

We conducted an extensive survey on published literature and GenBank database searching for archaeal 16S rRNA gene sequences that matched the following criteria: (i) sequences obtained from freshwater environments; (ii) high quality sequences (defined as sequences of >300 bp each and with the minor number of ambiguous positions); and (iii) sequences obtained with the same PCR primers in order to cover the same gene region. As a result, a database of nearly 1000 archaeal 16S rRNA sequences recovered from 32 environmental freshwater habitats was obtained. Different methodologies and sampling efforts were homogenized by clustering sequences at 97% identity cut-off (Shaw 2008). The final database comprised 775 archaeal 16S rRNA sequences from 23 globally distributed lacustrine sites (Table 3.4).

The different studies were grouped into four distinct habitat typologies (understood as a group of environments sharing a close geo-physico-chemistry) as follows: Meso- to Eutrophic stratified lakes (EU), Oligotrophic lakes (OL), Saline lakes (SL) and finally a mixed typology group (UC) containing diverse lacustrine environments of difficult classification according to their diverse physico-chemical characteristics.

Table 3.4. Freshwater archaeal studies analysed in the *in silico* survey.

Location	Lake name	Typology[a]	Number of sequences	References
Europe				
	Arcas	EU	6	GenBank (unpublished data)
	Charca verde	EU	28	Briée et al 2007
	Coromina	EU	33	Llirós et al (umpublish data)
	Cruz	EU	7	GenBank (unpublished data)
	Grube prinz	EU	17	Ochsenreiter et al 2003
	Pavin	EU	10	Lehours et al 2007
	Priest pot	EU	28	Whitby et al 2004
	Tejo	EU	3	GenBank (unpublished data)
	Tobar	EU	4	GenBank (unpublished data)
	Vilar	EU	148	Llirós et al 2008
	Banyoles	OL	51	GenBank (unpublished data)
	Bourget	OL	21	GenBank (unpublished data)
	Ladoga	OL	2	Keough et al 2003
	Llebreta	OL	87	Auguet & Casamayor 2008
	Monegros	SL	10	GenBank (unpublished data)
	Boreal fen	UC	9	Juottonen et al 2005
	Finland fen	UC	22	Galand et al 2003
			486	
America				
	Alkali	SL	6	GenBank (unpublished data)
	Mono	SL	8	GenBank (unpublished data)
	Silver	SL	5	Navarro et al 2009
	Superior	OL	2	Keough et al 2003
	Mackenzie	OL	23	Galand et al 2008
	Tucurui	OL	83	GenBank (unpublished data)
			127	
Africa				
	Fazda	SL	42	Mesbah et al 2007
	Manzallah	SL	16	Elsaied & Maruyama 2008
	UmRisha	SL	29	Mesbah et al 2007
	Kivu	OL	60	Llirós et al 2009
	Rift	OL	14	Rees et al 2004
	Victoria	OL	1	Keough et al 2003
			162	

[a] lacustrine environments were grouped in different habitat typologies according to close geo-physico-chemistry as follows: Meso- to Eutrophic stratified lakes (EU), Oligotrophic lakes (OL), Saline lakes (SL) and an outlier group (UC) containing environments of difficult classification.

Next, a semi-quantitative environmental matrix was constructed according to the range of several environmental parameters in the defined habitats: latitude, longitude, depth, surface (square kilometers), volume (cubic kilometers), oxygen presence (from oxic to anoxic), salinity (from freshwater to hypersaline waters), trophic state (from oligotrophic to hypereutrophic waters), and lake typology (according to the habitat classification described above). Note that the determination of some parameters was only qualitative and based on sampling site descriptions available in the literature rather than on direct measurements. All these parameters were obtained from both public sources (GenBank and published papers) and/or directly form the authors (personal communications).

All 16S rRNA gene sequences retrieved were automatically aligned using the NAST aligner web server (DeSantis et al 2006a) and imported into a Greengenes database (DeSantis et al 2006b) compatible with the ARB software package (Ludwig et al 2004). Two base frequency filters ("*Positional Variability by Parsimony*" and "*Archaeal filter by Maximum Frequency*") were applied to exclude highly variable positions before sequences were added to the original Greengenes database using the *parsimony quick add marked* tool from ARB. The final 16S rRNA tree contained 775 archaeal sequences.

3.4.8. Community phylogenetic analyses

To quantify the community similarity using phylogenetic relatedness, a β diversity metric (Unifrac; http://bmf.colorado.edu/unifrac) was used (Lozupone et al 2006; Lozupone & Knight 2007). A distance matrix was constructed using the UniFrac metric on the 775 lacustrine archaeal environmental sequences (Auguet et al, in press). To assess the sources of variation in the UniFrac environmental matrix we used permutational manova based on 999 permutations (McArdle 2001) with function adonis in vegan package (Oksanen et al 2008).

Phylogenetic diversity (PD) is a quantitative measure to analyse biodiversity on populations or taxa level rather than species level based on phylogenetic information (Lozupone & Knight 2008). PD index for each of the four distinct habitat typologies was calculated as the sum of the branch length associated with the 16S rRNA gene sequences within those habitat typologies (Faith 1992). To correct for unequal number of sequences, we calculated the mean PD of 1,000 randomized subsamples of each habitat (Barberan & Casamayor, submitted). The phylogenetic structure for each habitat topology was calculated with the phylogenetic species variability (PSV) estimate (Helmus et al 2007). The degree of phylogenetic relationship among species, or higher

taxonomic levels, in a given communtity is sumarised by the PSV. A PSV value of 1 refers to a large distribution of species within a given environment, so species in that environment are phylogenetically unrelated. On the contrary, a PSV close to 0 reflected a more related species community in a given environment. To statistically test whether habitats were composed of species that are more or less related to each other than expected by chance, we compared the mean observed PSV to distributions of mean null values (1,000 iterations) using two different randomization procedures. Null model 1 maintains species occurrence, while null model 2 maintains habitat species richness (Helmus et al 2007). Analyses were run with the R package picante (Kembel et al 2008).

3.4.9. Lineage based analyses

Archaeal lineages were named according to the labelled clusters or divisions provided by default in the Greengenes main tree immediately subordinate to the *Crenarchaeota* or *Euryarchaeota* phyla and also following the cluster grouping and naming proposed by Teske & Sorensen (2008). However, several sequences appeared not related to any cluster and would have remained unaffiliated at the lineage level. Grouping at a lower phylogenetic level was ruled out due to poor taxonomic affilitation in *Archaea*, and especially within the Kingdom *Crenarchaeota*, due to the lack of close culture representatives. Finally, in order to identify those archaeal lineages analogous to the ecological concept of "indicator species" for each habitat typology, a table of abundances was constructed to calculate IndVal index (Auguet et al, in press). This index value combines relative abundance and relative occurrence frequency of a given trait (Dufrene & Legendre 1997).

3.5. Prokaryotic cell counts

Water samples (100 ml) were fixed in situ with paraformaldehyde (PFA, final conc. 0.4%, w/v) and stored at 4 °C for 24 h. Planktonic cells were collected on white 0.22 μm pore-size polycarbonate filters (25 mm filter diameter; Millipore, Eschborn, Germany), washed twice with phosphate-buffered saline (PBS) buffer (pH 7.6), dried and stored at –20 °C until analysis.

Total cell numbers were determined by epifluorescence counting after staining with 4′, 6-diamidino-2-phenylindole (DAPI) as previously described (Bruns et al 2002) while *Bacteria*, *Archaea* and *Cytophaga* were enumerated by fluorescence in situ hybridization with catalyzed-reported deposition (CARD-FISH; Pernthaler et al 2004)

using specific probes (Table 3.2) and a modification of the protocol described by Teira and co-workers (2004) to improve cell wall permeabilization. Cell losses during permeabilization and filter processing were minimized by dipping the filters in low-gelling-point agarose (0.1 % w/v, in Milli-Q water) and drying them upside down on a glass Petri dish at 37 °C. The filters were subsequently dehydrated in 95% ethanol (v/v) and let air dry. To inhibit potentially present intracellular peroxidases, filters were incubated with 0.01M HCl at room temperature (RT) for 20 min, washed with 1× PBS buffer, Milli-Q water and dried. For cell wall permeabilization, filters were incubated with lysozyme solution (10 mg ml^{-1} in 0.05M EDTA and 0.1M Tris-HCl at pH 8.0; Fluka) during 30 min at 37 °C and, afterwards, gently rinsed with Milli-Q water and absolute ethanol. Filters were then incubated with proteinase K (0.25 U mg^{-1}, conc. 0.25 mg ml^{-1} in 0.05M EDTA and 0.1M Tris-HCl (pH 8.0); Roche) during 5 min at 50 °C and washed with Milli-Q water. Subsequently, the filters were incubated with 4% PFA (w/v, final conc.) during 5 min at RT, washed with Milli-Q water, dehydrated with absolute ethanol, and air dried. Probe hybridization, washing, signal amplification and filter preparation were done as described previously (Teira et al 2004). Filter sections were finally air dried, embedded in Citifluor antifading solution (Citifluor Ltd., UK) and examined under an Axioskop epifluorescence microscope (Zeiss, Germany) equipped with a 50-W Hg bulb and appropriate filter sets for DAPI and Alexa-Fluor 488. Triplicate filters were processed independently for each depth. At least 40 microscopic fields were randomly selected to count DAPI-stained and probe-hybridized cells.

3.6. Biological uptake of dissolved inorganic carbon

To measure the uptake of inorganic carbon by the bulk prokaryotic community, 70 ml plastic flasks were fulfilled with water samples and spiked with 50 to 750 µl of radiolabeled bicarbonate ($NaH^{14}CO_3$; 1 mCi ml^{-1}; DHI, Denmark) at a final concentration of 0.15-2.0 µCi ml^{-1}. All water samples were incubated at *in situ* conditions for 4–6 hours around noon using a hand-made incubator hanger. To keep anoxic conditions in samples from the hypolimnion, these flasks were carefully filled avoiding aeration. For each water sample, the incubation set included two clear (light) and two dark incubation flasks and one additional formaldehyde-killed flask (37% fin. conc.) to be used as dead-control. After incubation, water samples were immediately fixed with formaldehyde (fin. conc. 3.7%) to stop microbial activity. The addition of this fixative was previously found to produce no interference in the experiments (A Camacho, pers. comm.). After fixation, water samples were passed through white 0.22 µm pore-size nitrocellulose filters (25-mm filter diameter; Millipore; Germany) at low vacuum pressure using a multiple vacuum filtration device (1225 Sampling Manifold,

Millipore, USA). Afterwards, filters were placed in scintillation vials and exposed overnight to HCl fumes to release precipitated bicarbonate. Scintillation cocktail (4 ml; Optiphase Hisafe 2) was added to each vial and the radioactivity of the filters was assessed in a Beckton-Dickinson LS6000 (Beckman, USA) scintillation counter. The disintegrations per minute (DPM) of the formol-fixed blank were subtracted from the corresponding samples (Pedrós-Alió et al 1993).

3.7. MICRO-CARD-FISH analyses

MICRO-CARD-FISH was carried out according to Alonso & Pernthaler (2005). Briefly, between 0.1 and 2.0 ml water subsamples from $NaH^{14}CO_3$ uptake incubations (final times) were fixed with formaldehyde (3.7% fin. conc.) overnight and passed through 0.22 µm pore-size white polycarbonate filters (25 mm filter diameter; Millipore; Germany) at low vacuum pressure using a multiple vacuum filtration device (1225 Sampling Manifold, Millipore; USA). Filters were subsequently hybridised following the CARD-FISH protocol described above and, afterwards, glued on glass slides by using epoxy adhesive (UHU plus, UHU GmbH, Germany). For autoradiography, slides were embedded in 46 ºC tempered photographic emulsion (KODAK NTB-2; melted at 46 ºC for 1h) 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 ºC until development. The optimal exposure time was determined for each sample and resulted in 14 and 24 days (data not shown). For development, exposed slides were developed and fixed following manufacturer specifications: 3 minutes immersion in photographic comercial developer (KODAK D19; 1:1 dilution with Milli-Q water), 30 seconds rinsing in Milli-Q water, 3 minutes of emulsion fixation (KODAK Tmax; 1:4 dilution with Milli-Q water) and two final 30 seconds rinsing steps with distilled and tap water. Slides were dried in a desicator overnight, counter-stained with DAPI (50 µl; fin. conc. 1µg ml^{-1}) and examined under an Axioskop epifluorescence microscope (Zeiss, Germany) equipped with a 50-W Hg lamp (OSRAM, Germany) and appropriate filter sets for DAPI and Alexa488. Cells actively uptaking the substrate were surrounded by silver grains detected in the autoradiograms. The presence of silver grains surrounding the cells was checked using the transmission mode of the microscope. From now on, for hibridised cells with presence of silver grains we will use EUB+MAR+, ARC+MAR+, CF+MAR+ and PROBE+MAR+ active cells, respectively to make the text less cumbersome.

4. High archaeal richness in the water column of a freshwater sulfurous karstic lake along an interannual study

4.1. Introduction

For the last two decades, the archaea have been the focus of intensive research in microbial ecology due to their ubiquity and abundance in almost every ecosystem on Earth and not only in those considered as extreme (Chaban et al 2006 and references therein). Research on marine and soil archaeal diversity, dynamics and activity has been particularly intense showing that archaeal phylogeny is more complex than expected previously and that biogeochemical cycles should be carefully revisited taking into account the archaeal contribution (Francis et al 2005, 2007; Schleper et al 2005; Nicol & Schleper 2006). The pool of data available so far for freshwater habitats is less comprehensive mainly due to the physico-chemical and limnological heterogeneity of the studied environments (e.g., alpine or polar lakes, tropical lakes and rivers, estuaries, and extreme habitats like hydrothermal springs or salt lakes; for a recent review see Casamayor & Borrego 2009) and to the fact that most studies consisted in a molecular snapshot of a selected microbial community in a particular habitat. In turn, long-term studies where a freshwater prokaryotic assemblage is seasonally monitored for changes in its structure (e.g., Bacteria) are scarce (e.g., Lindström 1998; Boucher et al 2006; Koch et al 2006, among a few other).

Within freshwater ecosystems, stratified lakes with seasonal or permanent oxic/anoxic interfaces constitute optimal study sites to monitor the changes experienced by the different microbial populations when facing the seasonal physicho-chemical perturbations in their habitats. The sulfurous temperate Lake Vilar has been the subject of an intensive research over the last 30 years due to its meromictic nature and the presence of an active sulfureta that strongly influences the structure and dynamics of the microbial populations found there. Historically, the lake has been the focus of a detailed research on the diversity, dynamics, and function of anoxygenic photosynthetic sulfur bacteria (Guerrero et al 1980, 1985; Bañeras & Garcia-Gil 1996; Bañeras et al 1999; Casamayor et al 2002). It has not been known until recently that the application of molecular techniques has shown that archaea also constituted a dynamic component of the planktonic assemblage in the lake (Casamayor et al 2000, 2001b). Of particular interest was the finding of a marked seasonality in the different archaeal groups found in the hypolimnion of the lake (methanogens, thermoplasmales, and *Crenarchaeota*), in the winter-summer transition, and the fact that only one single crenarchaeotal phylotype was recovered throughout the study period (Casamayor et al 2001b). The analysis was focused on the anoxic, sulfide-rich monimolimnion, although weak positive PCR signals were obtained in the

oxygenated epilimnetic waters from unknown archaea that were not explored (Casamayor et al 2001b). Recently, abundant archaeal populations have been reported in surface-oxygenated waters from high mountain lakes (Auguet & Casamayor 2008) with potential autotrophic activity (Auguet et al 2008).

In the present work, we have surveyed temporal changes on the planktonic archaeal community richness in Lake Vilar along an interannual long-term study (from summer 2001 to autumn 2005). A detailed physico-chemical profile of the lake comparing oxic *vs.* anoxic water compartments was also carried out at each sampling date. Thus, we attempted to detect potential seasonal and interannual trends in the free-living planktonic Archaea composition, and answer some questions such as do archaea constitute or not a stable component in the planktonic prokaryotic assemblage of the lake, do archaeal phylotypes segregate above and below the oxic/anoxic interphase as bacteria do, and whether or not anoxic sulfide-rich hypolimnia harbour a high archaeal richness.

4.2. Results

Physico-chemical characterization of the water column

Lake Vilar has been traditionally considered a meromictic lake due to the continuous incoming flux of sulphate-rich water by bottom springs that maintained a permanent chemical stratification throughout the year (Guerrero et al 1980, 1985). This situation, however, has changed recently mainly due to the pronounced decreased in the local precipitation regime that has reduced incoming water fluxes (J. Colomer, pers. commun.). Thus, during the present survey period (2001–2005), the lake showed an unexpected complete mixing (holomixis) and oxygenation of the water column in winter, and a thermal and chemical stratification lasting from late spring to autumn (Figure 4.1). On some occasions, a thin (0.5 m in height) high-conductivity deep-water layer was detected at the bottom of the sampled basin after winter water mixing (e.g., January 2002) having high sulfide concentrations (400 µmol l^{-1} of H_2S).

During stratification periods, the water column split into two well-defined water compartments separated by a broad thermocline (ranging from 4 to 7 m depth) and an oxycline extending from 3 to 5 m depth (Fig. 4.1). Oxygen minimum values were detected at the chemical transition zones (*ca*. from 4 to 5 m depth), whereas below 5 m depth the water became anoxic and sulfide accumulated, reaching values up to 1,500 µmol l^{-1}.

Representative water samples from different depths were collected to compare the planktonic archaeal assemblage thriving in both water compartments.

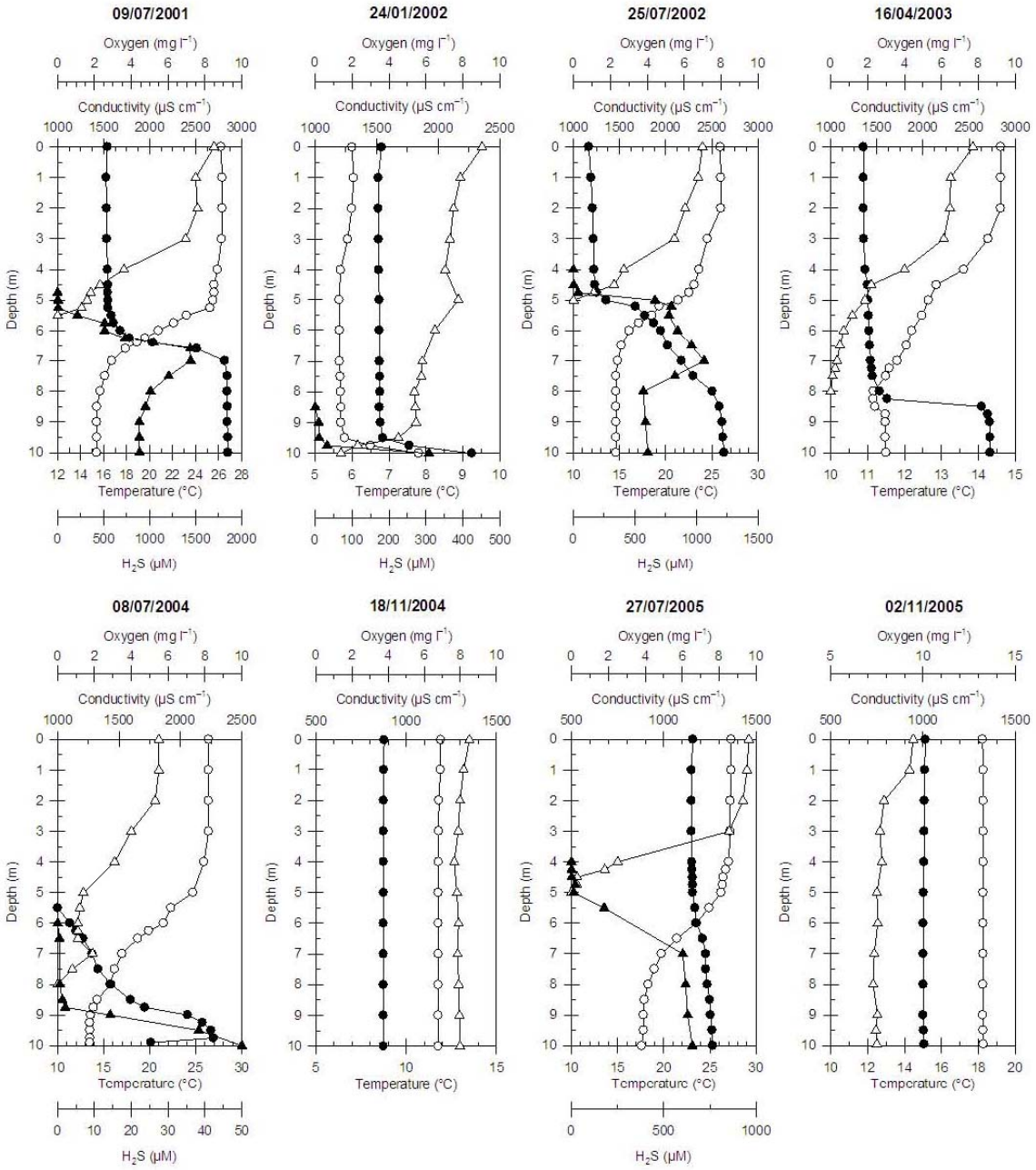


Figure 4.1. Physico-chemical depth profiles for temperature (open circles), conductivity (closed circles), oxygen (open triangles), and sulfide (closed triangles) in the water column of Lake Vilar. Dates have been selected to illustrate periods of summer stratification and winter mixing.

Molecular analysis

We used a PCR-DGGE approach using three primer combinations (Table 3.2 of Material and Methods section) to retrieve the maximal archaeal richness within the planktonic assemblage of the lake. DGGE was chosen based on both the high number of samples to be analyzed (140) and its convenience for profiling and comparing microbial communities to obtain gene sequences after band excision from the gel (Muyzer et al 1993, 1998). In all cases, nested amplification yielded positive products and well-defined DGGE fingerprints except those loaded with amplicons obtained with PAIR-1 primer combination, which resulted in smear and fuzzy bands (not shown). Using PAIR-1 only two clean 16S rRNA gene sequences could be retrieved and further used for phylogenetic analysis (Table 4.1 and Fig. 4.2). Both phylotypes grouped within the *Deep Hydrothermal Vents Euryarchaeota* (DHVE, Takai & Horikoshi 1999). In turn, when the PAIR-2 combination was used in the nested step, up to 63 sequences from both oxic and anoxic water samples were recovered, all of them affiliated to *Crenarchaeota* (Table 4.1). Fifty six percent of these sequences (35 out of 63) clustered within Group1.2 of the *Miscellaneous Crenarchaeota Group* (MCG), a large cluster of environmental crenarchaeotal sequences retrieved from different environments (Inagaki et al 2003) (Fig. 4.3, sequences in red). The remaining sequences (44%) were distributed among different subclusters of the MCG (Table 4.1), mainly grouping with clones retrieved from very different environments (subsurface habitats, deep marine sediments, geohydrothermal vents, etc.). Only two phylotypes grouped with the subcluster MCG:MCG1, which includes some clones recovered from freshwater habitats. The application of the PAIR-3 combination resulted in the detection of additional unexpected archaeal richness within the planktonic assemblage, recovering 104 sequences from different water depths (Table 4.1). From this total, 38 and 33 sequences were affiliated within the DHVE-3 and the MCG Group 1.3 clusters, respectively (Figs. 4.2 and 4.3, sequences in green). The remaining sequences (20 belonging to the *Euryarchaeota* and 13 to the *Crenarchaeota*) were distributed within five euryarchaeotal (SAGMEG, HV-1, pMC1, WSA-2, and DHVE-6) and 13 crenarchaeotal clusters (several subclusters within the MCG and Soil1.1b:SCA1154) (Table 4.1).

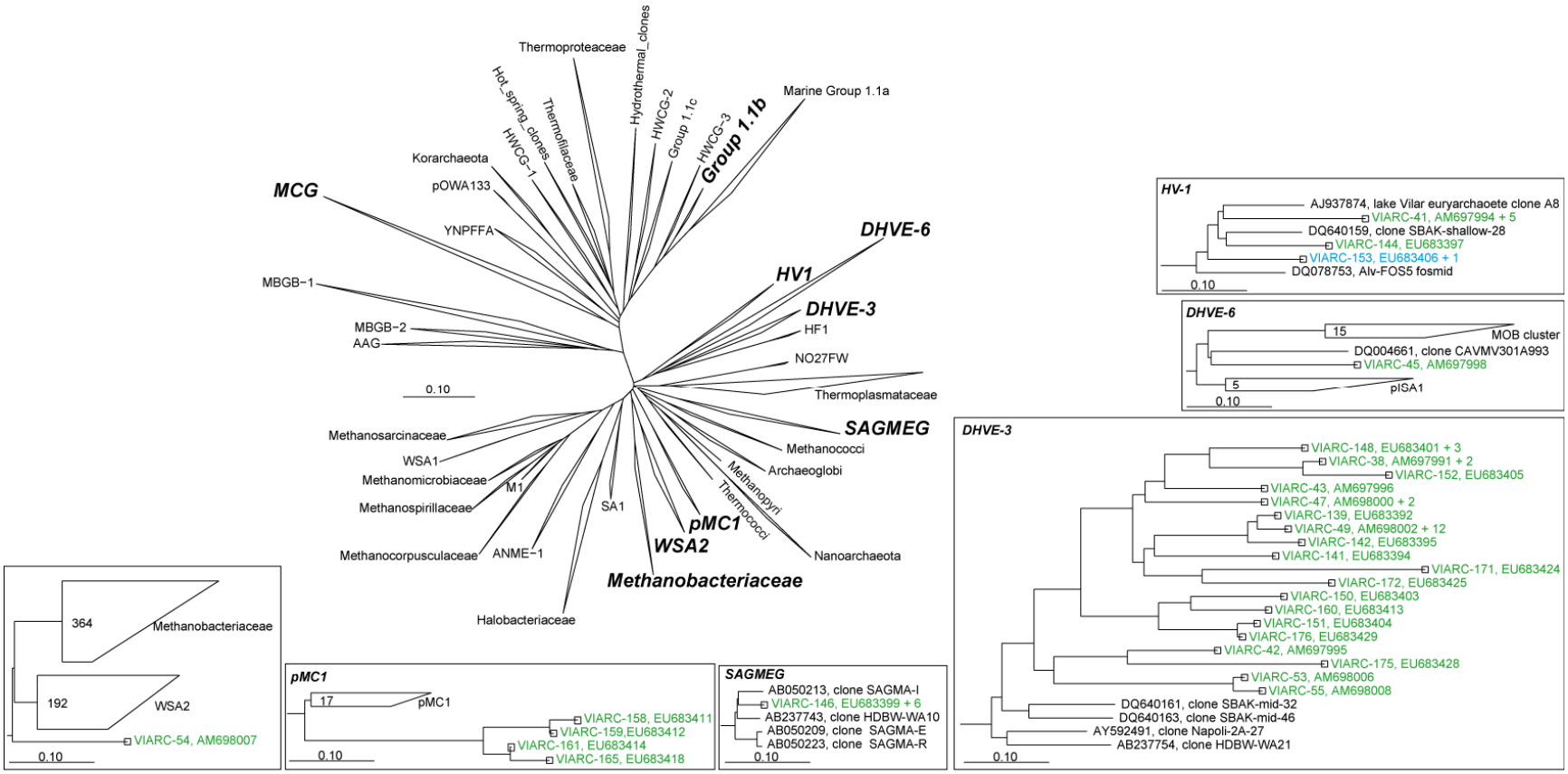


Figure 4.2. Phylogenetic tree showing the affiliation of *Euryarchaeota* partial 16S rRNA sequences retrieved from the water column of Lake Vilar during the study period. Sequences recovered using PAIR-1 and PAIR-3 are shown in blue and green colour, respectively. Database sequences are shown in black. When possible, only one representative sequence of each phylotype (≥98% identity) is shown together with the number of additional sequences ascribed to the same phylotype (see Table 4.2). Reference sequences are described by "clone name and accession number". The scale bar indicates 10% estimated sequence divergence.

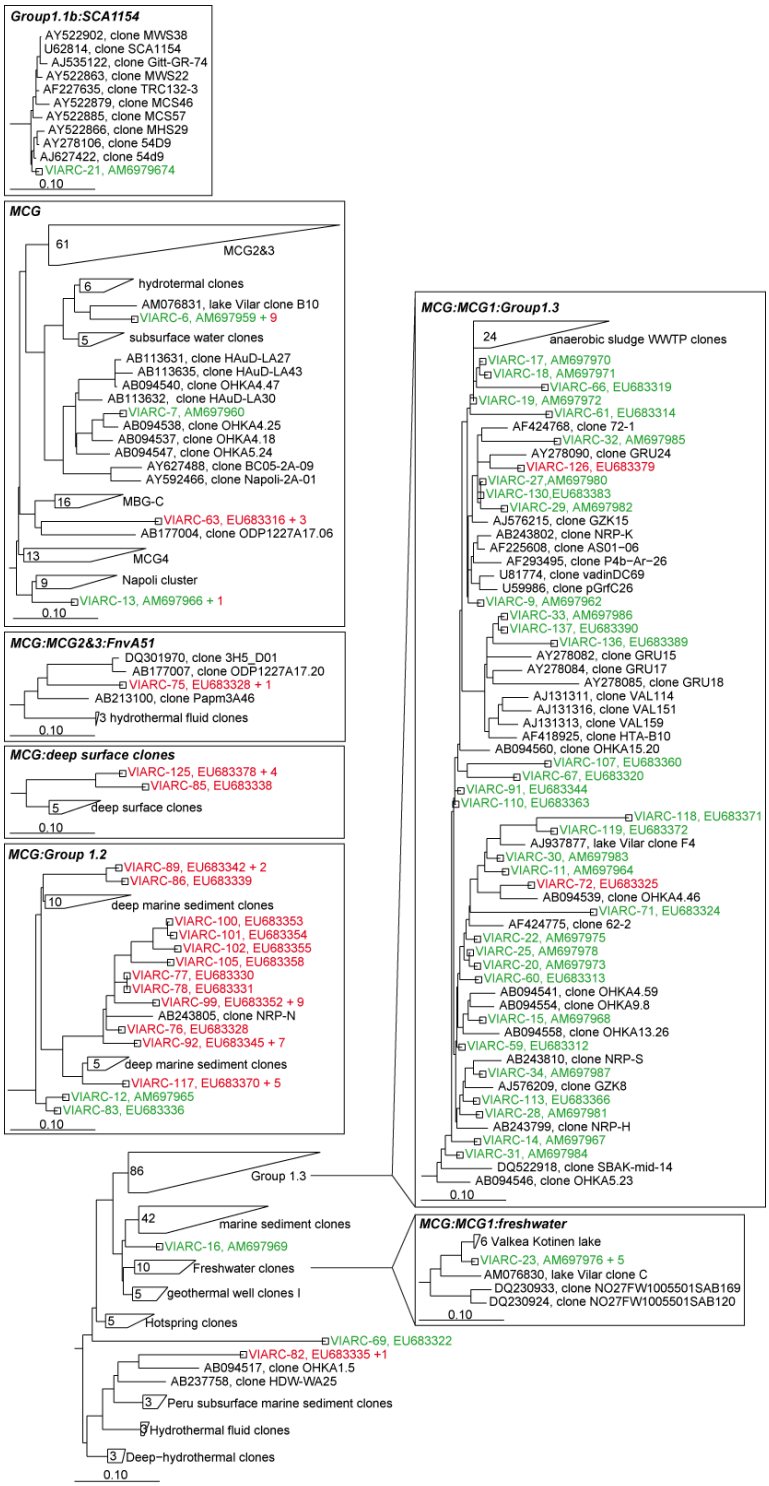


Figure 4.3. Same as Figure 4.2 but for the Kingdom *Crenarchaeota*. Sequences recovered using PAIR-2 and PAIR-3 are shown in red and green colour, respectively. When possible, only one representative sequence of each phylotype (≥98% identity) is shown together with the number of additional sequences ascribed to the same phylotype (see Table 4.3). Reference sequences are described by "clone name and accession number". The scale bar indicates 10% estimated sequence divergence.

Altogether, up to 169 16S rRNA gene archaeal sequences from the water column of Lake Vilar were recovered throughout the study period (July 2001–November 2005). The retrieved sequences spread over 14 *Crenarchaeota* (109 sequences) and six *Euryarchaeota* (60 sequences) clusters (Table 4.1). Interestingly, almost all the *Crenarchaeota*-related sequences (107 out of 109, 98%) clustered within the MCG whereas most of the euryarchaeotal sequences (48 out of 60, 80%) affiliated to clusters with sequences from hydrothermal vent environments (HV-1, DHVE-3 and DHVE-6). Accordingly, MCG and hydrothermal vent clusters contributed to 92% of the detected archaeal richness in the lake. The remaining sequences (two *Crenarchaeota* and 12 *Euryarchaeota*) distributed into five less abundant taxa, three of them (SAGMEG, pMC1 and WSA-2) belonging to the *Euryarchaeota* (Table 4.1). Interestingly, most of the sequences (34 *Crenarchaeota* and 15 *Euryarchaeota*) were recovered from anoxic layers during summer stratification (Fig. 4.4). In turn, phylotypes retrieved during winter mixing were less abundant (three *Crenarchaeota* and one *Euryarchaeota*). Only phylotypes related to the *Euryarchaeota* were found during the entire study period (11% of the total *Euryarchaeota* found during both winter mixing and under stratification conditions).

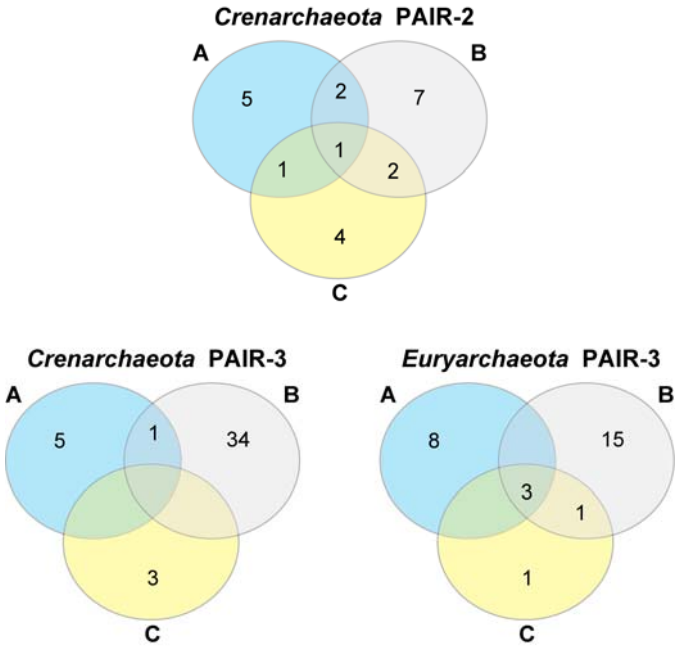


Figure 4.4. Venn diagram representation of total phylotypes affiliated to *Crenarchaeota* and *Euryarchaeota* retrieved using different primer combinations from specific water compartments of Lake Vilar during the study period. A, phylotypes exclusively found in oxic water layers during summer stratification; B, same as A but for anoxic water layers; C, phylotypes found during winter mixing. As an example, using PAIR-2 primer combination 5, 7 and 4 phylotypes belonging to *Crenarchaeota* were unique in oxic, anoxic, and whole water column, respectively.

Table 4.1. Archaeal sequences retrieved from the water column of Lake Vilar during the study period (July 2001–November 2005) using the different PCR primer pairs

	Primer combination[a]			Total Seq.	% of Total	% within Kingdom
	PAIR-1	PAIR-2	PAIR-3			
Euryarchaeota						
Hydrotermal Vent Cluster						
HV-1	2	0	7	9	5.3	15
DHVE-3	0	0	38	38	22.5	63
DHVE-6	0	0	1	1	0.6	2
SAGMEG	0	0	7	7	4.1	12
pMC1	0	0	4	4	2.4	6.
WSA-2	0	0	1	1	0.6	2
Subtotal	2	0	58	60	35.5	100
Crenarchaeota						
Miscellaneous Crenarchaeota Group[b]						
MCG:MCG1	0	0	1	1	0.6	1
MCG:MCG1:Group1.3	0	2	33	35	20.7	32
MCG:MCG1:MS	0	0	1	1	0.6	1
MCG:MCG1:FW	0	2	4	6	3.5	5
MCG:MCG1:GHT	0	2	0	2	1.2	2
MCG:MCG2&3	0	1	1	2	1.2	2
MCG:MCG2&3:GHT	0	9	1	10	5.9	9
MCG:MCG2&3:SSM	0	0	1	1	0.6	1
MCG:MCG2&3:MBG-C	0	4	0	4	2.4	4
MCG:MCG2&3:FnvA51	0	2	0	2	1.2	2
MCG:MCG5:DMS	0	6	0	6	3.5	5
MCG:MCG6:Group1.2	0	35	2	37	21.9	34
Soil1.1b:SCA1154	0	0	1	1	0.6	1
Other	0	0	1	1	0.6	1
Subtotal	0	63	46	109	64.5	100
Total	2	63	104	169	100.0	

[a]: For primer pair details and PCR conditions see Table 3.2 and Materials and Methods section.

[b]: Clusters are named using codes in ARB database, when available. For unnamed clusters, a code indicating the main environment from which cluster sequences were retrieved has been used, as follows: MS: marine sediments; FW: freshwater; GHT: geohydrothermal; SSM: subsurface marine; DMS: deep marine surface.

Besides, when the number of sequences *vs.* the taxon was plotted (Fig. 4.5), we obtained a curve similar to that reported previously to illustrate the large microbial biodiversity present in any natural environment (Pedrós-Alió 2006). The sequence *vs.* taxon distribution indicated that some taxa were more abundant and stable through seasons, while others constituted occasional taxa that were only present in certain periods (Magurran & Henderson 2003; Pedrós-Alió 2006, 2007).

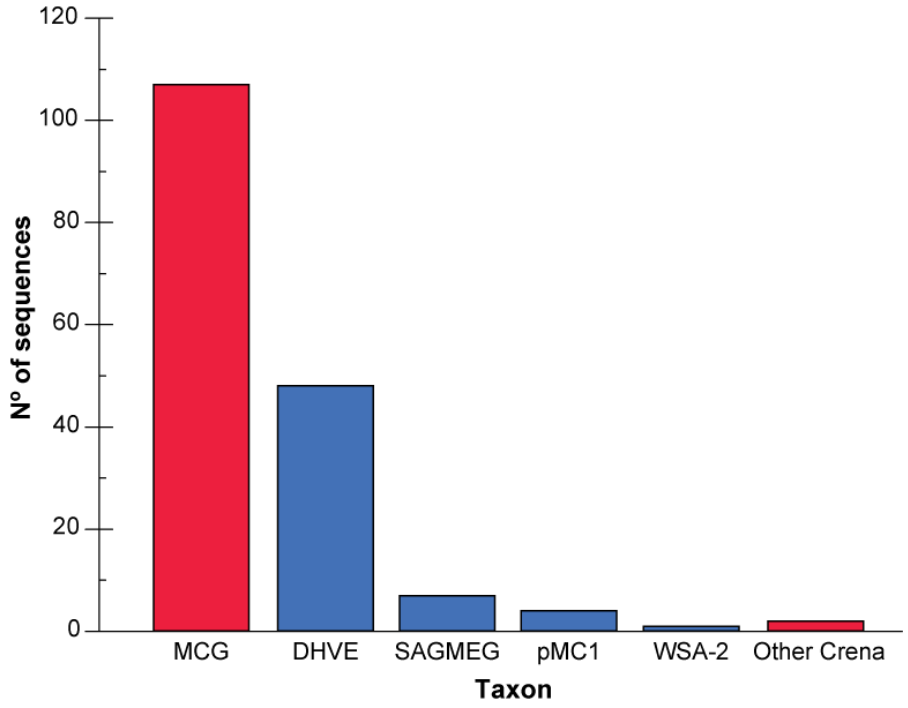


Figure 4.5. Distribution of the total number of sequences retrieved from the water column of Lake Vilar *vs.* Taxon, with taxa ranked according to their respective sequence abundance. *Crenarchaeota* and *Euryarchaeota* clusters are shown in red and blue, respectively. All sequences affiliated to MCG subclusters (see text) and hydrothermal vent clusters (HV-1, DHVE-3 and DHVE-6) have been grouped together.

Despite these apparent differences in richness distribution and seasonality, no clear correlations were obtained when multivariate statistical analyses (using the CANOCO software package; Biometris-Plant research International, The Netherlands) were performed comparing the retrieved archaeal phylotypes with the prevalent physico-chemical conditions (i.e., temperature, conductivity, and both oxygen and sulfide concentrations) at the date of sampling.

Table 4.2. List of *Euryarchaeota* partial 16S rRNA sequences from Lake Vilar used to construct Figure 4.2.. Sequences are listed by phylogenetic group, name of the sequence and accession number.

Cluster	Sequence		accession number
DHVE-3			
	VIARC-148		EU683401
		VIARC-156	EU683409
		VIARC-164	EU683417
		VIARC-174	EU683427
	VIARC-38		AM697991
		VIARC-37	AM697990
		VIARC-147	EU683400
	VIARC-152		EU683405
	VIARC-43		AM697996
	VIARC-47		AM698000
		VIARC-162	EU683415
		VIARC-166	EU683419
	VIARC-139		EU683392
	VIARC-49		AM698002
		VIARC-36	AM697989
		VIARC-46	AM697999
		VIARC-48	AM698001
		VIARC-157	EU683410
		VIARC-163	EU683416
		VIARC-167	EU683420
		VIARC-168	EU683421
		VIARC-169	EU683422
		VIARC-170	EU683423
		VIARC-140	EU683393
		VIARC-143	EU683396
		VIARC-149	EU683402
	VIARC-142		EU683395
	VIARC-141		EU683394
	VIARC-171		EU683424
	VIARC-172		EU683425
	VIARC-150		EU683403
	VIARC-160		EU683413
	VIARC-151		EU683404
	VIARC-176		EU683429
	VIARC-42		AM697995
	VIARC-175		EU683438
	VIARC-53		AM698006
	VIARC-55		AM698008
HV-1			

	VIARC-41		AM697994
		VIARC-39	AM697992
		VIARC-44	AM697997
		VIARC-155	EU683408
		VIARC-56	AM698009
		VIARC-173	EU683426
	VIARC-144		EU683397
	VIARC-153		EU683406
		VIARC-154	EU683407
SAGMEG			
	VIARC-146		EU683399
		VIARC-35	AM697988
		VIARC-40	AM697995
		VIARC-138	EU683391
		VIARC-50	AM698003
		VIARC-51	AM698004
		VIARC-52	AM698005
pMC1			
	VIARC-158		EU683411
	VIARC-159		EU683412
	VIARC-161		EU683414
	VIARC-165		EU683418
DHVE-6			
	VIARC-45		AM697998
WSA-2			
	VIARC-54		AM698007

Table 4.3. List of *Crenarchaeota* partial 16S rRNA sequences from Lake Vilar used to construct Figure 4.3.. Sequences are listed by phylogenetic group, name of the sequence and accession number.

Cluster	Sequence	accession number
MCG1		
	VIARC-148	EU683401
MCG1:group 1.3		
	VIARC-17	AM697970
	VIARC-18	AM697971
	VIARC-66	EU683319
	VIARC-19	AM697972
	VIARC-61	EU683314
	VIARC-32	AM697985
	VIARC-126	EU683379
	VIARC-27	AM697980
	VIARC-130	EU683383
	VIARC-29	AM697982
	VIARC-9	AM697962
	VIARC-33	AM697986
	VIARC-137	EU683390
	VIARC-136	EU683389
	VIARC-107	EU683360
	VIARC-67	EU683320
	VIARC-91	EU683344
	VIARC-110	EU683363
	VIARC-118	EU683371
	VIARC-119	EU683372
	VIARC-30	AM697983
	VIARC-11	AM697964
	VIARC-72	EU683325
	VIARC-71	EU683324
	VIARC-22	AM697975
	VIARC-25	AM697978
	VIARC-20	AM697973
	VIARC-60	EU683313
	VIARC-15	AM697968
	VIARC-59	EU683312
	VIARC-34	AM697987
	VIARC-113	EU683366
	VIARC-28	AM697981
	VIARC-14	AM697967
	VIARC-31	AM697984
MCG1:MS		
	VIARC-16	AM697969

MCG1:GHT			
	VIARC-82		EU683335
		VIARC-81	EU683334
MCG1:FW			
	VIARC-23		AM697976
		VIARC-10	AM697963
		VIARC-24	AM697977
		VIARC-26	AM697979
		VIARC-104	EU683357
		VIARC-112	EU683365
MCG2&3:Napoli			
	VIARC-13		AM697966
		VIARC-90	EU683343
MCG2&3:GHT			
	VIARC-6		AM697959
		VIARC-65	EU683318
		VIARC-73	EU683326
		VIARC-74	EU683327
		VIARC-79	EU683332
		VIARC-80	EU683333
		VIARC-84	EU683337
		VIARC-120	EU683373
		VIARC-121	EU683374
		VIARC-122	EU683375
MCG2&3:SSM			
	VIARC-7		AM697960
MCG2&3:MBG-C			
	VIARC-63		EU683316
		VIARC-64	EU683317
		VIARC-68	EU683321
		VIARC-106	EU683359
MCG2&3:FnvA51			
	VIARC-75		EU683328
		VIARC-111	EU68683364
MCG5:DMS			
	VIARC-125		EU683378
		VIARC-57	EU683310
		VIARC-58	EU683311
		VIARC-123	EU683376
		VIARC-124	EU683377
	VIARC-85		EU683338
MCG6:group1.2			
	VIARC-89		EU683342
		VIARC-87	EU683340
		VIARC-88	EU683341

	VIARC-86		EU683339
	VIARC-100		EU683353
	VIARC-101		EU683354
	VIARC-102		EU683355
	VIARC-105		EU683358
	VIARC-77		EU683330
	VIARC-78		EU683331
	VIARC-99		EU683352
		VIARC-95	EU683348
		VIARC-96	EU683349
		VIARC-97	EU683350
		VIARC-103	EU683356
		VIARC-108	EU683361
		VIARC-109	EU683362
		VIARC-127	EU683380
		VIARC-133	EU683386
		VIARC-134	EU683387
	VIARC-76		EU683328
	VIARC-92		EU683345
		VIARC-70	EU683323
		VIARC-93	EU683346
		VIARC-94	EU683347
		VIARC-98	EU683351
		VIARC-128	EU683381
		VIARC-131	EU683384
		VIARC-132	EU683385
	VIARC-117		EU683370
		VIARC-114	EU683367
		VIARC-115	EU683368
		VIARC-116	EU683369
		VIARC-129	EU683382
		VIARC-135	EU683388
	VIARC-12		AM697965
	VIARC-83		EU683336
Soil1.1b			
	VIARC-21		AM697974
Other			
	VIARC-8		AM697961

4.3. Discussion

Selective recovery of archaeal phylotypes

The application of different specific primer combinations revealed unexpected archaeal richness in the water column of Lake Vilar, beyond the reach of single general archaeal primers. These results were obtained after extensive nested PCR and DGGE runs to obtain comparable archaeal fingerprints between sampling dates and depths. Previous studies performed in Lake Vilar used direct amplification with universal archaeal primers in the DGGE (Casamayor et al 2001b). Probably there was lower abundance of archaea during the present study than in the study carried out in 1996. Although the application of nested-PCR on environmental samples has some bias in priming the selectivity and specificity of internal primers used (Suzuki et al 1998; Mahmood et al 2006), it offers some advantages such as characterization of nondominant phylotypes (Benlloch et al 2002, Pedrós-Alió 2006). Besides, the use of newly designed primers as well as the systematic use of distinct primer combinations (targeting general or specific groups) may increase chances to recover the maximal microbial biodiversity within a given environment (Teske & Sorensen, 2008). Despite the previously mentioned limitations of primer ARC337f (see Material and Methods), its use in nested reactions in combination with ARC915r appeared to be a valid way to enhance the detection of freshwater phylotypes of archaea. When compared with PAIR-2 (Cren28F/Cren457r), PAIR-3 (ARC337f/ARC915r) showed a higher efficiency in recovering sequences from the MCG Group 1.3 (2 *vs.* 33, respectively), a cluster mainly dominated by phylotypes retrieved from freshwater environments, but the opposite was obtained for MCG Group 1.2 (35 *vs.* 2, respectively), a cluster grouping marine phylotypes. PAIR-2, however, recovered most of the crenarchaeotal phylotypes (58% of the total *Crenarchaeota* sequences obtained) probably because of the high priming specificity towards Kingdom *Crenarchaeota* (Schleper et al 1997). It is of interest to note that all sequences recovered by PAIR-2 affiliated to MCG clusters related to marine environments, whereas the sequences clustering into MCG:MCG4:Deep Marine Sediments; MCG:MCG2&3:Marine Benthic Group C, and MCG:MCG2&3:FnVA51 were not efficiently detected by PAIR-3 (Table 4.1). These results suggest that both primer combinations showed certain selective specificity, PAIR-3 toward lacustrine- and PAIR-2 towards marine *Crenarchaeota*. The bias of this latter primer combination, originally designed to detect *Crenarchaeota* 16S rRNA gene signatures in anoxic freshwater-lake sediments, probably arise from the sequences set used for primer design (Teske & Sorensen 2008) although no detailed information about their origin has been provided so far (Schleper et al 1997). At any rate,

the large set of *Crenarchaeota* sequences recovered in our study may be useful to redesign primer ARC337f carefully to improve its specificity towards lacustrine *Crenarchaeota*.

Richness and structure of the planktonic archaeal assemblage

The mesophilic archaea have been reported to be present in the planktonic prokaryotic assemblage of different stratified freshwater lakes worldwide, for example Lake Saelenvannet (Ovreas et al 1997), Lake Cadagno (Bosshar et al 2000), Mono Lake (Humayoun et al 2003), Mariager Fjord (Ramsing et al 1996; Teske et al 1996), and Lake Pavin (Lehours et al 2005, 2007). Previous studies carried out in Lake Vilar with samples collected in 1996 also detected planktonic archaea in the anoxic, sulfide-rich monimolimnion (Casamayor et al 2001b). In that year's survey, up to five archaeal phylotypes were detected grouped within the DHVE-6 cluster (VIARC-1 and VIARC-5, originally assigned to Thermoplasmales) and the methanogenic M1 cluster (VIARC-3 and VIARC-4). Interestingly, the kingdom *Crenarchaeota* was represented by a unique phylotype (VIARC-2, belonging to the MCG:MCG2&3), which showed a marked seasonal dynamics, with the maximal relative abundance in winter. After comparing this community structure with that found during the current study, we may draw as a first conclusion that the planktonic archaeal community in Lake Vilar has experienced a drastic change in both richness and relative abundance. The fact that we were able to detect archaeal 16S rRNA gene signatures only after nested amplifications suggested a low abundance of archaea in the lake during the period 2001-2005, a very different situation with regard to the past (Casamayor et al 2000, 2001b). Unfortunately, microscopic counts using the universal archaeal probe 915 (Stahl & Amann 1991) by FISH and catalyzed reporter deposition (CARD)-FISH failed even after several attempts and modifications on the original protocol. It is rather difficult, however, to ascertain the causes that led to this low abundance, especially without a detailed knowledge of the physiological requirements and metabolic capabilities of the different archaeal lineages in nonextreme environments. Recent studies demonstrate that Archaea, and especially *Crenarchaeota*, are able to act either as chemolithotrophs using ammonia (Francis et al 2005, 2007; Könneke et al 2005; Coolen et al 2007; Beman et al 2008) or other reduced inorganic compounds (Auguet et al 2008) as an energy source, or as chemoorganotrophs using simple (Ouverney & Furhman, 1999; Herndl et al 2005; Teira et al 2006) or complex organic matter (Biddle et al 2006). All these putative metabolisms can be present or dominant in Lake Vilar at certain depths and dates considering the vertical physico-chemical gradients, their seasonal variations and the microniche formation derived. However, the low abundance of archaea in the lake suggests either that the detected archaeal phylotypes are not well adapted to the prevalent conditions or that their growth rates are too low to favor accumulation and blooming of any dominant phylotype in the changing environment imposed by the lake dynamics. In

fact, long-term enrichment cultures inoculated with water from Lake Vilar support the idea of a slow growth even under controlled incubation conditions (Plasencia A & Borrego CM, unpublished results). In addition, the new seasonal dynamics of Lake Vilar, switching from anoxia to complete oxygenation, may limit the development of higher archaeal abundances. It is also interesting to note that phylotypes related to known ammonia-oxidizing archaea were not found during the study, indicating that the conditions of Lake Vilar, with high eutrophy and an active sulfur cycle (Guerrero et al 1980, 1985; Bañeras & Garcia-Gil 1996), were far from those prevailing in environments where these nitrifying archaea are usually found, i.e. oligotrophic marine waters (Francis et al 2005, 2007; Nicol & Schleper 2006; Coolen et al 2007; Beman et al 2008; Varela et al 2008).

Two final remarks concerning the dominance of phylotypes related to the MCG and DHVE in Lake Vilar deserve some discussion. First, the large amount of sequences recovered within these groups is another evidence of its wide habitat and strongly supports the need of a careful revision of their phylogeny as suggested previously (Teske & Sorensen 2008). Second, despite the fact that MCG- and DHVE-related populations probably did not reach high cell abundances, their long-term persistence throughout the seasons suggests that they constituted an archaeal seed-bank with low population losses waiting for better conditions to growth. Unfortunately, few data are available to envisage what these better conditions would be. The members of the MCG Archaea have been considered as heterotrophic anaerobes based on their capability to take up organic carbon in buried sediments (Biddle et al 2006). The fact that most of the MCG phylotypes were recovered in the eutrophic anoxic hypolimnion of Lake Vilar agrees with this putative heterotrophic metabolism. However, the cosmopolitan distribution of MCG and their complex phylogeny suggests a metabolic diversity larger than previously assumed, probably ranging from mixo- and heterotrophy to chemolithoautotrophy. The same applies for members of the DHVE because although they were originally retrieved from hydrothermal vents (Takai & Horikoshi 1999), now the cluster has been expanded with clones from cold and terrestrial environments (Takai et al 2001a). The largest fraction of DHVE was retrieved from the anoxic water layers of lake Vilar, where reduced organic and inorganic compounds (e.g. ammonia, sulfide, H_2, etc.) accumulate, providing different energy sources for either chemolitho- (as in hydrothermal habitats) or chemoorganotrophic metabolisms. The recent maintenance of several DHVE and MCG phylotypes from Lake Vilar in long-term enrichment cultures incubated under anoxic conditions and with CO_2 as the sole carbon source (Plasencia A & Borrego CM, unpublished results) suggests that chemolithoautotrophic members of these clusters may be important components in the hypolimnetic planktonic archaeal assemblage of the lake. As stated recently by different authors (Giovannoni & Stingl 2007; Nichols 2007; Donachie et al 2008), molecular environmental surveys must be synergistically

complemented by culture-dependent techniques to properly interpret molecular data in a more ecological context. This is especially true for the study of archaea inhabiting non-extreme environments, because only after isolates are studied, will we succeed in the ecophysiological interpretation of environmental 16S rRNA gene data.

5. Segregation of archaeal populations along the epipelagic waters of the stratified oligotrophic freshwater Lake Kivu (East Africa)

5.1 Introduction

Lake Kivu is a meromictic lake located in a volcanic region between Rwanda and the Kivu Province (Democratic Republic of Congo) and is the smallest of the African Great Rift Lakes. Kivu contains a large amount of dissolved CO_2 and methane in the monimolimnion as a result of geological and biological activity (Tietze et al 1980; Schöell et al 1988). The accumulation of methane converts Lake Kivu in one of the largest methane reservoirs in the world and in a unique ecosystem for geomicrobiologists interested on the methane cycle, risk assessment and management (Deuser et al 1973; Jannasch 1975; Tietze et al 1980; Schöell et al 1988; Schmid et al 2002, 2005; Tassi et al 2009). Besides, comprehensive studies on the diversity and activity of planktonic populations of both large and small eukaryotes and their trophic interplay operating in the lake are available (de Iongh et al 1983; Kilham et al 1986; Marshall 1991; Kaningini et al 2003; Isumbisho et al 2004, 2006). In clear contrast, very few data exist on the composition, diversity and spatial distribution of microbial communities although recent studies have provided information on seasonal variations on photosynthetic and heterotrophic picoplankton (Sarmento et al 2006, 2008). For *Archaea*, all the studies conducted so far have been focused on the occurrence and activity of methanogens (Deuser et al 1973; Schöell et al 1988) and methanotrophs (Jannasch 1975) but no one have addressed the presence of other archaeal populations.

Molecular surveys conducted in aquatic and terrestrial habitats during the last two decades have revealed that mesophilic archaea are ubiquitous and abundant (DeLong 1998; Chaban et al 2006; Casamayor & Borrego 2009). Besides, the discovery of genes encoding enzymes related to nitrification and denitrification in archaeal metagenomes from soil and marine waters (Venter et al 2004; Treusch et al 2005; Hallam et al 2006) and the isolation of the first archaea able to fix CO_2 using ammonia as energy source (Könneke et al 2005) have provided evidences that some lineages of mesophilic archaea may have a large impact on global cycles of carbon and nitrogen (Schleper et al 2005; Nicol & Schleper 2006). In fact, Ammonia Oxidizing *Archaea* (AOA) of the *Crenarchaeota Marine Group I.1a* have consistently been found as the dominant phylotypes both in the deep water masses of several oceanic regions (Herndl et al 2005; Herfort et al 2007; Varela et al 2008; Beman et al 2008; Galand et al 2009) and in the metalimnetic suboxic layers of several stratified marine environments (Lin et al 2006; Coolen et al 2007). Although less information is available for freshwater habitats, recent studies carried out in oligotrophic high-mountain lakes showed an important contribution

of AOA both in the planktonic and in the neustonic microbial assemblages (Urbach et al 2001, 2007; Auguet & Casamayor 2008).

The peculiar physico-chemistry of Lake Kivu composes a unique environment mainly dominated by the methane cycle. The main goal of this study was to ascertain if archaeal populations, other than methanogenic lineages, were relevant components of the planktonic microbial community. Results have shown that the archaeal assemblage of the lake is richer than previously expected and that methanogenic archaea are not among the most common phylotypes in the epipelagic waters. In turn, the recovery of phylotypes related to marine and terrestrial archaeal ammonia oxidizers in oxic and suboxic water layers suggest a direct role of these microorganisms in nitrification processes occurring at these depths and provide some clues that will allow a better understanding of the processes and players beneath the carbon and nitrogen cycles operating in the lake.

5.2. Results

Physico-chemical profiles

The physico-chemical depth profiles of the four sampled stations showed the characteristic downward stepwise oxic and thermal stratification patterns for the rainy season (Figure 5.1). The low mixing conditions during this season allowed the establishment of a temporary stratification in the mixolimnion, which expands from the surface to 35–65 meters depth depending on the station. Below 65 m depth the permanent anoxic waters expand to the bottom of the lake (489 m at its maximal depth). Two well defined oxycline patterns can be clearly distinguished between basins located at the open lake area (Northern (NB) and Eastern (EB)) and those located at the southern, more wind-protected, side of the lake (Southern Basin (SB) and Bukavu Bay (BB)). Whereas "open" basins (NB and EB) showed a steep oxycline between 30 and 40 m depth, the oxygen concentration profiles in SB and BB decreased more smoothly, with an oxycline extending from 10 to 50–60 m depth. These differences in oxygen profiles are consistent with chemical differences measured in the water masses of basins located at the Main lake basin and those placed at the southern side (Tassi et al 2009). The fact that deep waters of BB are isolated from SB by the presence of a shallow sill (Fig. 3.1 of Material and Methods section) and that the maximum depth of the bay is around 100 m lead to fewer gas accumulation in deep waters than in other basins. Water layers with oxygen

concentrations < 2 mg l^{-1} were considered as suboxic and defined as transition zone throughout the text.

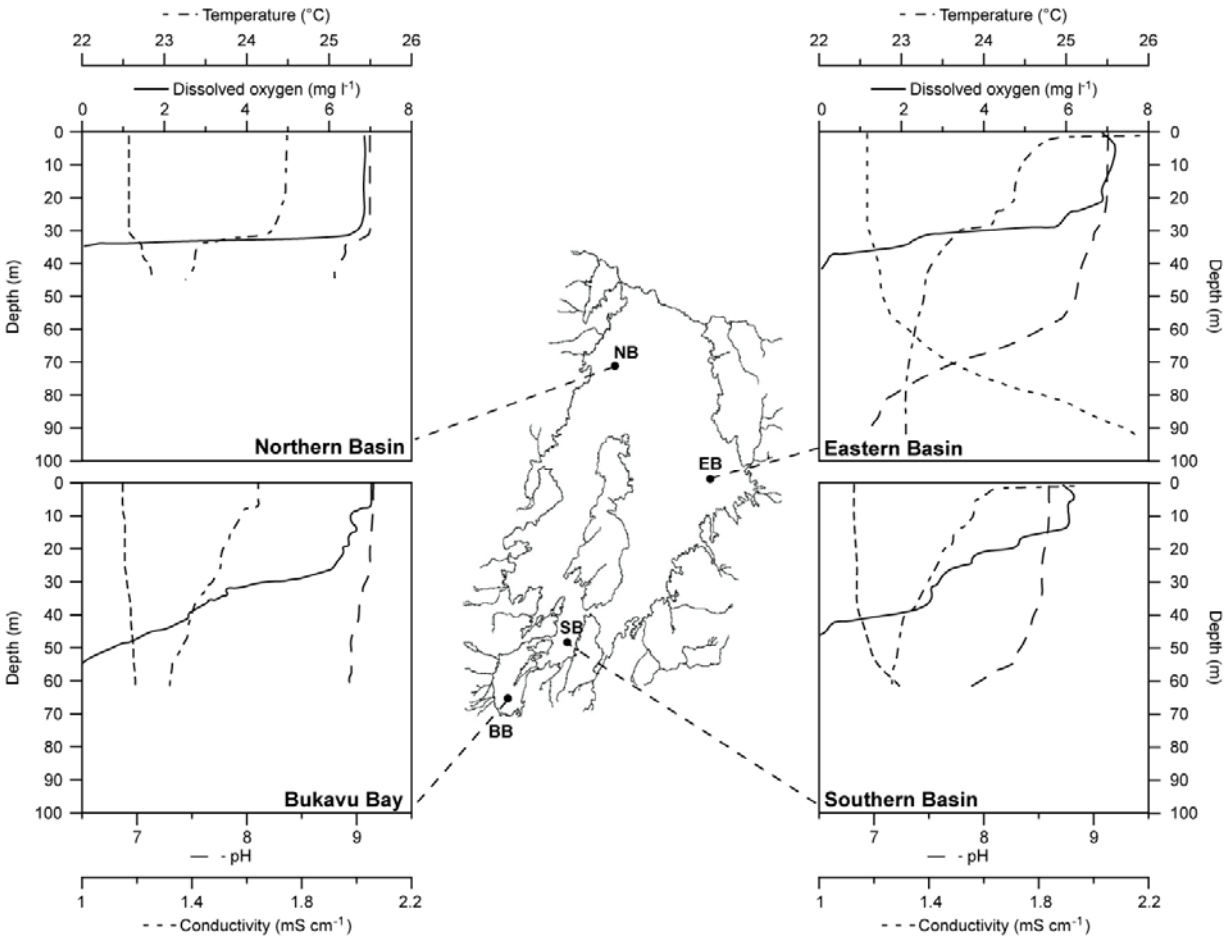


Figure 5.1. Depth profiles of temperature, conductivity, dissolved oxygen, and pH in the different basins at the day of sampling.

Bacterial and Archaeal abundance

The total cell numbers determined by DAPI staining ranged from $1.80\pm0.39\times10^5$ to $9.26\pm5.80\times10^5$ cells ml^{-1} and from $8.90\pm0.58\times10^5$ to $2.57\pm0.77\times10^6$ cells ml^{-1} in NB and BB, respectively (Fig. 5.2, right panels). These abundances are within the same range of those previously reported by Sarmento and co-workers using flow cytometry (Sarmento et al 2008). Only samples from NB and BB were collected to measure archaeal and bacterial abundances and for this reason no direct cell counts for EB and SB are available. However, we have assumed that both the distribution and cell numbers in these basins would be fairly similar to those measured for NB and BB according to: (i) the similar physico-chemical depth profiles measured for "open" basins (NB and EB) and "protected" ones (SB and BB) (Fig. 5.1); (ii) the similar distribution patterns for heterotrophic bacteria and cyanobacteria observed in the epilimnion of Northern and Eastern basins of Lake Kivu during the rainy season (Sarmento et al 2008); and (iii) the identical band patterns obtained for both archaeal 16S rRNA and *amoA* genes fingerprints from all basins (see next subsections) which suggest a very similar, if not identical, distribution of the planktonic archaeal populations in all basins.

The *Bacteria*-specific probe EUB338 and the *Archaea*-specific probe ARCH915 were used to quantitatively estimate the contribution of both domains in the microbial planktonic community throughout the water column. The sum of abundances for *Bacteria* and *Archaea* ranged from 52 to 96% and from 64 to 94% in NB and BB, respectively. Similar values have been reported for different water masses (Teira et al 2004; Herndl et al 2005; Auguet & Casamayor 2008) where the sum of different groups ranged from 40 to 102% of the total DAPI-stained cells. Recovery efficiencies lower than 100% of DAPI-stained cells might be due to low cell-permeability (Pernthaler et al 2004) or unspecific DAPI staining of a large fraction of death or non-nucleoid-containing cells (ghosts) (Zweifel & Hagström 1995). In both basins members of the Domain *Bacteria* were dominant, with relative abundances ranging from 51.4±15.8 to 95.7±3.5% and from 63.1±10.1 to 93.2±9.5% in Northern Basin and Bukavu Bay, respectively (Fig. 5.2, left panels), whereas archaeal cells ranged from 0.3±0.1 to 4.3±2.2% and from 0.6±0.1 to 4.5±1.7% in the same basins. Contrary to the bacterial distribution, the relative contribution of archaeoplankton to the total cell numbers increased with depth, reaching their maxima at the oxic-anoxic boundary layer (~40 m depth) in both sampling stations.

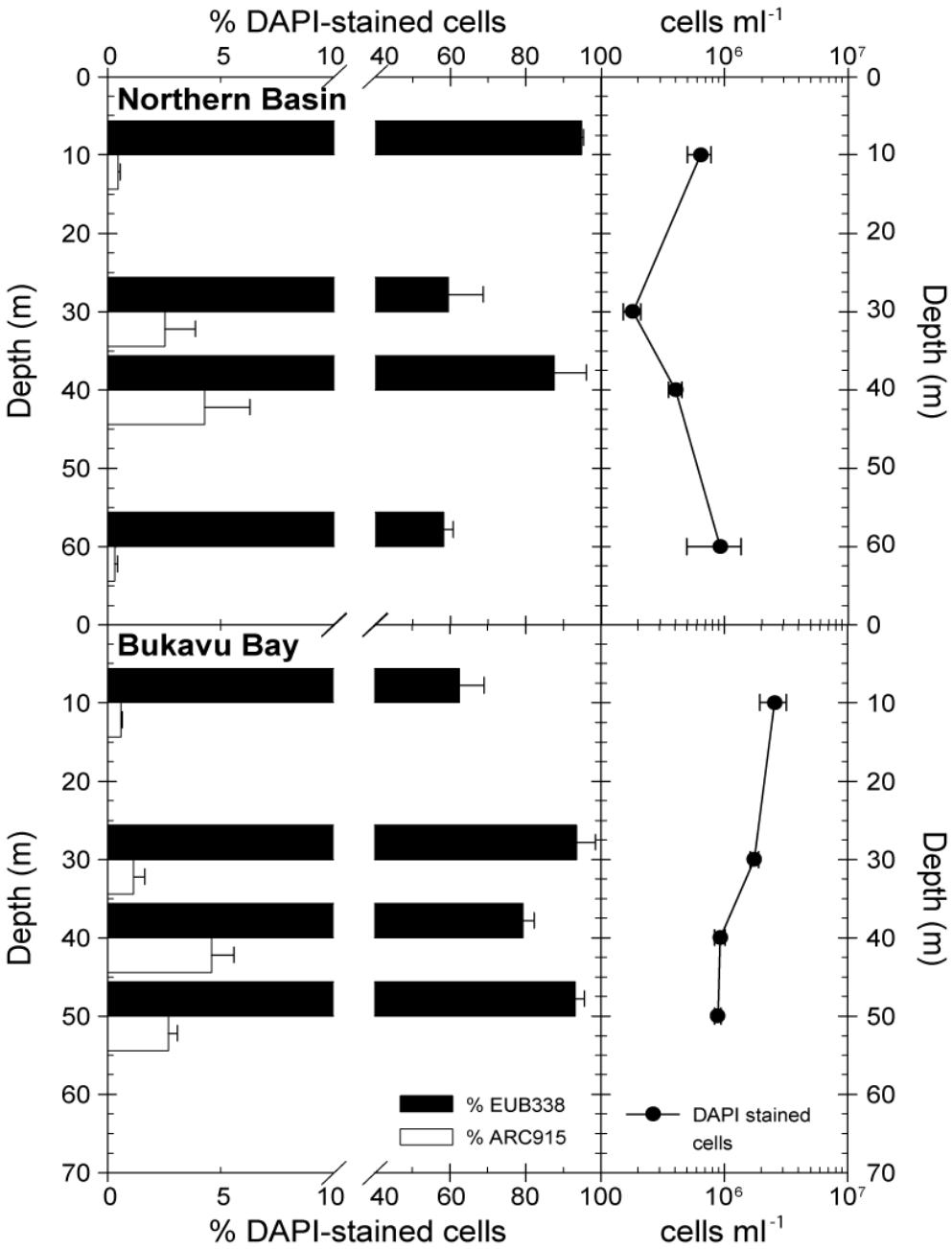


Figure 5.2. Relative abundances of *Bacteria* and *Archaea* obtained after CARD-FISH counts (left) and total cell numbers (DAPI staining, right) in Northern Basin (NB) and Bukavu Bay (BB). Error bars show the standar deviation from triplicate filters (see Material and Methods section for more information).

Phylogenetic fingerprint of the archaeal planktonic assemblage

Direct amplification of archaeal 16S rRNA gene failed in all samples tested and nested reactions were needed to obtain suitable amplicons from all sampling depths collected at the four basins to carry out further DGGE fingerprinting. The two primer combinations used allowed an independent and specific amplification of most members of the Domain *Archaea* (PAIR-1) and the Kingdom *Crenarchaeota* (PAIR-3) (Table 3.2 of Material and Methods section). The resulting DGGE fingerprints showed great similarities for all basins regardless of the primer pair used (Figs. 5.3 and 5.4). Slight differences in band migration observed in fingerprints from NB and BB compared with those from EB and SB were attributed to irregularities along the vertical denaturant gradient produced during casting the gels, affecting the migration of some bands. The use of an internal ladder for each DGGE run allowed the proper comparison of the gels using image analysis. The analyses of sequences obtained from bands at similar melting positions excised from different gels (i.e., basins) further confirmed their identity regardless of the mismatch in band position.

The analysis of the recovered sequences revealed remarkable aspects on both the structure of the planktonic archaeal assemblage and the recovery efficiency for both primers. Most of the sequences detected in all basins and sampling depths were recovered using both primer combinations. This observation suggested both that no substantial biases were introduced by the primer pair used (Table 5.1) and that the differences in the archaeal assemblage above and below the oxic-anoxic transition were consistent throughout the lake. Some bands halted at different positions in the gels resulted in almost identical sequences (≥98% similarity, ***e.g.*** ***a***K1, ***a***K2, ***a***K9 in Fig. 5.3; or ***b***K1, ***b***K2, ***b***K3 and ***b***K4 in Fig. 5.4) and hence they were ascribed to the same phylotype (Table 5.1). This anomaly has been previously observed by other authors when profiling microbial communities by DGGE and it has been related to variable melting behaviours or multiple ribosomal gene copies in a single organism (Casamayor et al 2000; Prat et al 2009).

Sequences recovered from the oxic water compartment (surface to 40 m depth) mainly affiliated within the *Crenarchaeota* Marine Group I.1a and Soil I.1b (Table 5.1, Figs. 5. 3; 5.4 and 5.5), two phylogenetic clusters grouping most of the ammonia oxidizing archaea (AOA) (Prosser & Nicol 2008). In particular, sequences affiliated to Marine Group I.1a showed sequence similarities ranging from 93 to 96% to *Candidatus* "Nitrosopumilus maritimus", the unique member of marine AOA isolated to date (Könneke et al 2005). PAIR-1 also retrieved 4 sequences related to the *Miscellaneous Crenarchaeotic Group* (MCG) and 8 sequences affiliated to *Euryarchaeota*. Most of the sequences within this latter kingdom were related to either methanogenic (2 sequences showing a 99.9%

similarity to *Methanosaeta concilii*, an acetoclastic methanogen; and 1 phylotype related to Methanobacteriales) or anaerobic methane oxidizers (3 sequences affiliated to ANME-2D cluster). The remaining 2 sequences were related to the WSA-1 cluster (Table 5.1 and Fig. 5.5). The PAIR-3 primer combination retrieved the same number and identity of AOA-related sequences than PAIR-1 (5). However, a larger recovery efficiency and phylogenetic identity was observed with sequences affiliated to non-marine crenarchaeotal clusters (the Soil Group I.1b (12), the MCG (5) and the *HotSpring* cluster (1); Table 5.1 and Fig. 5.5) indicating that PAIR-3 is biased towards lacustrine crenarchaeota (Llirós et al 2008). PAIR-3 only retrieved one sequence within the Kingdom *Euryarchaeota* that affiliated to the *Hydrothermal Vent Euryarchaeota* cluster (band *b*B9 in Fig. 5.4).

Table 5.1. Melting types of archaeal 16S rRNA gene sequences retrieved from the plankton of Lake Kivu (0–100 m depth) using the two PCR primer combinations.

	***Primer combination*[a]**		
Phylogenetic affiliation	**PAIR-1**	**PAIR-3**	***Phylotypes*[b]**
Euryarchaeota			
Methanosaeta cluster	2	0	2
ANME-2D cluster	3	0	3
WSA-1 cluster	2	0	1
Methanobacteriales cluster	1	0	1
HV1 cluster	0	1	1
SubTotal	8	1	8
Crenarchaeota			
Marine 1.1a cluster	5	5	1
Soil 1.1b cluster	6	12	17
MCG cluster	4	5	9
HotSpring cluster	0	1	1
SubTotal	15	23	29
Total	23	24	37

[a]: For primer pair details and PCR conditions see Table 3.2 and Material and Methods section.
[b]: Phylotypes were defined at 98% cut-off.

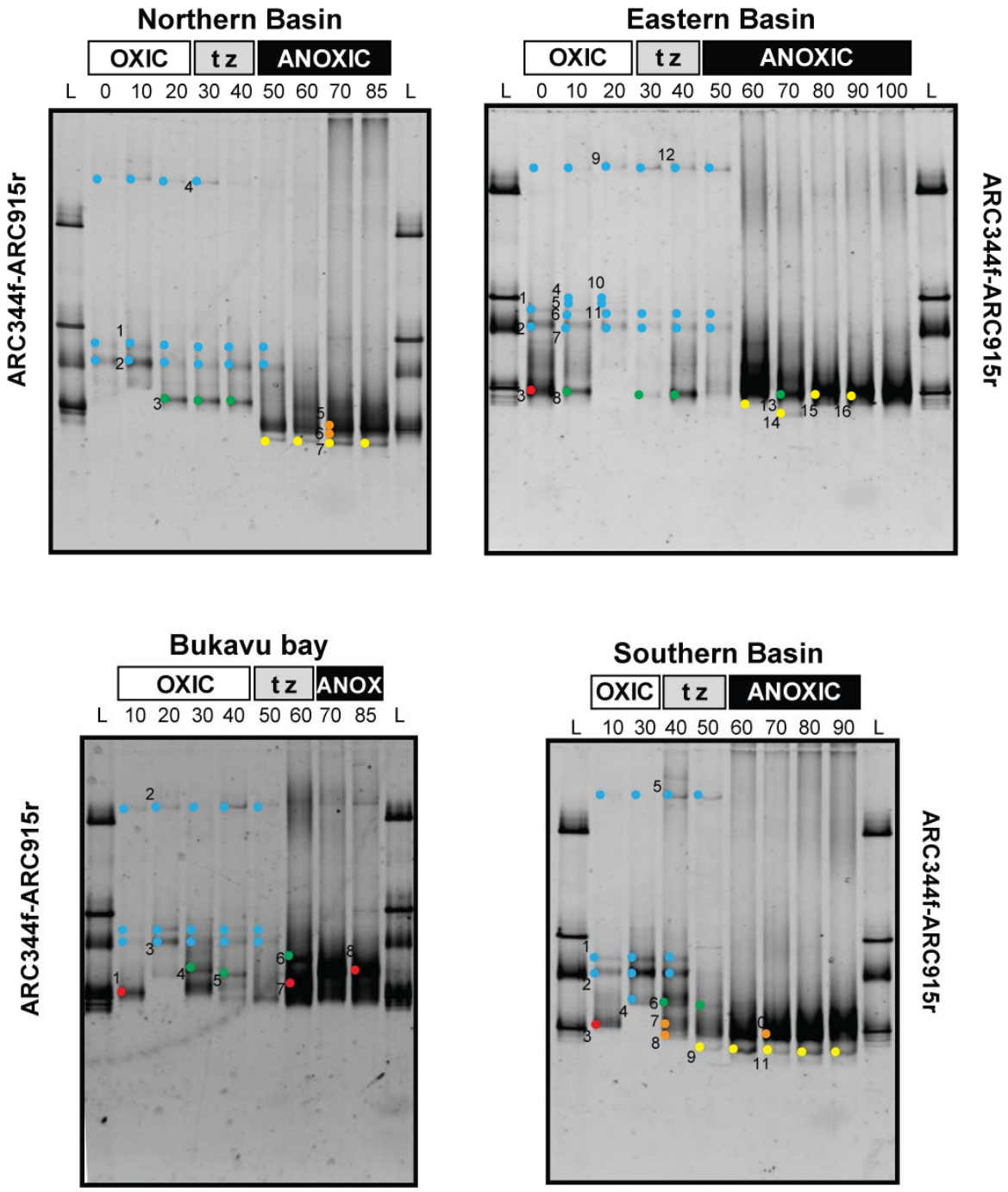


Figure 5.3. DGGE fingerprints of archaeal 16S rDNA fragments obtained from Lake Kivu samples after nested PCR amplification using PAIR-1 primer combination (ARC344f/ARC915r, Casamayor et al 2001). Only representative, and consecutively numbered, sequences from each fingerprint are shown and used for further phylogenetic analyses (Fig. 5.5). Colour codes refer to phylogenetic affiliation of the sequences obtained from representative bands of each melting type (Fig. 5.5): *Crenarchaeota* Marine Group I.1a (blue); *Crenarchaeota* Soil Group I.1b (green); Methanogenic archaea (red); ANME groups (orange); *Miscellaneous Crenarchaeotic Group* (yellow). L: DGGE ladder; tz: oxic-anoxic transition zone.

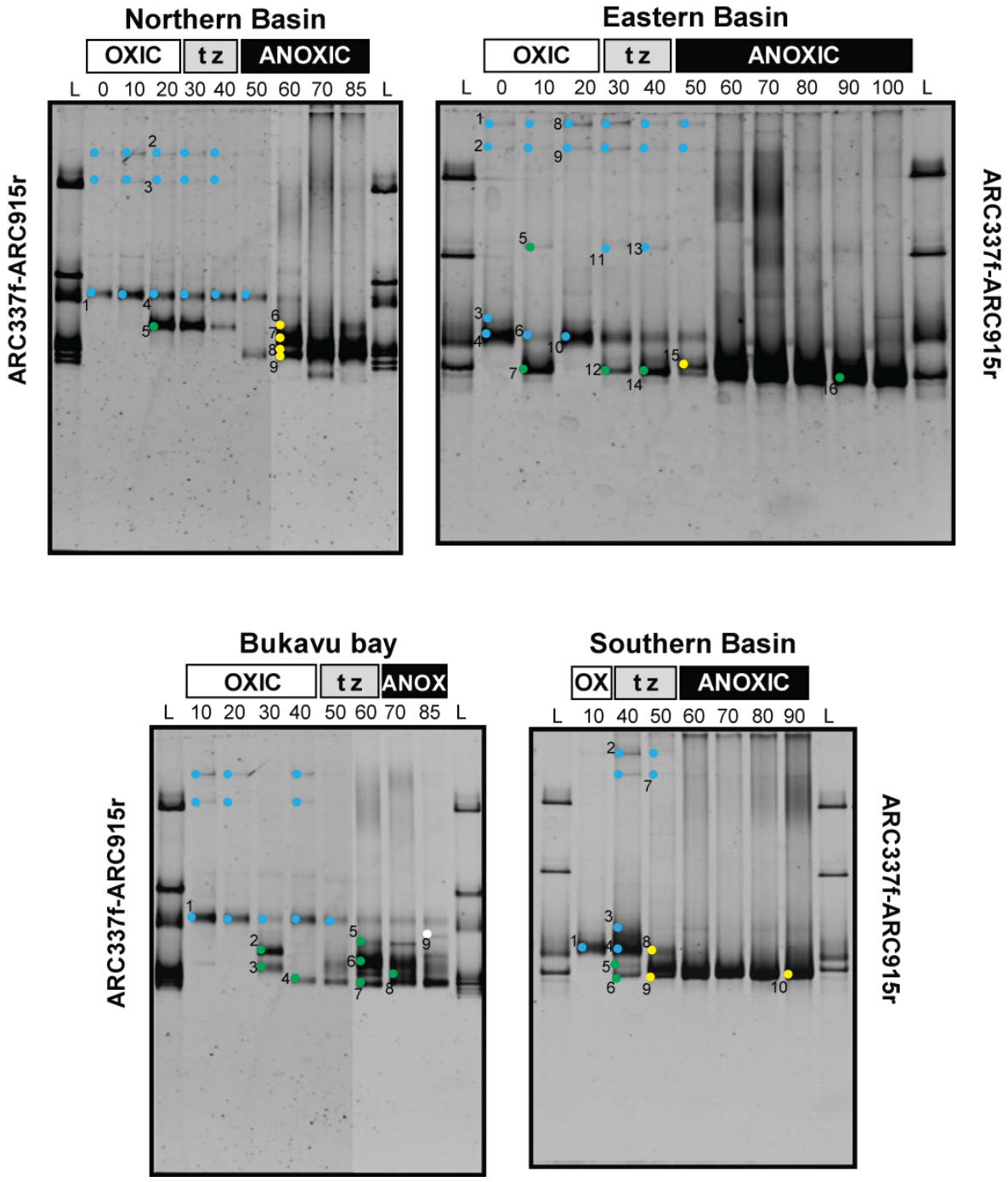


Figure 5.4. Same as Fig. 5.3 but using PAIR-3 primer combination (ARC337f/ARC915r; Llirós et al 2008). L: DGGE ladder; tz: oxic-anoxic transition zone.

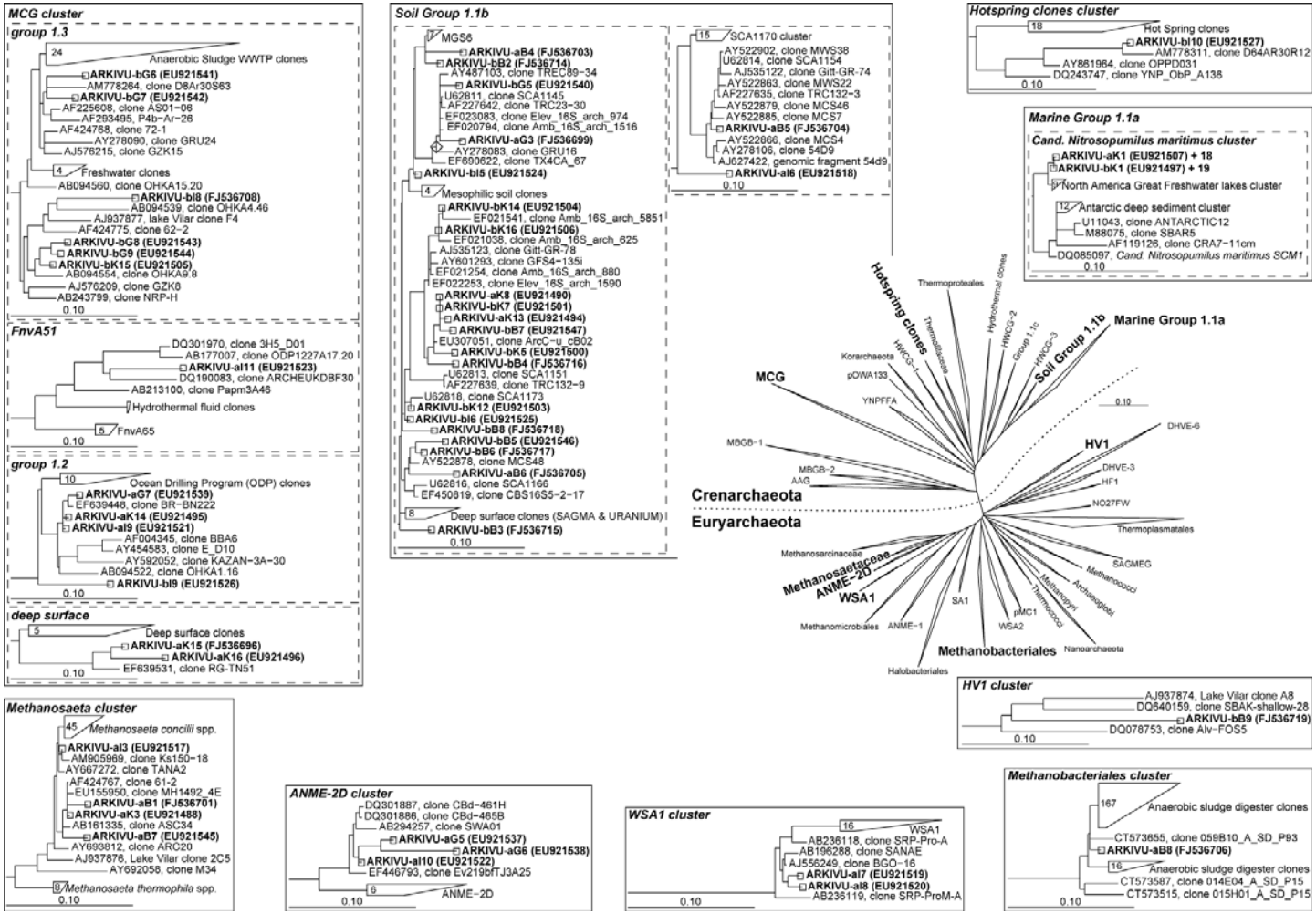


Figure 5.5. Phylogenetic tree for *Archaea* showing the affiliation of representative partial 16S rRNA gene sequences retrieved from Lake Kivu. Sequences are named according to the primer pair used (a or b for primer PAIR-1 or PAIR-3, respectively), the capital letter of the corresponding basin (K for Kibuye (Southern basin), I for Ishungu (Eastern basin), G for Goma (Northern basin), B for and Bukavu (Bukavu Bay)), and the band number from each basin (see Figs. 5.3 and 5.4). The scale bar indicates 10% estimated sequence divergence.

Fingerprint of archaeal amoA

To further explore the presence and vertical distribution of AOA in the epipelagic waters of Lake Kivu, samples were analyzed for the presence of the α-subunit of archaeal ammonia monooxygenase (*amoA*), the well-known functional marker for archaeal ammonia oxidation (Francis et al 2005; Treusch et al 2005). Positive amplification was obtained in all cases with very intense signals in samples collected at the oxic-anoxic transition (data not shown). DGGE fingerprints obtained from these amplicons showed an identical band profile for all basins, with a clear four-band pattern especially intense at those depths where distinct maxima of nitrite and nitrate were measured (Fig. 5.6). The comparison of the sequences retrieved from all *amoA* bands revealed a striking similarity between them (99.9 to 100% of sequence identity) and also a high similarity with the *amoA* gene sequence from *Candidatus* "Nitrosopumilus maritimus" (99.61% and 99.49% similarity at DNA and amino-acid level, respectively). Similar banding patterns with four highly similar *amoA* nucleotide sequences (99% similarity) were found by Herfort and co-workers when analyzing the spatial and temporal distribution of crenarchaeota in the southern North Sea (Herfort et al 2007). The phylogenetical analysis of *amoA* sequences recovered from Lake Kivu and other from public databases grouped the former in a distinct subcluster within the freshwater clade previously defined by Francis and co-workers (2005) and clearly separated from other archaeal *amoA* sequences recovered from both marine and terrestrial environments (Fig. 5.7).

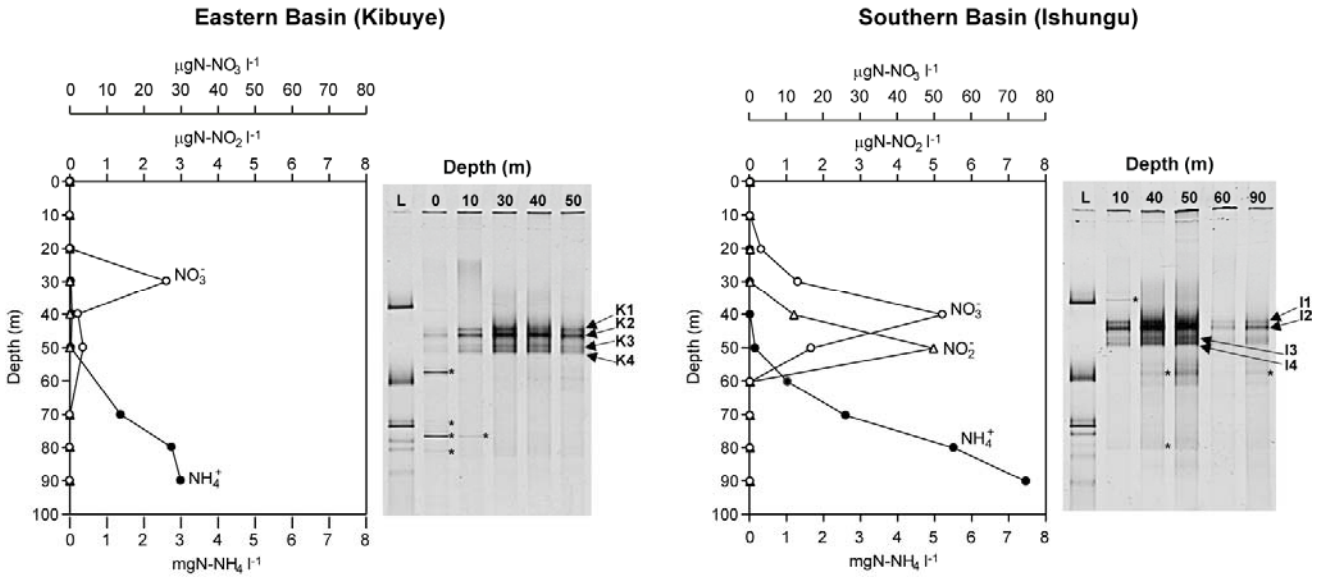


Figure 5.6. Vertical profiles of ammonium, nitrate and nitrite and DGGE fingerprints of *amoA* gene fragments obtained from water samples collected at Eastern (left panel) and Southern (right panel) basins of Lake Kivu. Identical *amoA* fingerprints were obtained with samples from NB and BB stations (data not shown).

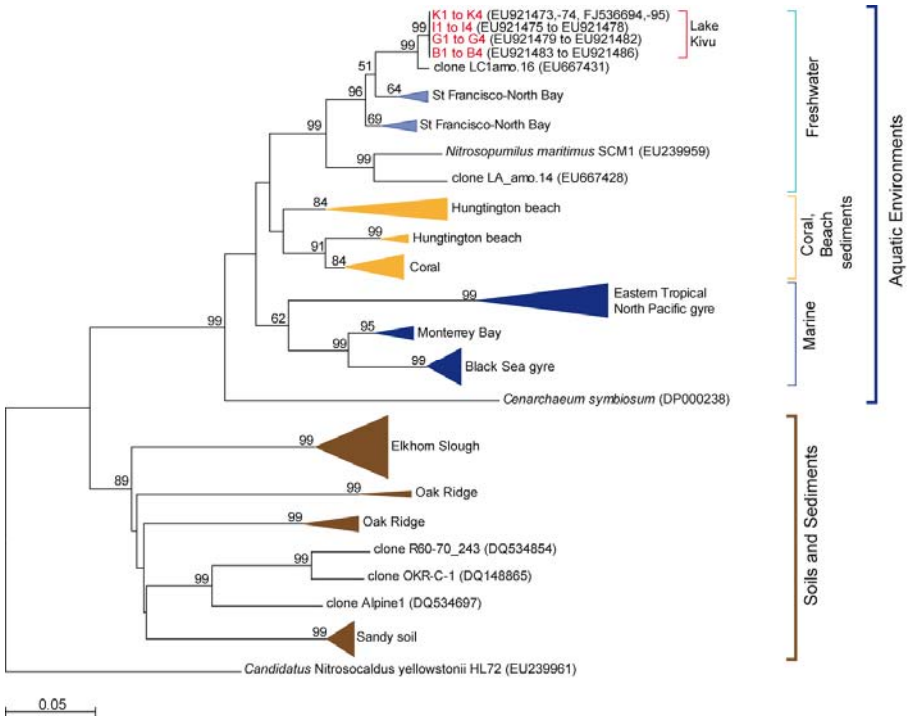


Figure 5.7. Neighbour-joining phylogenetic tree for *amoA* sequences constructed using Tamura-Nei corrected distances with a bootstrap value of 1000. Bootstrap values higher than 50% are indicated at branch points. Wedge size is proportional to sequences condensed on them. Brackets highlight environmental clusters. The scale bar indicates 5% sequence dissimilarity.

5.3 Discussion

The contribution of archaea to planktonic microbial assemblages in stratified freshwater lakes is highly variable depending on the studied ecosystem but values reported so far are usually lower than those measured for marine environments (Casamayor & Borrego 2009). In Lake Kivu the relative abundance of planktonic archaea (0.3 to 4.5% of total DAPI counts) fairly agrees with values reported for other freshwater environments. Particularly, a survey based on dot blot quantification of archaeal nucleic acids from great lakes in different continents reported that archaea accounted for a 5.9% of the total nucleic acids in the nearby located Lake Victoria and up to 10.1% in the rest of the lakes studied (Keough et al 2003). Similar archaeal abundances (between 1 and 7% of DAPI-stained cells) have been found in samples from other freshwater lakes using FISH or CARD-FISH (Pernthaler et al 1998; Jurgens et al 2000) although higher values have recently been reported for oligotrophic high mountain lakes (3 to 37%, Auguet & Casamayor 2008) and polar lakes (22%, Urbach et al 2001, 2007). In stratified marine environments such as the Cariaco Basin and the Black Sea, the archaeal planktonic fraction ranged between 1 to 9% and from 10 to 30% of total DAPI counts, respectively, peaking at the redoxcline and coinciding with depth maxima of nitrite and nitrate (Lin et al 2006; Coolen et al 2007; Lam et al 2007). Similar distribution patterns have also been observed in arctic lakes (Pouliot et al 2009). Although the archaeoplankton abundance in Lake Kivu is at the lower range of the values reported so far, the substantial increase in archaeal cell numbers at the oxic-anoxic transition zone (30–50 m depth) and the coincident vertical distribution of both the molecular signatures for AOA (16S rDNA and *amoA*) and the nitrate and nitrite maxima suggest a link between the planktonic archaea and nitrification at these depths.

The phylogenetic structure of the archaeal assemblage in Lake Kivu appears to be fairly homogeneous in all sampling basins, with a clear segregation imposed by the oxic/anoxic transition (Fig. 2.8). Whereas in oxic and suboxic water layers most of the recovered sequences affiliated with AOA lineages (both *Crenarchaeota* Marine Group I.1a and Soil Group I.1b), sequences from the anoxic water compartment were mainly related to MCG, ANME groups and methanogens. According to the high amounts of methane that accumulate in the anoxic monimolimnion, the low contribution of methanogens to the archaeal community (5.8% of the total identified phylotypes) might be surprising. The fact that all samples analyzed were collected from the upper 100 m of the lake and that active methanogenesis mainly occurs at deep water layers (Deuser et al 1973; Tietze et al 1980; Schöell et al 1988) may account for this low contribution. The five

sequences related to methanogenic euryarchaeota grouped with either acetoclastic (99.9% similarity to *Methanosaeta concilii*) or hydrogenotrophic clusters (the WSA1 cluster within the Methanomicrobiales). This affiliation fairly agrees with the biological origin of methane in Lake Kivu and with the common methanogenic archaeal groups found in other stratified lakes (Lehours et al 2007). The recovery of some sequences related to methanogens from oxygenated water layers (bands *a*K3, *a*I3 and *a*B1 in Fig. 5.3 and 5.5) is not in accordance to the strictly anaerobic metabolism assumed for these microorganisms. Notwithstanding this, the reported tolerance to oxygen of some members of the *Methanosaeta* cluster (Hirasawa et al 2008) or the occurrence of water mixing processes that transported microorganisms from the upper part of the monimolimnion to shallow depths might explain these findings. On the other hand, the recovery of three phylotypes related to anaerobic methane oxidizers (ANME-2D cluster, with similarity values ranging from 93 to 98%) and the high amounts of dissolved methane in the lake might suggest that anaerobic oxidation of methane (AOM) probably occurs in the water column of Lake Kivu although it remains unknown the relative contribution of this process to the overall methane oxidation. Although they were originally found in methane seep marine sediments, archaea involved in AOM (either from ANME-1, ANME-2 or ANME-3 clades) have also been detected in anoxic water layers of other stratified environments (Valentine 2002; Wakeham et al 2003; Durisch-Kaiser et al 2005) and, accordingly, they might be involved in methane consumption processes in Lake Kivu.

Almost all sequences recovered from oxic and suboxic water layers affiliated within *Crenarchaeota* Marine I.1a and Soil I.1b lineages, which contain almost all AOA representatives known so far (Prosser & Nicol 2008). The diversity within Marine Group 1.1a was very low (only one phylotype) but in contrast Soil Group 1.1b contained up to 17 different phylotypes (98% cut-off). In this latter case, however, only sequences grouping with clone ArcC-u-cB02 (aK8, aK13, bK5, bK7, bB4 and bB7 in Fig. 2.5) could be indirectly related to archaea involved in ammonia oxidation (Hansel et al 2008). Unfortunately, neither samples were preserved to allow analysis of *amoA* gene expression nor nitrification activities were determined during our sampling campaign. In this regard, any consideration about the potential activity of crenarchaeota in Lake Kivu as ammonia oxidizers is speculative but the good correlation found between archaeal cell counts, nitrite and nitrate maxima and the intense signals from archaeal *amoA* at suboxic water layers (30–50 m depth) is significant and agrees with results found in other stratified environments (Francis et al 2005, 2007; Lin et al 2006; Coolen et al 2007; Pouliot et al 2009).

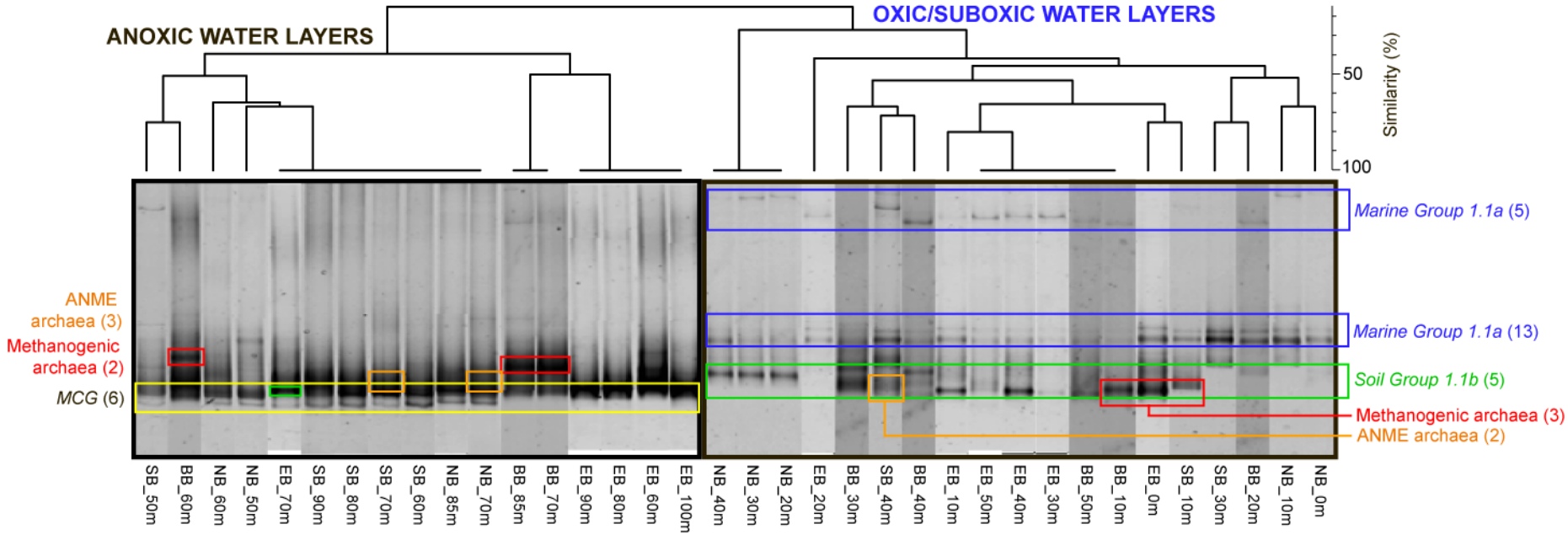


Figure 5.8. Negative image composition of SYBR Gold-stained DGGE gels of archaeal 16S rDNA gene fragments from all basins (PAIR-1 primer combination) showing the low similarity level (ca. 30%) between samples from oxic/suboxic water layers and from the anoxic water compartment. The dendrogram on top is based on a similarity matrix (Dice index) calculated from a presence-absence binary matrix of bands. Grouping was based on the UPGMA method. Colour codes refer to phylogenetic affiliation of the sequences obtained from representative bands of each melting type (see Figs. 5.3 and 5.5): *Crenarchaeota* Marine Group I.1a (blue); *Crenarchaeota* Soil Group I.1b (green); Methanogenic archaea (red); ANME groups (orange); *Miscellaneous Crenarchaeotic Group* (yellow).

The phylogenetical analysis between the Kivu phylotype affiliated within the Marine Group I.1a and other marine archaeal nitrifiers represented by *Candidatus* "Nitrosopumilus maritimus" (92 to 96% sequence similarity) suggests a distant relation that may arise from its freshwater origin. Besides, all *amoA* sequences recovered from Kivu group together within the freshwater clade, providing further support to the idea of sequence clustering according to habitat (Francis et al 2005, 2007; Beman et al 2007, 2008; Pouliot et al 2009).

A final concern refers to the potential impact of the ammonia-oxidizing archaeal populations may have on N and C cycles in Lake Kivu, especially considering their small contribution to the microbial planktonic assemblage according to the abundances measured. It should be noted however that small numbers do not necessarily imply low activity. Recent reports on the deep marine subsurface biosphere highlight that some important microbial activities can be performed by a small, but very active, subset of community members (Sogin et al 2006; Fry et al 2008). Indeed, other authors have indicated that high archaeal abundances do not necessarily mean a proportional contribution on biogeochemical processes (Lipp et al 2008). Further work is then needed to elucidate the precise contribution of nitrifying Archaea (and Bacteria) in N cycle in Lake Kivu and to resolve their potential spatial and seasonal variations. Besides, the proper determination of prokaryotic players involved in the aerobic and anaerobic oxidation of methane should also be addressed. These studies may help to provide a better picture of the processes and players beneath the microbial cycling of C and N not only in Lake Kivu but also in other Great African lakes.

6. Contribution of planktonic *Archaea* to inorganic carbon fixation processes in a hypereutrophic sulfurous stratified lagoon

6.1. Introduction

Permanent or seasonally stratified lakes constitute very interesting systems in which investigate the links between microbial communities and their metabolic roles under biogeochemical and ecological frameworks. The presence of distinct physico-chemical gradients determine the generation of different water compartments harbouring well-defined prokaryotic communities (e.g., van Gemerden & Mas 1995; Garcia-Gil et al 1999; Casamayor et al 2000, 2002; Camacho et al 2001; Selig et al 2004; Boucher et al 2006; Llirós et al 2008) playing different roles in the ecosystem. Regarding sulfurous stratified lakes, their chemoclines are very active sites where different microbial populations face opposite gradients of oxygen, reduced compounds (as sulfate, ammonia, and ferrous iron) and intensity and quality of incident light (Borsheim et al 1985; Vila & Abella 1994; Fischer et al 1996). Available reduced conmpounds generated within the sediments are mobilised to the water column throughout seasonanl mixing episodes. These compounds can either be used as energy sources or electron donors in diverse metabolic processes carried out by both planktonic bacteria and archaea.

Within the metabolic processes, primary production (CO_2 incorporation into organic matter) is one of the essential processes in nature depending on light and nutrient supplies. Light carbon fixation is carried out by oxygenic (algae and cyanobacteria) and anoxygenic (purple and green sulfur bacteria, PSB and GSB respectively) photosynthetic microorganisms. In some lakes, a significant contribution of photoautotrophic bacteria other than cyanobacteria has been found exceeding carbon fixation carried out by phytoplankton (García-Cantizano et al 2005). In turn, dark carbon fixation processes have been traditionally ignored although their significant importance in quantitative terms in both the redoxcline and the anoxic waters of some freshwater environments (e.g., Culver & Brunskill 1969; Jorgensen et al 1979; Casamayor et al 2001a; García-Cantizano et al 2005). In stratified lakes, integrated dark carbon fixation rates in the whole water column have been recently reported to be equivalent to values of carbon fixation in the light suggesting the implication of a large repertory of chemoautotrophic metabolisms (Casamayor et al 2008). A few studies analysing and partitioning CO_2 incorporation into distinct microbial guilds present in stratified lakes have been carried out (Camacho et al 2001; García-Cantizano et al 2005; Casamayor et al 2008) although none of them focused on the contribution of the archaeal component of the planktonic assemblage which can be particularly rich in those environments (Casamayor et al 2001b; Llirós et al 2008).

Carbon fixation processes mediated by *Archaea* may cover a wide range of metabolisms involving both N and S cycles (Berg et al 2007). Besides, it has been demonstrated that mesophilic *Archaea* could use inorganic carbon as sole carbon source (Kuypers et al 2001; Pearson et al 2001; Wuchter et al 2003; Herndl et al 2005; Ingalls et al 2006). In this regard, analyses on isotopic composition of membrane lipids from *Crenarchaeota* (Hoëfs et al 1997; Kuypers et al 2001) revealed that these microorganisms can incorporate inorganic carbon to their cell structures acting as autotrophs (Schouten et al 2000; Sinninghe-Damsté et al 2002a, b). Recently, the presence of biotin carboxylase subunit (encoded in the *accC* gene) — a subunit of the acetyl-CoA-carboxylase, key enzyme in the archaeal 3-hydroxypropionate/4-hydroxybutyrate CO_2 fixation pathway — has been used as a functional biomarker for archaeal CO_2 fixation and consistently found in a series of enrichment cultures of *Archaea* inoculated with water from a small stratified karstic lake from the Banyoles area (Auguet et al 2008). Despite their potential autotrophic metabolism, planktonic *Archaea* can also function as heterotrophs (Agogué et al 2008; Schleper 2008). Particularly, it has been suggested the implication of members of Crenarchaeota Marine Benthic Group-B (MGB-B) and Miscellaneous Crenarchaeotic Group (MCG) in methane oxidation without carbon assimilation (Biddle et al 2006). Interestingly, MCG phylotypes have been found to be common members of the planktonic assemblage in the eutrophic anoxic hypolimnetic waters of Lake Vilar during an interanual study (Llirós et al 2008).

Despite the widespread distribution of uncultured *Archaea*, most of the studies available so far have been carried out in marine environments (e.g., Herndl et al 2005; Teira et al 2006; Wuchter et al 2006b; Varela et al 2008). Therefore, the current knowledge on their ecological roles and metabolic processes carried out by these lacustrine *Archaea* beyond methanogens is scarce (Auguet et al 2008; Llirós et al, submitted). Here we investigated the links between the bacterial and archaeal community structure and the inorganic carbon uptake in a holomictic eutrophic lagoon located in the Banyoles karstic lacustrine system using single cell analyses (MICRO-CARD-FISH) and 16S rRNA gene sequencing.

Results

Physico-chemical profiles

In May, the water column of Coromina lagoon was thermally stratified and three distinct water compartments were defined: an oxic epilimnion ranging from surface to 1.75 m depth, a thin (*ca.* 0.5 m) oxic-anoxic transition zone (metalimnion) located at 2.0 m depth and an anoxic hypolimnion from 2.25 to maximum depth (labelled as I, II and III respectively in Figure 6.1A). Oxygen concentrations decreased from *ca.* 17 mg l^{-1} at surface to extinction at 2.25 m depth with an apparent oxygen peak at 1.0 m depth (17.2 mg l^{-1}). Sulfide increased with depth reaching values of 381 µM at the bottom (4.75 m) with maximum values (*ca.* 450 µM) at 4.0 m. The slight increase in conductivity values along depth was due to the accumulation of reduced compounds resulting from microbial activities generating a sort of mild biogenic meromixis (Borrego & García-Gil 1994a). This soft chemical gradient persisted during fall (October 2007), also splitting the water column in three water compartments, while no thermal stratification was observed in this season (Fig. 6.2A). In this case, the three water compartments were recognized on the basis of oxygen profile which clearly distinguished the oxic epilimnion from the anoxic hypolimnion (I and III in Fig. 6.2A, respectively) by a oxic-anoxic transition zone, which was wider than that of May (*ca.* 1 m; II in Fig. 6.2A). Low oxygen concentrations (3.5 mg l^{-1}) were observed which rapidly extinguished at 1.5 m depth. Sulfide concentrations increased with depth from 7 µM (at 2.5 m) to 173 µM at the bottom (4.0 m).

Overall, the lagoon maintained a well-defined stratified pattern with a redoxcline of variable thickness. Representative samples from the different water compartments were collected to analyse changes in the structure of the planktonic assemblage and in those cells actively uptaking inorganic carbon.

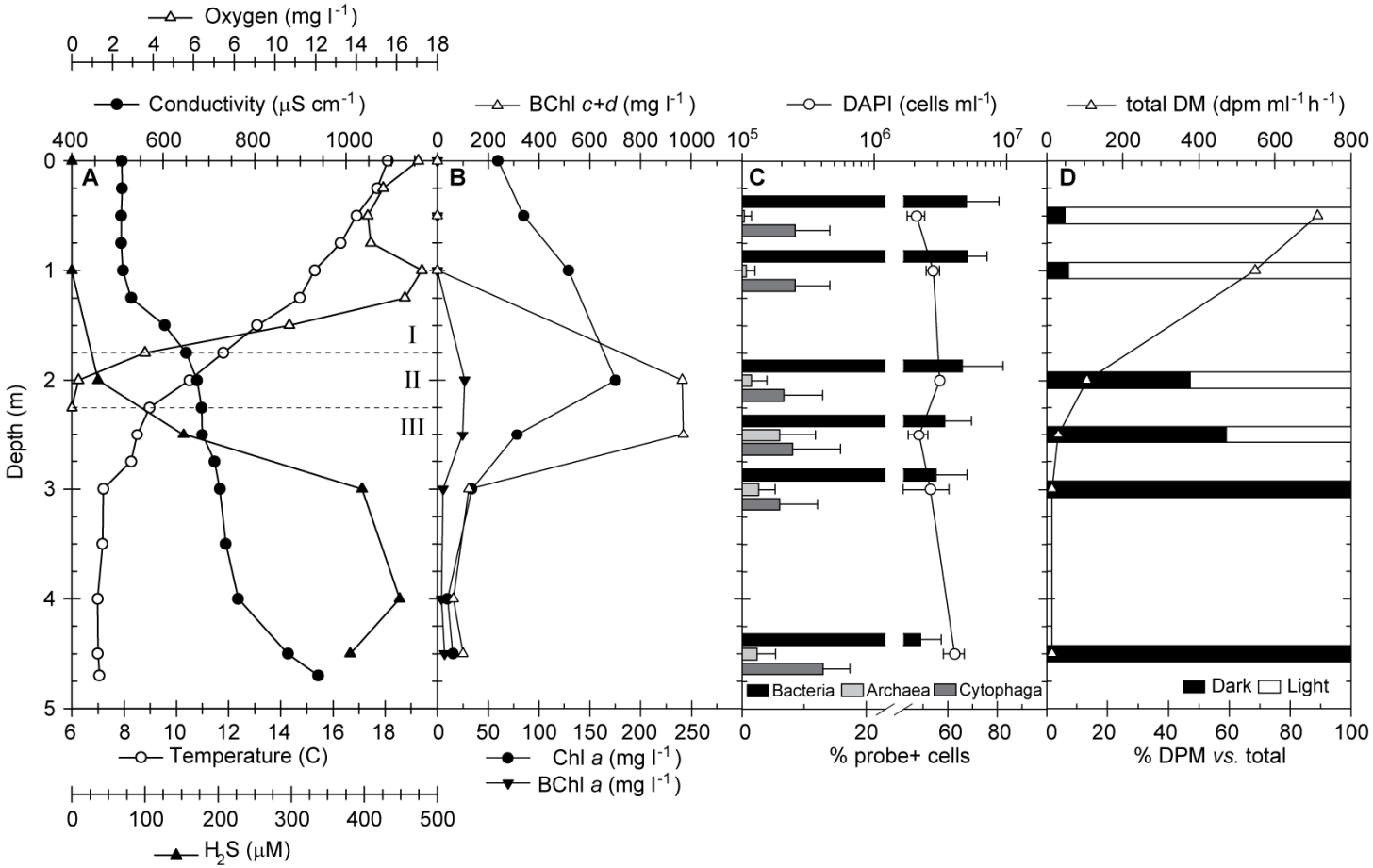


Figure 6.1. Vertical depth profiles in May 2007 for Coromina lagoon. (A) temperature, conductivity, oxygen and sulphide profiles; (B) photosynthetic pigments (Chlorophyll *a*, Chl *a*; Bacteriochlorophyll *a*, BChl *a*; and Bacteriochlorophyll *c* and *d*, BChl *c*+*d*) profiles; (C) DAPI-stained cells and relative proportions of prokaryotic groups detected by CARD-FISH with specific probes for Bacteria, Cytophaga and Archaea, respectively; and (D) bulk radiolabeled bicarbonate uptake and relative proportions in the dark and light incubations.

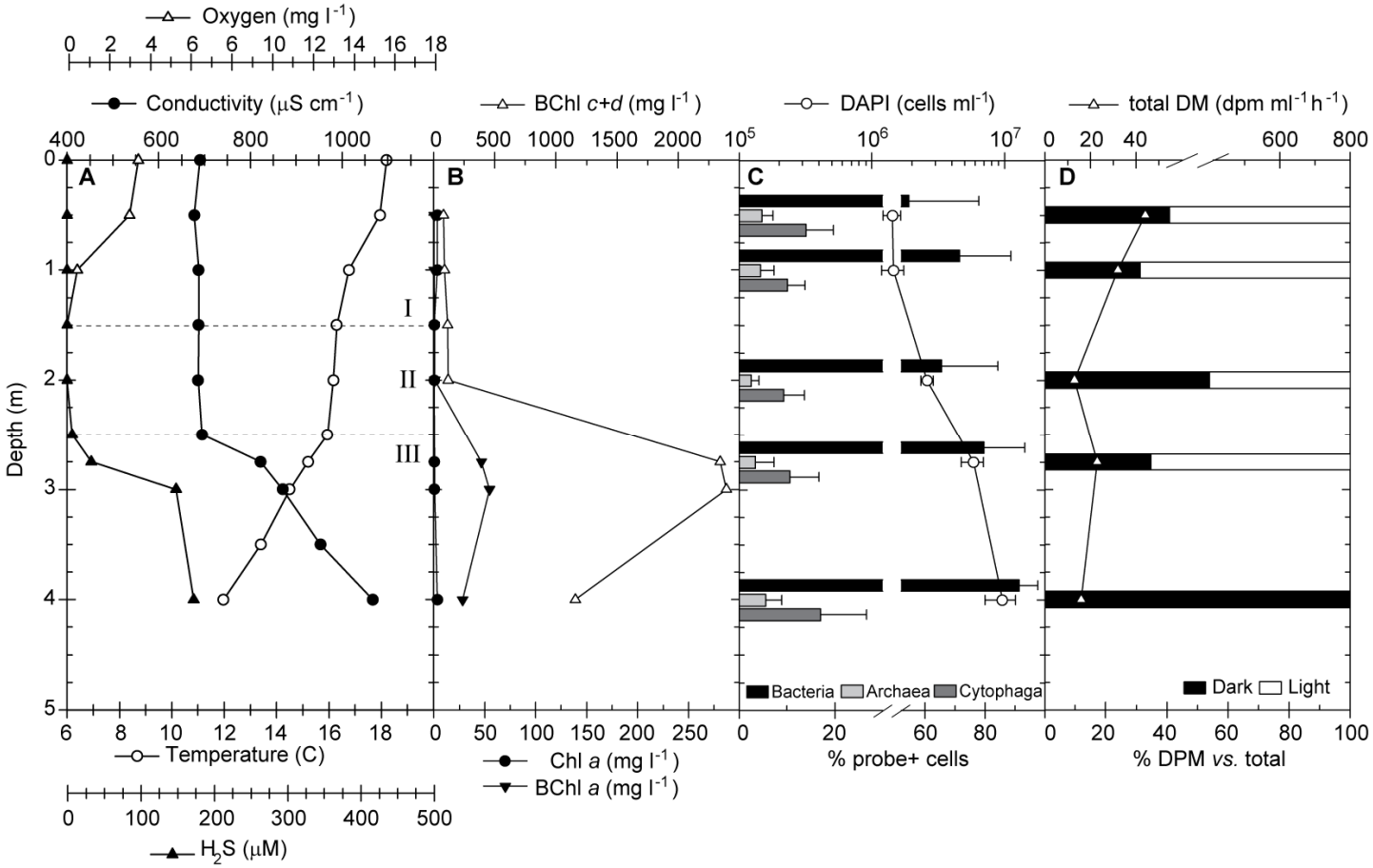


Figure 6.2. Vertical depth profiles in October 2007 for Coromina lagoon. (A) temperature, conductivity, oxygen and sulphide profiles; (B) photosynthetic pigments (Chlorophyll *a*, Chl *a*; Bacteriochlorophyll *a*, BChl *a*; and Bacteriochlorophyll *c* and *d*, BChl *c*+*d*) profiles; (C) DAPI-stained cells and relative proportions of prokaryotic groups detected by CARD-FISH with specific probes for Bacteria, Cytophaga and Archaea, respectively; and (D) bulk radiolabeled bicarbonate uptake and relative proportions in the dark and light incubations.

Vertical distribution of microbial populations

During the present study well-developed assemblages of anoxygenic photosynthetic bacteria and oxygenic phytoplankton were found in the lagoon (Figs. 6.1B and 6.2B). High chlorophyll *a* (Chl *a*) concentrations were measured in the oxic epilimnion in May (Fig. 6.1B), ranging from maximal values at the oxic-anoxic interface (175.4 µg l^{-1}) to minimal ones at the bottom (9.7 µg l^{-1}), probably due to settled algae cells. The depth profile of Chl *a* and oxygen concentration suggested a high photosynthetic activity in epilimnetic waters. In October, Chl *a* values were comparatively low, never exceeding *ca.* 3.5 µg l^{-1} (Fig. 6.2B). The anoxic conditions and the accumulation of conspicuous amounts of sulfide in most of the water column favoured blooming of anoxygenic sulfur bacterial populations. At both sampling dates, Green Sulfur Bacteria (GSB) and Purple Sulfur Bacteria (PSB) constituted stable planktonic populations in the oxic-anoxic interfase and in the anoxic hypolimnion. The GSB population was mainly composed by *Pelodictyon clathariforme* which constituted an abundant population as deduced by the high values of bacteriochlorophyll (BChl) measured (*ca.* 970 and 2,400 µg BChl *c+d* l^{-1} in May and October, respectively). These green-coloured species contains a heterogeneous antena composed of a mixture of BChl *c* and *d* (BChl *c+d*; Gich et al 2001). In turn, PSB were detected at lower abundances as indicated by the lower concentration of BChl *a* measured (26.9–54.7 µg BChl *a* l^{-1}). In this case, the dominant member of the PSB community was *Amoebacter purpureus*, as revealed by microscopic observations (data not shown). Other prokaryotic groups were analysed by specific staining and *in situ* hybridization. Total and relative abundances of the different prokaryotic groups analysed are shown in Figures 6.1C (May) and 6.2C (October). Total prokaryotic abundances (DAPI-stained cells) ranged from $2.08\pm0.31x10^6$ to $4.05\pm0.73x10^6$ cells ml^{-1} in May, and from $1.42\pm0.21x10^6$ to $9.62\pm2.50x10^6$ cells ml^{-1} in October. No clear trends on vertical distribution of either total cell numbers or relative abundances of hybridised Bacteria (EUB+), Archaea (ARC+) and Cytophaga (CF+) were observed in May. Maximal relative abundances of archaea were observed below the oxicline (6%) whereas Cytophaga were more abundant at the bottom (13%). In October, total prokaryotes and relative abundances of EUB+ cells increased with depth while Archaea and Cytophaga cells appeared more equally distributed along the vertical gradient with relative abundances ranging from 2% to 5% and from 9% to 17%, respectively. Overall, the bacterial contribution (49% to 91%) to total planktonic assemblage was higher than that for Archaea at both sampling dates.

Active incorporation of inorganic carbon

We selected representative samples from each water compartment at each sampling date to study qualitative vertical distribution patterns of inorganic carbon incorporated by different microbial groups (percentage of dark activity *vs.* light incorporation; Figs. 6.1D and 6.2D). Incubations carried out in May in the epi- and metalimnion showed maximal contribution of light-driven incorporation processes (*ca.* 94% of total DPMs). Dark incorporation processes contributed a 59% of total carbon incorporation within the upper part of the hypolimnion (3.0 m) and 100% at the bottom, where no light was available. Similar results were obtained in October with light-driven carbon incorporation predominating at the epilimnion (between 60 and 70% of total DPMs) while dark processes being dominant at the redoxcline (44%) and at the bottom of the lagoon (100%). In October, however, photosynthetic carbon incorporation (45%–70% of total DPMs) might be performed by anoxygenic phototrophs which dominated the water column, as indicated by higher BChl *c*+*d* values (2,400 µg l^{-1}) and low Chl *a* values (below 3.5 µg l^{-1}) (Fig. 6.2B and 6.2D). Overall, total DPMs measured in epilimnetic water samples from October were *ca.* 10 times lower than those from May water samples, whereas those values measured from hypolimnetic water samples at both sampling dates were within the same range (Figs. 6.1D and 6.2D).

DGGE fingerprinting analyses

Changes in the composition of the bacterial and the archaeal community composition (from now on BCC and ACC, respectively) were studied with DGGE fingerprinting and 16S rRNA gene sequencing of excised bands. Overall, fifteen samples from the two sampling dates were analysed. In most cases only few very intense bands (1 to 4) were found, suggesting a microbial community dominated by few abundant phylotypes. Both BCC and ACC fingerprints showed banding patterns clearly segregated above and below the physico-chemical gradients at both sampling dates (Fig. 6.3). When analysed using gel-image software, the BCC showed two different patterns above and below the oxic-anoxic transition zone whereas the ACC fingerprint revealed a primary segregation according to sampling date (Fig. 6.4). In turn, archaeal fingerprints obtained from the hypolimnia of May and October clustered together suggesting a stable archaeal community below the redoxcline (Fig. 6.4).

The BCC was analysed with a universal bacterial primer combination revealing between 13 and 21 different bacterial DGGE bands per sample and showing low band richness in the hypolimnia of both sampling campaigns (Fig. 6.3A). Up to 36 representative DGGE bands were excised from the gels, further sequenced and used in phylogenetic analyses.

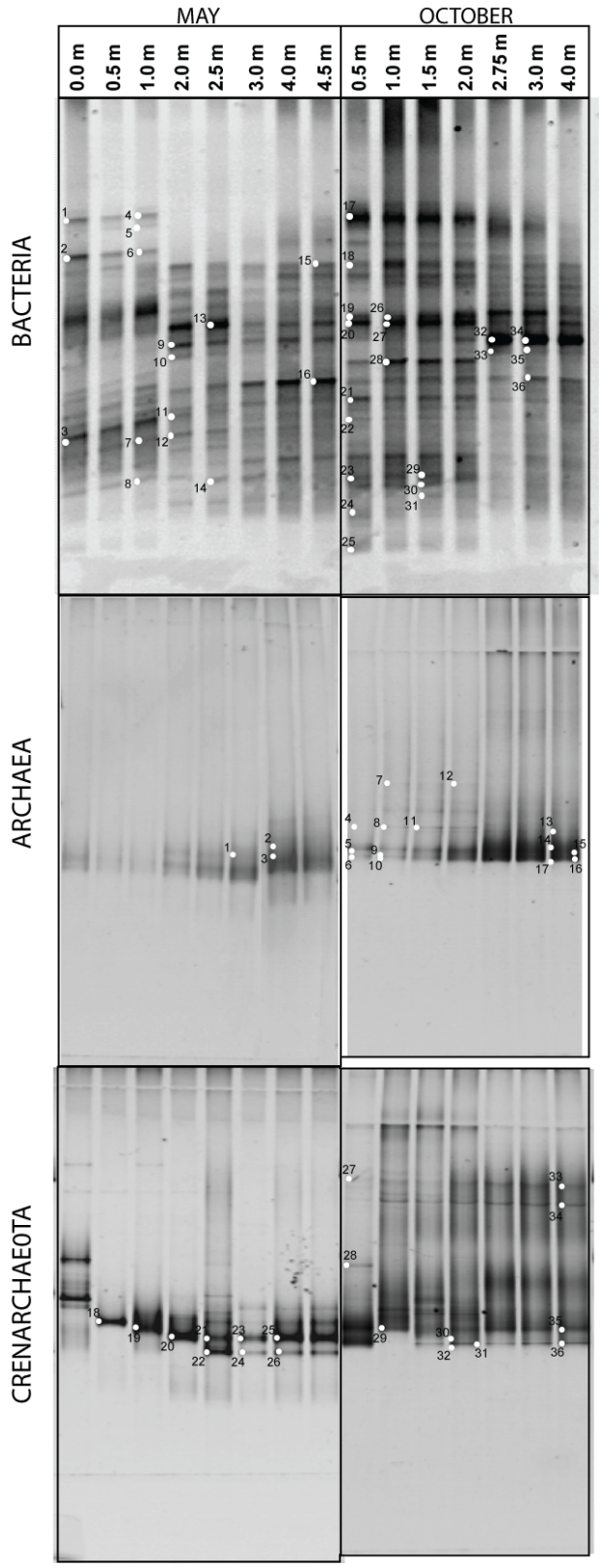


Figure 6.3. DGGE fingerprints of bacterial (upper) and archaeal (middle and lower) 16S rDNA gene fragments obtained after PCR amplification using 341f/907r; PAIR-1; and PAIR-3 primer pairs for Bacteria, Archaea and Crenarchaeota; respectively. Dots and numbers indicate sequenced DGGE bands and correspond to 16S rRNA sequence numbers shown in Figs. 6.5 and 6.6.

The analyses of the ACC using a general primer pair combination (PAIR-1) unveiled a low number of bands (between 2 and 9 DGGE bands per sample; Fig. 6.3B) in the whole water column. In this case, up to 17 bands representing different band positions were excised, sequenced and used for phylogenetic analyses. In turn, when the primer combination biased towards lacustrine *Crenarchaeota* (PAIR-3) was used, between 1 and 10 different DGGE bands per sample were retrieved (Fig. 6.3C), and 19 different sequences suitable for phylogenetic analyses were obtained.

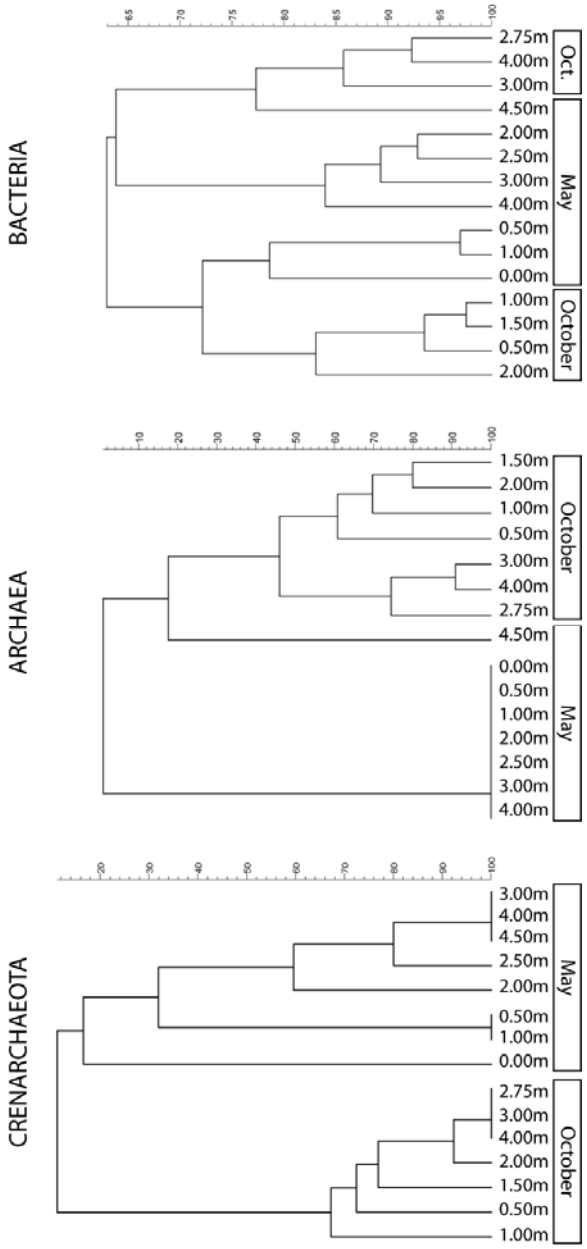


Figure 6.4. Dendrograms corresponding to DGGE gels from Fig. 6.3 based on a similarity matrix (Dice index) calculated from a presence-absence binary matrix of bands. Grouping was based on the UPGMA method.

Phylogenetic affiliations of the BCC and ACC

Bands exhibiting the same melting behaviour in the gels corresponded to the same phylotype, and thus we ended with 30 out of 36 and 17 out of 37 unique 16S rRNA gene sequences for *Bacteria* and *Archaea*, respectively.

The bacterial sequences grouped within six phylogenetic groups i.e., *Alpha-Proteobacteria* (3% of total bacterial phylotypes), *Beta-Proteobacteria* (43%), *Bacteroides* group (17%), Green Sulfur Bacteria (GSB; 10%), High-GC Gram-positive bacteria (i.e., *Actinobacteria*; 23%) and Low-GC Gram-positive bacteria (i.e., *Firmicutes*; 3%) (Table 6.1 and Fig. 6.5). A few phylotypes (3) affiliated with plastids closely related to the diatom *Nitszchia* sp. (Fig. 6.5C) were also recovered. Most of the sequences of the Division *Proteobacteria* belonged to the *Beta-Proteobacteria* and only one to the *Alfa-Proteobacteria*. Furthermore, 3 out of 13 *Beta-Proteobacteria* retrieved sequences belonged to the *Polynucleobacter* clade. In addition, 7 sequences affiliated within the Class *Actinobacteria*. With the exception of *Alpha-Proteobacteria* and *High-* and *Low-GC Gram-positive* bacteria, all the dominant members of the Coromina lagoon bacterioplankton were present all over the water column at both sampling dates (Table 6.1 and 6.2). *Bacteroidetes* clade and *Beta-Proteobacteria* were detected all over the water column of the lagoon with relative DGGE band intensities ranging from 3% to 18% and from 2% to 34%, respectively (Table 6.2). Whereas, GSB showed maximal relative band intensities in suboxic and anoxic water layers (between 12% and 28%; Table 6.2). Maxima relative contribution of GSB to the BCC directly correlated with BChl *c+d* maxima within the oxic-anoxic transition zone (Table 6.2 and Figs. 6.1B and 6.2B). *High-GC Gram-positive* bacteria largely contributed to suboxic BCC in October (25% of total band intensity at 1.0 m depth). Moreover, *Low-GC Gram-positive* bacteria were only detected in the hypolimnion (from 10% to 14% of total band intensity in May; Table 6.2) of the lagoon.

Archaea obtained with the general archaeal primer set (PAIR-1) exclusively belonged to the Kingdom *Euryarchaeota*, mainly affiliated within methanogens (*Methanosaeta*, *Methanoregula* and *Methanospirilli*; 73% of total phylotypes retrieved with this primer set) but also within the *Deep Hydrothermal Vent Euryarchaeota* clusters DHVE-3 and DHVE-5 (Tables 6.1 and 6.3A; and Fig. 6.6). The whole water column was dominated by methane-related phylotypes, exclusively by *Methanosaeta* in May while *Methanosaeta*, *Methanoregula* and *Methanospirilli* phylotypes were recovered in October. *Archaea* retrieved with the specific primer set (PAIR-3) affiliated within both *Euryarchaeota* (WSA-2 and HV-1 clusters; 33.3% of total phylotypes retrieved with the PAIR-3) and *Crenarchaeota* (Soil I.1b and the Miscellaneous Crenarchaeotic Group (MCG) group 1.3;

Tables 6.1 and 6.3B; and Fig. 6.6). In May, the fingerprint of the oxic epilimnion was clearly dominated by *Euryarchaeota* (WSA-2 cluster) and *Crenarchaeota* (Soil I.1b) (100% of relative band intensity at 0.5 and 1.0 m depth, respectively) while both May anoxic hypolimnion and October whole fingerprints were clearly dominated by MCG phylotypes (between 47% to 100% of relative band intensity; Table 6.1 and 6.3B).

Table 6.1. Bacterial and archaeal clusters retrieved after sequencing the DGGE bands (absence/presence), and general distribution in the water compartments of Coromina lagoon.

		Epilimnion[b]		Metalimnion		Hypolimnion	
Cluster	**N[a]**	**May**	**October**	**May**	**October**	**May**	**October**
Bacteria							
Alpha-Proteobacteria	1	+	-	-	-	-	-
Beta-Proteobacteria	13	+	+	+	+	+	+
Bacteroidetes	5	+	+	-	+	+	-
GSB[c]	3	-	-	+	+	+	+
High-GC Gram positive	7	-	+	-	+	+	-
Low-GC Gram positive	1	-	-	+	-	+	-
Euryarchaeota							
Methanosaeta	4	+	+	+	+	+	+
Methanoregula	3	-	+	-	-	-	-
Methanospirilli	1	-	+	-	-	-	-
DHVE-3[d]	2	-	+	-	+	-	-
DHVE-5	1	-	+	-	-	-	-
DHVE-1	1	-	+	-	-	-	-
WSA-2	1	+	-	-	-	-	-
Crenarchaeota							
Soil I.1b	1	+	-	-	-	-	-
MCG:group1.3[e]	3	-	+	+	+	+	+

[a]: number of bacterial and archaeal phylotypes within each cluster.
[b]: presence or no detection of each cluster.
[c]: *Green Sulphur Bacteria*.
[d]: *Deep Hydrothermal Vent Euryarchaeota* group.
[e]: *Miscellaneous Crenarchaotic Group*.

Table 6.2. Richness (number of DGGE bands) and relative abundances for each bacterial clade obtained using 341f-907r primer pair in May and October water samples.

Depth	Richness	Nitzchia	GSB	CFB	Proteobacteria		Gram-positive		
(m)					Alpha-	Beta-	Low GC	High GC	n.i.
May		%	%	%	%	%	%	%	%
0.0	14	5	-	14	3	16	-	-	62
0.5	16	6	1	11	4	3	-	-	75
1.0	17	8	1	4	2	19	-	-	65
2.0	14	-	34	8	2	13	14	-	28
2.5	14	-	24	7	-	8	10	3	48
3.0	14	-	14	10	-	18	12	6	41
4.0	13	-	12	12	-	18	13	4	41
4.5	13	-	14	9	-	16	12	-	49

Depth	Richness	GSB	CFB	Proteobacteria	Gram-positve	
(m)				Beta	High GC	n.i.
October		%	%	%	%	%
0.5	18	6	17	31	18	28
1.0	20	2	21	23	25	30
1.5	21	2	18	28	21	31
2.0	18	5	28	34	8	26
2.75	13	35	3	2	-	60
3.0	15	30	4	3	-	62
4.0	13	38	6	7	-	49

n.i. not identified.

Table 6.3A. Richness (number of DGGE bands) and relative abundances for each archaeal cluster obtained using PAIR-1 primer pair in May and October water samples.

Depth	Richness	*Euryarchaeota*	
(m)		***Methanosaeta* spp.**	**n.i.**
May		%	%
0.0	2	100	0
0.5	2	100	0
1.0	2	100	0
2.0	2	100	0
2.5	2	100	0
3.0	2	100	0
4.0	2	100	0
4.5	2	100	0

Depth	Richness	*Euryarchaeota*					
(m)		***Methanosaeta* spp.**	***Methanoregula* spp.**	***Methanospirilli* spp.**	**DHV-3**	**DHV-5**	**n.i.**
October		%	%	%	%	%	%
0.5	5	14	71	14	-	-	0
1.0	7	5	44	30	3	10	8
1.5	6	-	43	40	11	-	6
2.0	9	2	35	29	10	-	23
2.75	6	94	-	-	-	-	6
3.0	5	94	-	-	-	-	6
4.0	6	93	-	-	-	-	7

n.i. not identified dgge bands.

Table 6.3B. Richness (number of DGGE bands) and relative abundances for each archaeal cluster obtained using PAIR-3 primer pair in May and October water samples.

Depth	Richness	*Euryarchaeota*	*Crenarchaeota*		
(m)		WSA-2	Soil I.1b	MCG:group1.3	n.i.
May		%	%	%	%
0.0	6	-	-	-	100
0.5	1	-	100	-	0
1.0	1	100	-	-	0
2.0	2	-	-	72	28
2.5	6	-	-	60	40
3.0	4	-	-	83	17
4.0	4	-	-	71	29
4.5	4	-	-	72	28

Depth	Richness	*Euryarchaeota*	*Crenarchaeota*	
(m)		DHVE-1	MCG:group1.3	n.i.
October		%	%	%
0.0	9	1	67	32
1.0	6	-	60	40
1.5	10	-	47	53
2.0	8	-	53	47
2.75	4	-	100	0
3.0	4	-	100	0
4.5	4	-	100	0

n.i. not identified dgge bands.

Prokaryotic uptake of radiolabeled bicarbonate

In May about 25% of total prokaryotes (EUB+MAR+ *vs.* DAPI+ cells; representing *ca.* 21% of total Bacteria) showed ^{14}C-labelling (Table 6.4). Bacteria (EUB+MAR+ cells) were very active in bicarbonate incorporation both in the light and in the dark (Table 6.4) and higher values (EUB+MAR+ cells) were found in the epilimnion than in the meta- and hypolimnion. A fraction of this bacterial activity was surprisingly carried out by Bacteroidetes, a group traditionally considered as strict heterotrophs. Up to 5% of the Bacteroidetes cells (CF+MAR+ *vs.* CF+) were actively incorporating radiolabeled bicarbonate in the oxic epilimnion under light and dark conditions whereas 1-2% of the cells were found labeled in the anoxic hypolimnion (Table 6.4). For Archaea, in May we observed between 9% and 12% of archaeal cells (ARC+MAR+ *vs.* ARC+) actively uptaking $NaH^{14}CO_3$ in the epi- and metalimnion and up to 3% in the anoxic hypolimnion (Table 6.4).

Table 6.4. Percentage of positively hibridised cells with probes for Bacteria (EUB338), Archaea (ARCH915) and Cytophaga/Bacteroidetes (CF319a) taking up radiolabeled bicarbonate ($NaH^{14}CO_3$; average±SD) as measured by MICRO-CARD-FISH during May 2007.

Depth	Bacteria[a]		Archaea[a]		Cytophaga[a]	
(m)	Light	dark	light	dark	light	dark
May	% *vs.* EUB+ cells		% *vs.* ARC+ cells		% *vs.* CF+ cells	
0.5	21.03±5.16	9.65±8.38	11.59±16.20	5.00±15.81	4.98±4.47	5.50±7.64
1.0	20.36±12.25	6.22±4.52	9.88±14.61	10.36±13.97	4.07±4.75	3.32±3.37
2.0	7.16±3.74	10.22±3.19	8.92±8.59	3.65±5.94	3.69±3.48	1.08±2.22
2.5	5.23±1.56	5.29±3.87	0.48±1.51	2.00±6.32	0.82±1.36	2.16±3.61
3.0	-[b]	4.62±4.23	-	1.67±5.27	-	1.51±2.57
4.5	-	4.00±4.52	-	3.53±4.79	-	1.53±1.84

[a]: probe+MAR+ cells in relation to probe+ cells for each treatment.
[b]: no light available

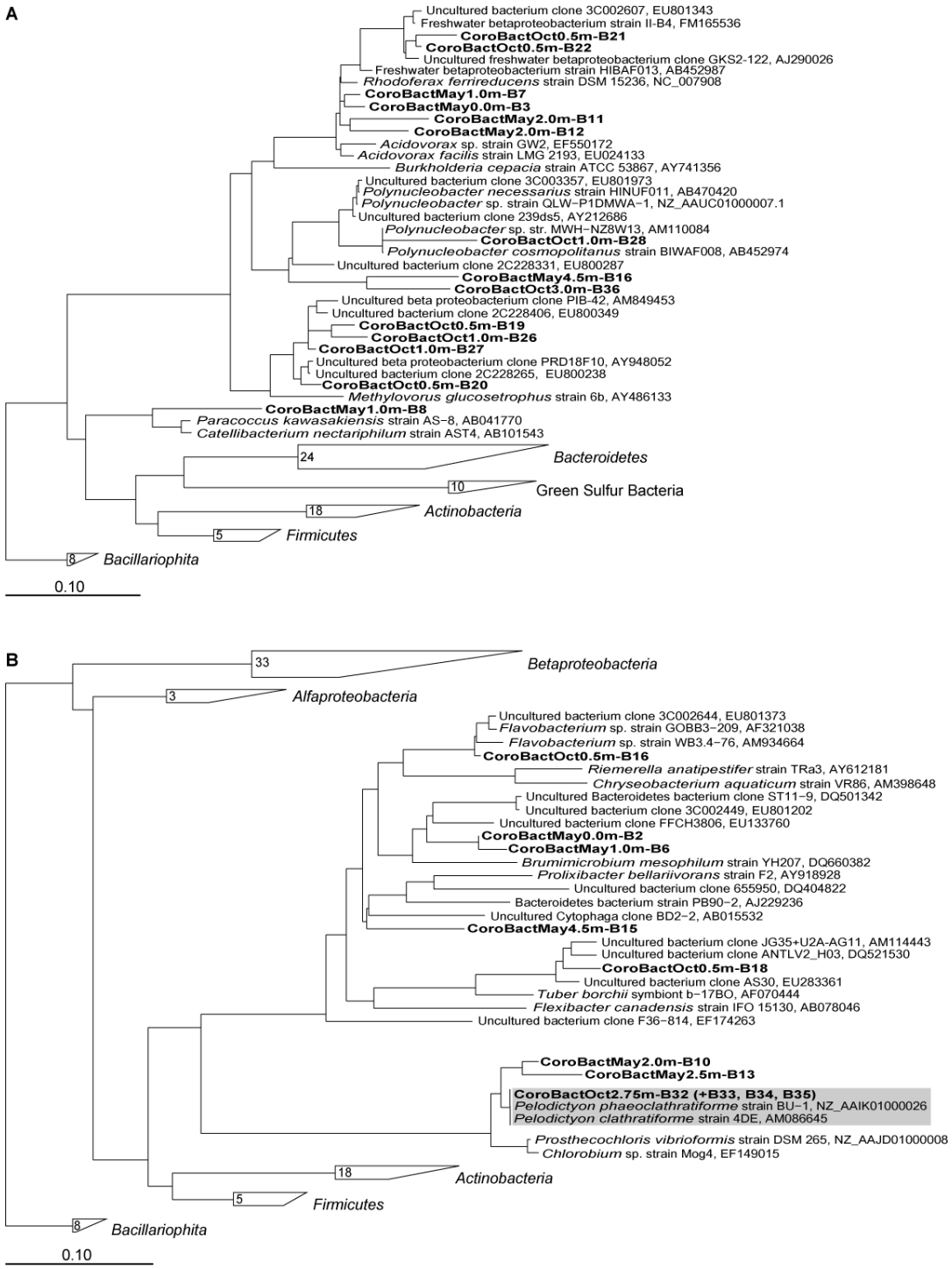


Figure 6.5. Phylogenetic trees showing the affiliation of bacterial partial 16S rRNA sequences retrieved from Coromina lagoon water column during May and October 2007. (A) *Alpha-* and *Beta-Proteobacteria* clades; and (B) *CFB* and *Chlorobi* clades. Sequences recovered in this study are bold highlighted. Sequences are coded as follows: Coro, Coromina lagoon; Bact according to Domain; May or Oct, May and October sampling date; 0.5m, sample depth; and "1", number assigned to the corresponding DGGE band (Fig. 6.3). Reference sequences are described by "accession number and clone name". The scale bar indicates 10% estimated sequence divergence.

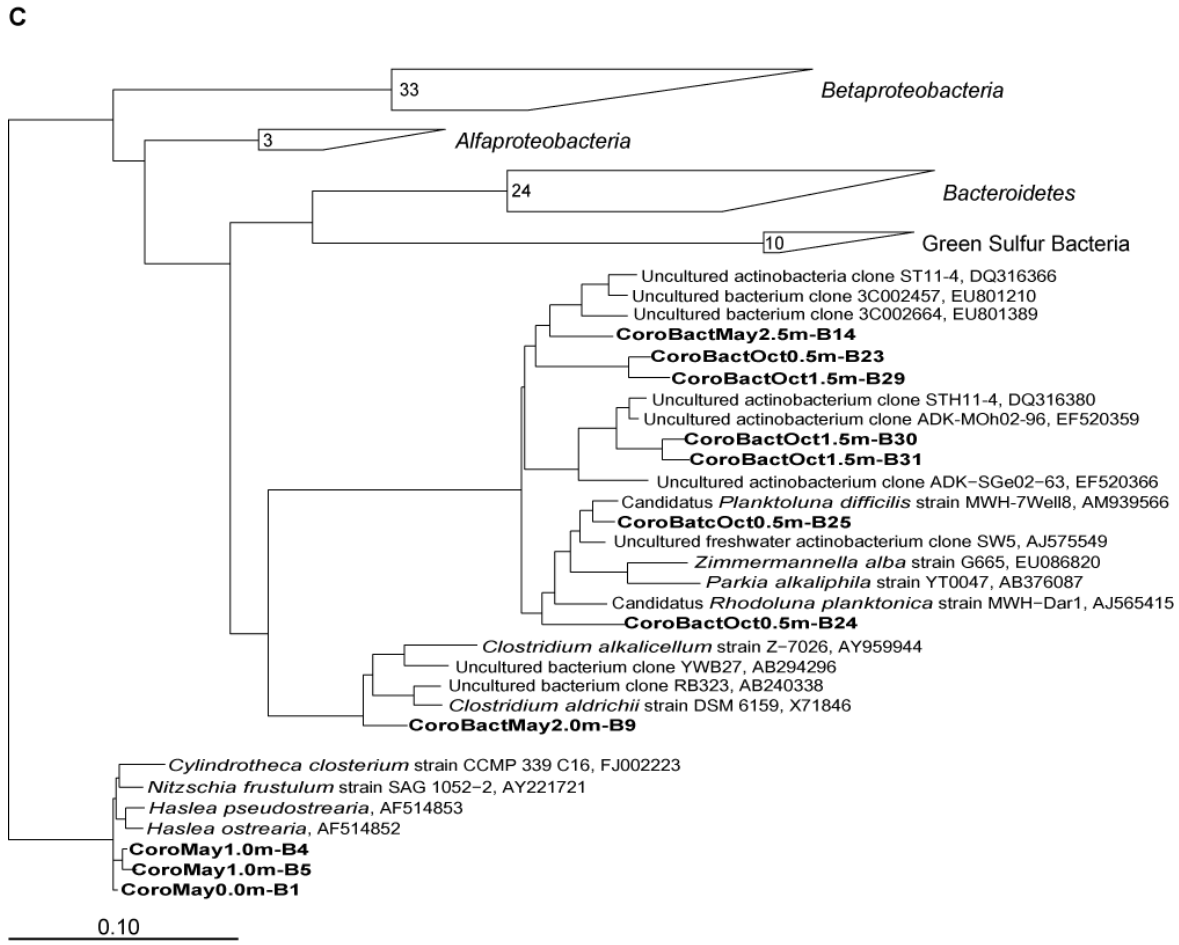


Figure 6.5 cont. Phylogenetic trees showing the affiliation of bacterial partial 16S rRNA sequences retrieved from Coromina lagoon water column during May and October 2007. (C) *High-*, *Low-GC Gram-positive Bacteria* and *Bacillariophyta* clades. Sequences recovered in this study are bold highlighted. Sequences are coded as follows: Coro, Coromina lagoon; Bact according to Domain; May or Oct, May and October sampling date; 0.5m, sample depth; and "1", number assigned to the corresponding DGGE band (Fig. 6.3). Reference sequences are described by "accession number and clone name". The scale bar indicates 10% estimated sequence divergence.

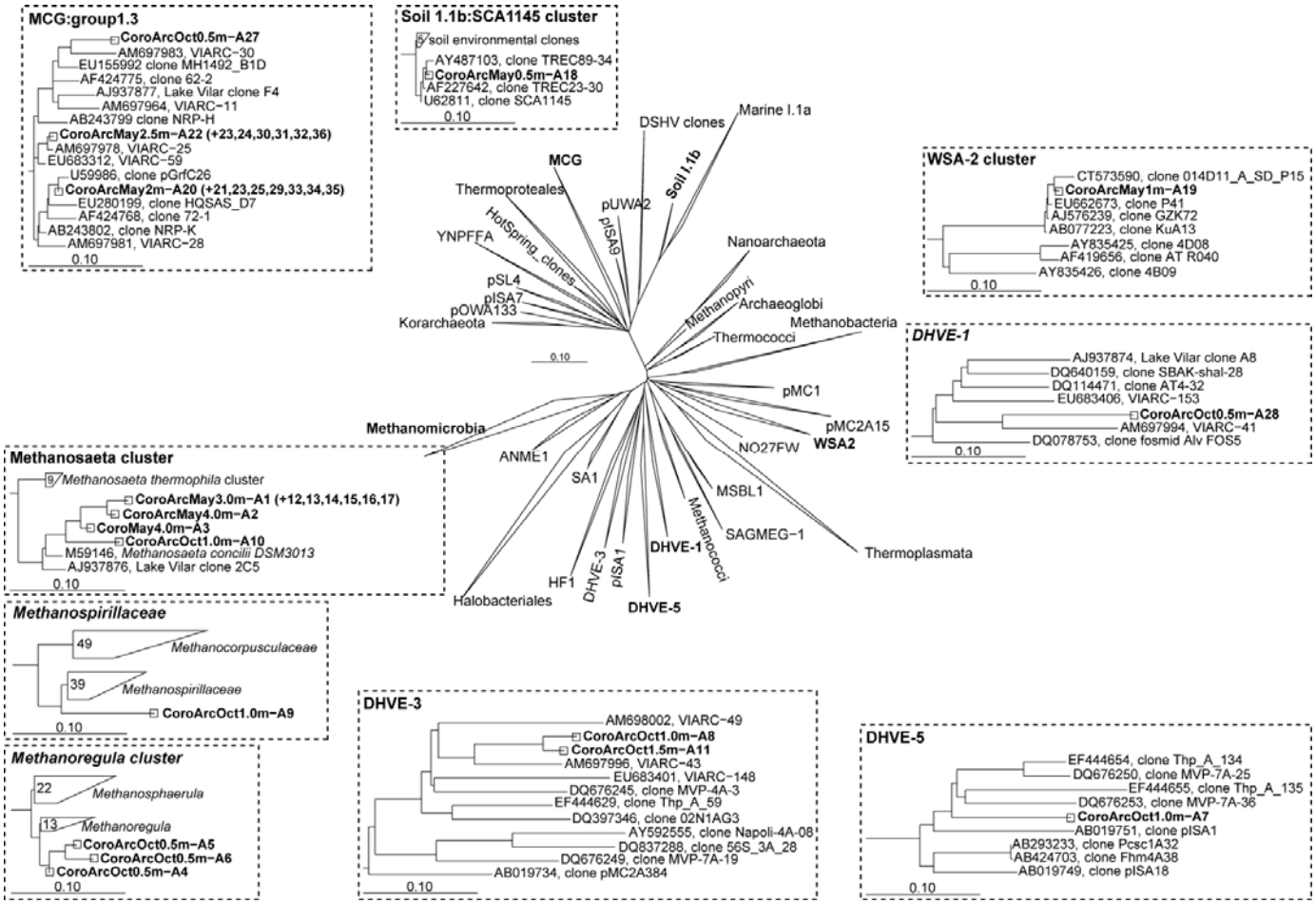


Figure 6.6. Phylogenetic tree showing the affiliation of archaeal partial 16S rRNA sequences retrieved from Coromina lagoon water column during May and October 2007. Sequences recovered in this study are bold highlighted. Sequences are coded Arc for Domain and "1", number assigned to the corresponding DGGE band (Fig. 6.3). Reference sequences are described by "accession number and clone name". The scale bar indicates 10% estimated sequence divergence.

6.3. Discussion

Archaea (both *Crenarchaeota* and *Euryarchaeota*) has been traditionally defined as obligate extermophiles thriving in environments too harsh for other organisms (e.g. Schleper et al 1995; Huber et al 2000; Kashefi & Lovley 2003). Accordingly, the physiological diversity of cultured representatives was limited by both the physico-chemical conditions imposed by this environmental constraits and the capability of researchers to produce new cultures from other environmental conditions. Widespread distribution and ubiquity of *Archaea* derived from culture-independent approaches (DeLong 1998; Schleper et al 2005; Chaban et al 2006; Casamayor & Borrego 2009) has changed this perception and revealed a metabolic diversity greater than previously expected. In this regard, several authors have demonstrated that Archaea can act as true chemoautotrophs fixing carbon in the dark using ammonia (Francis et al 2005; Könneke et al 2005; Coolen et al 2007; He et al 2007; Beman et al 2008; de la Torre et al 2008; Hatzenpichler et al 2008) or other reduced inorganic compounds (Auguet et al 2008; Kozubal et al 2008) as energy source; or as heterotrophs using simple (Ouverney & Fuhrmann 1999; Herndl et al 2005; Teira et al 2006) or complex organic matter (Biddle et al 2006). To date, cultivated *Archaea* are all extremophilies (with respect to growth temperature, osmolarity, pH and oxygen) with the unique exception of Cand. *Nitrosopumilus maritimus*, a mesophilic chemoautotrophic isolate from a saltwater aquarium able to growth aerobically using ammonia (Könneke et al 2005). The high abundance of *Archaea* in the prokaryoplankton of several aquatic ecosystems (e.g., Karner et al 2001; Auguet & Casamayor 2008) together with the close links established with the carbon (CO_2 fixation) and the nitrogen (nitrification) biogeochemical cycles (e.g., Herndl et al 2005; Nicol & Schleper 2006) has enlarged the interest on the ecology of *Archaea*. Furthermore, *Archaea* also actively participate in the sulfur cycle in thermal springs by the oxidation and reduction of inorganic sulfur compounds (Amend & Shock 2001; Kletzin et al 2004; Kletzin 2007).

The physico-chemistry of stratified lakes drives the stratification of both the planktonic assemblage and metabolic processes in narrow water layers. Oxygen-sulfide interfaces concentrate most of the autotrophic activity in these environments (e.g., Pedrós-Alió et al 1993) and anoxygenic photosynthetic sulfur bacteria (mainly GSB and PSB) and other non-photosynthetic micoorganisms found optimal growth conditions at the chemocline due to the occurence of increassing sulfide and low oxygen concentrations (Jorgenssen et al 1979; Borsheim et al 1985; Gich et al 2001; García-Cantizano et al 2005; Casamayor et

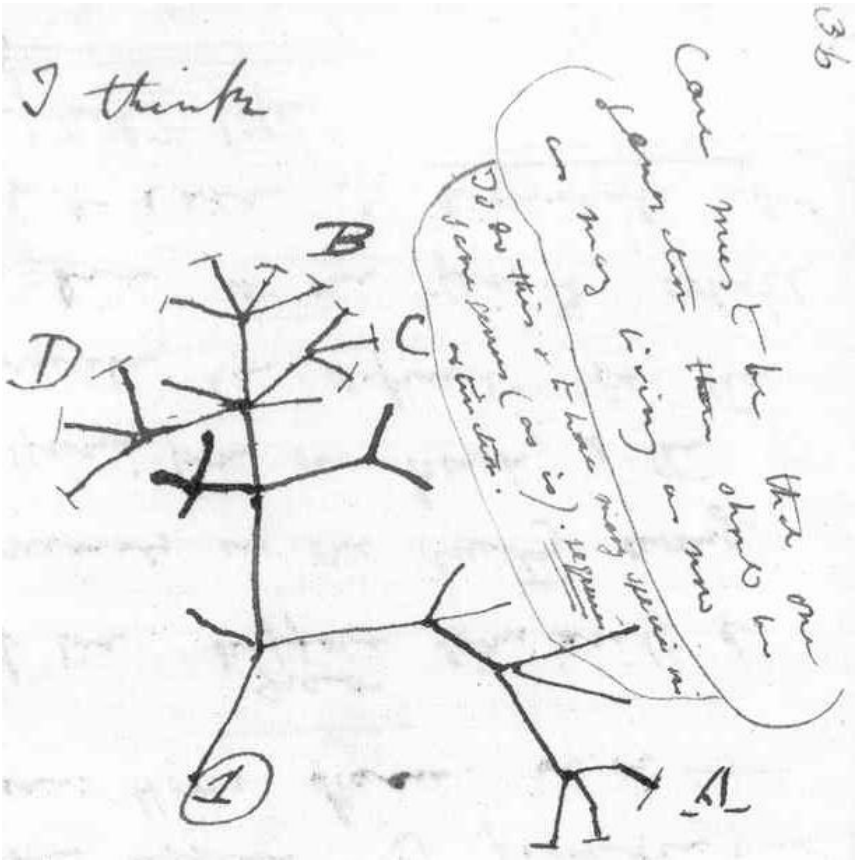
al 2008) as it happens in Coromina lagoon. The BCC of the lagoon was characterised by typical freshwater bacterial clades, similarly to other lagoons of the region (Casamayor et al 2000). All recovered sequences in the present study represent new phylotypes except those GSB-related sequences that exactly matched cultured representatives (*Pelodictyon* (*Pld*.) *chathratiforme* and *Pld. phaeochlathratiforme*) agreeing with previous results carried out in the same lagoon (Gich et al 2001). Nevertheless, no previous reports on the ACC present in the lagoon are available in the literature. In this sense, the ACC in Coromina lagoon was dominated by methanogens and MCG-related phylotypes resembling that of other lagoons of the region as the BCC do (Casamayor et al 2001b; Llirós et al 2008). Moreover, no phylotypes related to known ammonia-oxidising Archaea (AOA) were found supporting the hypothesis that small karstic hypereutrophic lakes with strong influence of the sulfur cycle are harsh environments for AOA presence (Llirós et al 2008; Erguder et al 2009). A low fraction of archaeal phylotypes (<11%) were related to soil and sediment phylotypes (Soil I.1b and WSA-2 clusters) corresponding to allochtonous *Archaea*, in spite of the closed behaviour of the lagoon. These allochtonous phylotypes might be originated by rain drainage of the surrounding area that accumulated in the basin as consequence of the shore slope.

Traditionally, methanogenesis has been considered a minor process in sulfate-rich environments (as marine sediments and karstic lagoons) due to higher affinities of sulfate-reducing bacteria (SRB) than methanogens for the main electron donors (as H_2 and/or acetate; Krisktjansson et al 1982; Lovley et al 1982). Sulfate-reducers and methanogens can coexist under certain environmental conditions (Purdy et al 2003a, 2003b) and methanogenesis could still take place in presence of SRB populations in freshwater environments using alternative single carbon compounds (as methylamines; Winfrey & Ward 1983) not being used by SRB (Lovley & Klug 1983). Interestingly, SRB-related phylotypes were neither retrieved in Coromina lagoon nor in other lakes of the region (Casamayor et al 2000). Moreover, some weak non-sequenced bands could correspond to these low abundant organisms at the time of sampling or might not be recovered because of the resolution of the technique. Methanogenic archaea but also acetogenic bacteria and some SRB, which are able to growth chemolitoautotrophycally in anoxic conditions (Shively & Barton 1991), might compite for electron donors available and being responsible for dark carbon fixation in the lagoon as previously reported in another stratified lake (Camacho et al 2001).

MCG-phylotypes were only recovered after a nested-PCR step, as for Lake Vilar (Llirós et al 2008), representing a minor fraction of the community (less than 1%; Muyzer & Smalla 1998; Muyzer 1999). Moreover, it has been recently reported some important drawbacks

concerning pour target coverage of both general archaeal primers (*viz.* ARC344f and ARC915r; Amann & Fuchs 2008; Teske & Sorensen 2008). Accordingly, those small stratified freshwater karstic lagoons with high influence of sulfur cycle could harbour low abundant but very rich archaeal communities with some phylotypes persisting in the water column over time (i.e., MCG and DHVE) as previously described (Llirós et al 2008). Furthermore, previous detection of MCG-related phylotypes by FISH or CARD-FISH approaches failed (Biddle et al 2006; Llirós et al 2008) supporting the need for nested-PCR approach when retrieving them by sequencing. Even the clear stratification patterns present in the lagoon, both *Methanosaeta* and MCG phylotypes were commonly retrieved all over the water column representing core phylogenetic groups for this kind of environments (see next section).

As expected due to photosynthetic pigment and bulk uptake carbon analyses, photosynthetic planktonic organisms, either oxygenic and anoxygenic, were the main primary producers in the lagoon. Furthermore, dark carbon fixation values reported so far for stratified freshwater environments (Cloern et al 1983; Pedrós-Alió & Guerrero 1991; Kuuppo-Leinikki & Salonen 1992; Hadas et al 2000; Camacho et al 2001) were higher than the ones observed in Coromina lagoon (between 1% and 10% of the targeted groups by the single-cell approach) suggesting a predominance of photoautotrophy over chemoautotrophy in the lagoon. Bulk dark incorporation values better correlated with EUB+MAR+ rather than ARC+MAR+, especially in May. In this sense, a key role on dark carbon fixation for *Archaea* could not be totally ruled out with our approach. Moreover, the observed active cytophaga-like cells (CF+MAR+) represented so far the first report of members of the *Bacteroidetes* actively uptaking ^{14}C-bicarbonate indicating a widder metabolic repertory in this larger bacterial group (Kirchman 2002). Altogether, dark carbon fixation results together with the accumulation of reduced compounds in the water column suggest that mesophilic *Archaea* present in the lagoon might possess potential hetero- or mixotrophic metabolisms (Jahn et al 2007; Auguet et al 2008). However, anaplerotic processes both in bacteria and archaea cannot totally be discarded (Menendez et al 1999; Herndl 2005; Ingalls et al 2006; Auguet et al 2008) and deserve more detailed studies.



7. Biogeographical distribution of lacustrine *Archaea*

7.1. Introduction

The Domain *Archaea* peerless illustrates to what extent the wide use of molecular techniques in microbial ecology has revolutionised the view of the microbial diversity (Woese 2000). *Archaea* are now considered abundant and ubiquitous microorganisms rather than rare and extreme bugs (Schleper et al 2005; Chaban et al 2006; Casamayor & Borrego 2009). Recently, a great abundance and ubiquity of mesophilic *Archaea* in different seas, oceans and sediments has been described (e.g., Schleper et al 2005; Francis et al 2005, 2007; Nicol & Schleper 2006 among others). In comparison, studies on freshwater *Archaea*, and specially *Crenarchaeota*, have been scarce (e.g., Jurgens et al 1997; Casamayor et al 2000; Auguet & Casamayor 2008; Llirós et al 2008; and for a recent review see Casamayor & Borrego 2009). Accordingly, the ecology and physiology of freshwater *Archaea* other than methanogens are pourly known.

The first archaeal phylogenetic reconstructions were based on cultured representatives (mainly hyperthermophiles, halophiles and methanogens). The resulting phylogenetic tree was composed of two main phyla, *Crenarchaeota* and *Euryarchaeota*, both containing a few branches. However, 16S rRNA gene surveys conducted on a wide variety of environments in the last two decades, have largely expanded our present view with the discovery of new widespread uncultured lineages. These new lineages deeply and divergently branch in the phylogenetic tree and are distantly related to hyperthermophilic lineages. Nowadays, 16S rRNA gene sequences from uncultured archaea are several orders of magnitude higher than those available from their cultured counterparts (*ca.* 80% are sequences from uncultured organisms; Robertson et al 2005). A precise taxonomic placement of these new sequences still remains uncertain especially considering the low culturability of *Archaea* that hindered the isolation of representative strains from a wider range of enviornments.

Another additional deficiency is the lack of environmental information associated with 16S rRNA gene sequences currently available in databases (DeSantis et al 2006a, b). Therefore, clade naming and clustering assignations are unclear, and sometimes lead to some confusing nomenclatures (Auguet et al, in press). At present, public databases contain a large number of archaeal 16S rRNA environmental sequences (*ca.* 40,000) from very different environments (Auguet et al, in press). To perform meta-analyses on global community patterns, accessory information associated to molecular data is compulsory to understand the environmental context of a given phylotype. Accordingly, a consensus towards a complete report of molecular and environmental data in public databases is

mandatory, both to support phylogenetic affiliations and to correctly interpret their ecological significance (Robertson et al 2005; Field et al 2008).

Microbial biogeography based on diversity and distribution patterns, especially for *Archaea*, has been largely overlooked and is nearly absent in general microbial ecology books (e.g., Bull 2004; Ogunsseitan 2005; Cavicchioli 2007; Garret & Klenk 2007). In order to elucidate which environmental forces primarily affect archaeal communities in lacustrine environments a global biogeographical analysis was conducted using molecular and statistical tools (Lozupone & Knight 2008; Auguet et al, in press).

7.2. Results

The application of Unifrac metrics to a home-made 16S rRNA gene lacustrine archaeal database revealed distinct environmental grouping according to the phylogenetic similarity of the different assemblages and to abiotic environmental parameters (Figure 7.1). Both 16S rRNA sequences and environmental sites grouped within 4 habitat typologies: Meso- to Eutrophic stratified lakes (EU), Oligotrophic lakes (OL) and Saline lakes (SL) and a final group comprised by unassigned sites and labelled as UC. From all the retrieved studies after literature and Genbank database searching, up to 10 different numerical and categorized environmental parameters were analysed (Table 7.1).

Salinity appeared as the environmental parameter showing the higher influence on the distribution of lacustrine archaeal communities (R^2=0.0510, $p<0.001$). With the exception of altitude, the rest of the analysed parameters also showed significance values ($p<0.01$), therefore contributing to some extent to modulate the distributional patterns of *Archaea* (Table 7.1). SL, EU and OL lakes were clearly segregated according to salinity and oxic/anoxic conditions. Those saline lacustrine environments included within SL category comprised lakes with strong periods of desiccation (e.g UmRisha, Fazda and Silver lakes) showing very distinct behaviour as compared with the remaing sites.

Oxygen (presence/absence) appeared as the second driving force (R^2=0.0473, $p<0.01$). In this regard, *ca.* 64% of all analysed sequences were retrieved from oxic waters while the rest came from anoxic or oxic-anoxic transition water masses. Nevertheless, the vast majority of sequences retrieved from EU lakes came from anoxic water compartments (data not shown).

Table 7.1. Statistical values for abiotic environmental parameters analysed.

Environmental parameter	R^2	Pr(>F)	Significance level[a]
Salinity	0.0510	0.001	***
Oxygen	0.0473	0.002	**
Longitude	0.0472	0.002	**
Lake typology	0.0469	0.003	**
Depth	0.0422	0.009	**
Latitude	0.0419	0.006	**
Trophic status	0.0404	0.009	**
Volume	0.0362	0.034	*
Surface	0.0356	0.020	*
Altitude	0.0319	0.185	ns[b]

[a]: the number of asterisks indicate its significance level: *** (<0.001), ** (<0.01), * (<0.1).
[b]: no significant.

None of the environmental parameters analysed in the present study could explain alone more than 5.1% (salinity; Table 7.1) of the variance from the environmental matrix. Furthermore, the strongest principal component axis only explained the 9.46% of variation (Fig. 7.1).

Table 7.2. Diversity estimators calculated for the different habitat typologies defined.

Habitat typology	PD[a]	PSV[b]
Meso- to Eutrophic lakes (EU)	8.62±0.41	0.832
Oligotrophic lakes (OL)	6.79±0.39	0.703
Saline lakes (SL)	5.78±0.00	0.400
Unclassified (UC)	5.40±0.22	0.660

[a]: Phylogenentic Diversity index.
[b]: Phylogenetic Species Variability index.

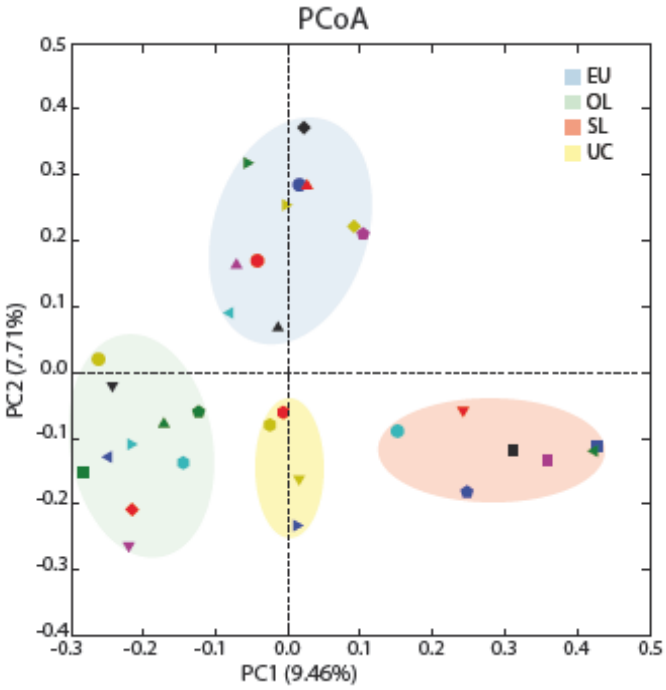


Figure 7.1. PCO (Principal Coordinates Analysis) obtained with a UniFrac distance matrix comparing the lacustrine environments summarized in Table 3.1 of Material and Methods section. Principal coordinate 1 (PC1) *vs.* principal coordinate 2 (PC2) is represented. Each symbol corresponded to each analysed site. Colour codes refer to habitat typologies as: light blue, Meso- to Eutrophic small meromictic Lakes (EU); light green, Oligotrophic Lakes (OL); light red, Saline Lakes (SL); and light yellow for the unclassified group (UC).

Phylogenetic Diversity and Phylogenetic Species Variability indicators

Phylogenetic diversity (PD) index was higher in EU (8.62±0.41) and OL (6.79±0.39) than in SL and the outliers (UC) (5.78±0.00 and 5.40±0.22, respectively; Table 7.2). Each habitat typology defined in the present study contained up to 8 distinct phylogenetic clusters (except for UC), with strong differences in their relative abundances (Fig. 7.2). When analysing the PSV (Phylogenetic Species Variability) for the four defined lacustrine habitat typologies, the highest PSV value was obtained for EU lakes (Table 7.2) revealing large community dispersion within this type of environments. Conversely, the lowest PSV value was obtained for saline environments (SL) reflecting a lower phylogenetic variability (Table 7.2). In fact, *Halobacteriales* represented up to 74% of all the sequences retrieved in SL environments while the relative abundance of a specific group in the remaing habitat typologies never exceed 57% (Fig. 7.2).

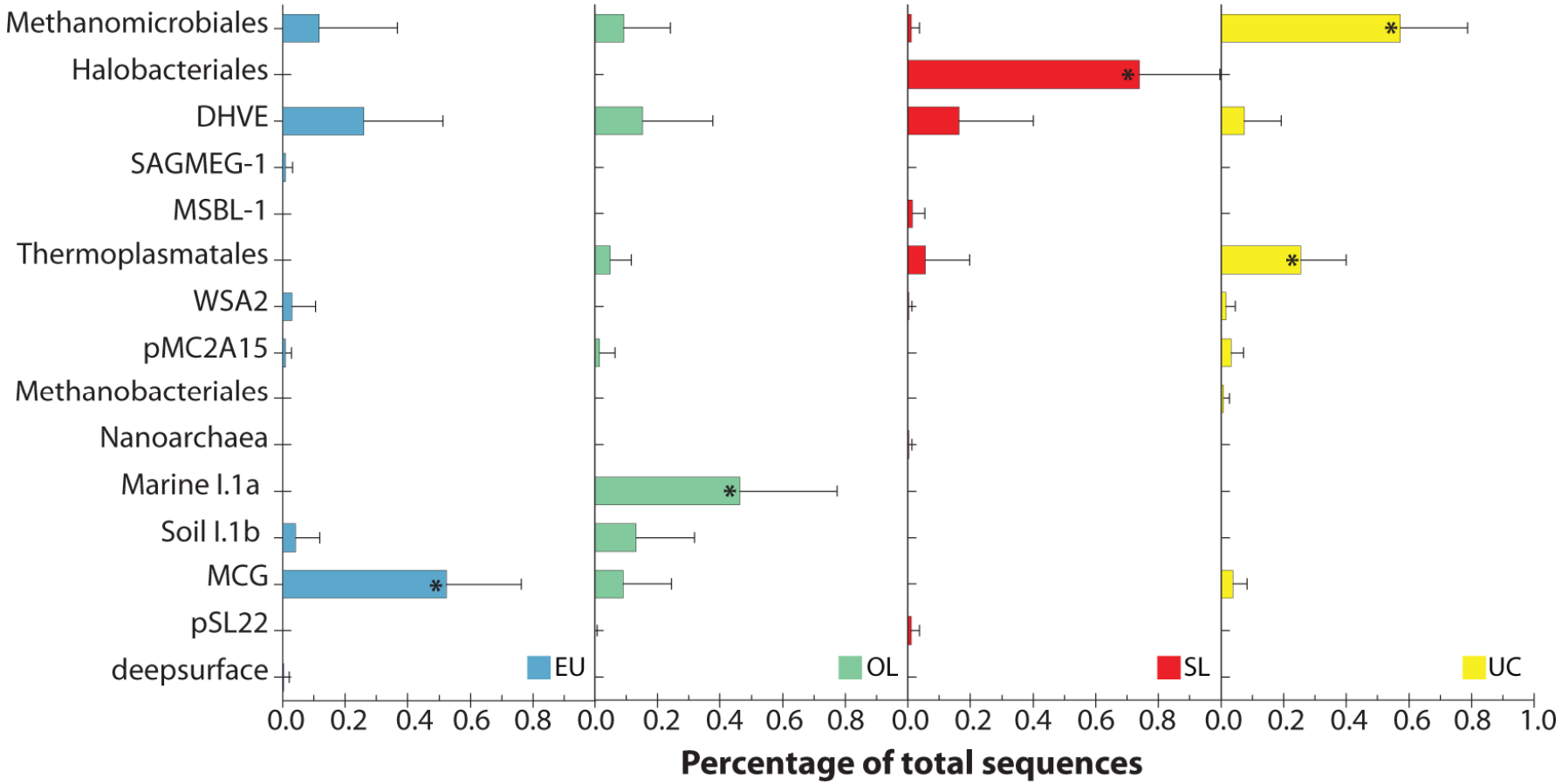


Figure 7.2. Bar plot representing the relative proportion of lacustrine archaeal lineages (based on sequence abundance) within each of the four identified lacustrine environmental typologies. Asterisks showed indicator archaeal lineages identified after the use of IndVal index at a significance level of p=0.01. Colour codes are the same as in Figure 7.1.

Table 7.3. Indicator taxa index (IndVal) for the different habitat typologies.

Habitat typology	Taxa	IndVal	Significance level
Meso- to eutrophic Lakes (EU)			
	MCG	80.50	**[a]
	DHVE	27.02	ns[b]
	SAGMEG-1	18.18	ns
	WSA2	11.18	ns
	deepsurface	9.09	ns
Oligotrophic Lakes (OL)			
	Marine I.1a	100	**
	Soil I.1b	49.50	?[c]
Saline Lakes (SL)			
	Halobacteriales	85.71	**
	MSBL-1	14.29	ns
	Nanoarchaeota	14.29	ns
	pSL22	8.40	ns
Unclassified (UC)			
	Methanomicrobiales	74.36	**
	Thermoplasmata	70.77	**
	Methanobacteriales	25.00	?
	pMC2A15	17.90	ns

[a]: significant ($p<0.01$).
[b]: no significant.
[c]: not enough data to test significance.

Indicator Value estimator

The 775 lacustrine archaeal 16S rRNA gene sequences analysed grouped in 15 clusters (Fig. 7.2). One third of them showed a significant indicator value (IndVal; $p<0.01$; labelled with an asterisks in Fig. 4.2). The *MCG*, *Marine I.1a*, *Halobacteriales*, *Methanomicrobiales* and *Thermoplasmata* had the higher IndVal values (from 70.77 to 100.00; see Table 7.3); while the remaining clusters showed non-significant IndVal values (<50) (Table 7.3). *DHVE* cluster was also predominantly retrieved in EU ecosystems (26%; Fig. 7.2), but no significant IndVal was observed ($p>0.01$; Table 7.3). Furthermore, *DHVE* sequences were detected in all habitat typologies, thus representing a putative cosmopolitan *Euryarchaeota* lineage. Conversely, *MCG*, *Marine I.1a* and *Halobacteriales* lineages were defined as indicator taxa for EU, OL and SL environment typologies, respectively (Table 7.3). None of these groups were significantly detected in the other habitats defined in the present study. *Marine I.1a* phylotypes, mainly related to the AOA *N. maritimus*, represented indicator taxa for those ecosystems categorised as OL.

Traditionally, these phylotypes were retrieved from marine environments were oligotrophy together with relaxed salinity influence took place. Recent analyses correlated the AOA presence with strict oligotrophy (Erguder et al 2009) as happened for those lacustrine environments classified within OL.

The lacustrine environments included within UC comprised different kinds of ecosystem, ranging from freshwater (Priest Pot) to saline (Manzallah) environments and from small lagoons to fens (Finland fen located near the Arctic), which represent a large physico-chemical heterogeneity. Even this heterogeneity, two indicator taxa (*Methanomicrobiales* and *Thermoplasmata*) were surprisingly identified but with the lowest significative IndVal values (Table 7.3 and Fig. 7.2). These taxa represented more than the 80% of phylotypes included in this habitat typology (UC).

7.3. Discussion

The use of molecular data (16S rRNA gene) without the culture component of a complete microbiology study is an obvious limitation when facing ecological studies (but see Ramette & Tiedje 2007; Auguet et al, in press). By now, only around 7,000 procaryotic species have been described and validated (International Committee on systematic of Prokaryotes) since the introduction of agar-based culture isolation procedures (Achtman & Wagner 2008) even though prokaryotes still represent "the unseen majority" (Whitman et al 1998). Within *Archaea*, about 18 out of the 49 currently accepted archaeal lineages contained one or more cultured representatives (Schleper 2007). Recently, it has been postulated that cultured archaeal phyla represented less than 2% of prokaryotic cultured phyla (Achtman & Wagner 2008). Moreover, with the increase in molecular information associated to *Archaea*, some readjustments on archaeal phylogeny will be performed (Robertson et al 2005; Schleper et al 2005; Brochier-Armanet et al 2008). A researcher's ability to make inferences and evaluate patterns from molecular and environmental data is limited by the quality of the available data. In this sense, 16S rRNA and other functional genes together with proper environmental descriptions are of interest to review archaeal nomenclatures and to correct phylogenetic classifications (Robertson et al 2005; Field et al 2008). Recently, it has been proposed a direct relationship between 16S rRNA lineages and environmental distribution at global scales for *Bacteria* and *Archaea* (Lozupone & Knight 2007; Auguet et al, in press; Barberan & Casamayor, submitted).

The use of indicator species (Dufrene & Legendre 1997) was succesfully applied to archaeal lineages supporting *Crenarchaeota group I.1a* as a marine planktonic group (Marine Planktonic Group I, DeLong 1998) and *group I.1b* as a soil group (Bintrim et al 1997; Jurgens et al 1997; Buckley et al 1998). Furthermore, these groups were the ones who better represent their environment segregation (Auguet et al, in press). Moreover a clear predominance of some characteristic archaeal lineages in lacustrine environments pointed towards a segregation of some of those lineages according to habitat (Llirós et al 2008, submitted; Auguet & Casamayor 2008; Auguet et al, in press). Therefore, if environmental forces select microorganisms on the basis of their functional capabilities, according to "everything is everywhere, but environment selects" reasoning (Bass-Becking 1934), indicator taxa should also be seen as key biogeochemical players in their characteristic habitats. The detection of *Crenarchaeota Marine I.1a* and *Soil I.1b* lineages (known groups that contain archaeal ammonia-oxidisers) in OL, where nitrification processes took place (Treusch et al 2005; Schleper et al 2005; Leininger et al 2006; Francis et al 2007; Llirós et al, submitted), supported this idea. Accordingly, recent molecular analyses of archaeal key genes involved in nitrification (*viz. amoA* gene) revealed a clear functional gene clustering according to habitat (Francis et al 2005, 2007; Beman et al 2007, 2008; Pouliot et al 2009; Llirós et al, submitted) mostly driven by oligotrophy (Erguder et al 2009). Therefore in oligotrophic marine and lacustrine environments but also in soil environments, ammonia-oxidising *Archaea* (AOA) predominate as evidenced by major recovery of *Crenarchaeota* groups *I.1a* and *I.1b*. Furthermore, *Halobacteriales* lineage is also a good example of phylogenetic and functional habitat concordance (e.g. Gasol et al 2004). Moreover, *DHVE* cluster appeared as the only phylogenetic group retreived in all defined habitats, hence revealing a cosmopolitan behaviour of these lineages. By contrast, indicator taxa could be also defined as endemic microorganisms in a given environment. Moreover, in clear contrast to marine bacterioplankton behaviour (Pommier et al 2007), cosmopolitan lacustrine archaea (*viz. DHVE*) showed lower relative abundances than the endemic or indicator ones (Fig. 7.2). Moreover, endemism revealed in the UC habitat type should be taken with care due to intrinsic heterogenity of this agrupation.

In relation to environmental drivers, salinity has been recently described as one of the major driving forces of bacterial and archaeal global distributions (Lozupone & Knight 2007; Auguet et al, in press), but also for lacustrine *Archaea* (this work). Moreover, the salinity influence for lacustrine *Archaea* must be associated to distinct lake dynamics of saline lakes in relation to the other lakes analysed in the present study. Furthermore, oxygen concentration appeared as the second environmental driver modulating lacustrine archaeal communities. Oxygen-richness, nutrient depletion and strong weather-related conditions are common traits for epilimnetic waters of stratified lakes, whereas the

hypolimnion is characterised by an opposite behaviour. It has been recently revealed a strong driving role of temperature and oxygen vertical gradients over the bacterial community composition within the water column of distinct stratified lakes (Shade et al 2008). It has been recently suggested a clear structuring effect of oxic-anoxic water column conditions for both a small stratified freshwater lagoon (Llirós et al 2008) and for a large oligotrophic stratified African lake (Llirós et al, submitted). Oxygen influence in lacustrine archaeal segregation could be observed after PCO analyses when lakes included in EU habitat typology segregated together with anoxic sequences from Lake Kivu (OL). At the same time, oxic-anoxic water column also structure the bacterial diversity patterns at global scales (Lozupone & Knight 2007).

Different biassing factors could somehow determine the described patterns. These biassing factors are not under the authors' control and have been minimised by the analytical tools applied. Even rely on molecular data (*viz.* PCR-based approaches), the selecting criteria applied in the present study should minimise this kind of bias. Moreover, one possible reason for the low relative abundance for lacustrine cosmopolitan Archaea could be the primming selectivity of archaeal primers as previously discussed (Teske & Sorensen 2008; Llirós et al 2008). Furthermore, results pointed towards an important bias due to poor sampling efforts in stratified lacustrine environments because of the own heterogeneity of stratified environments but also because of molecular surveys consisted on snapshots of their microbial communities or water compartments. Accordingly more sampling efforts are needed to obtain a fully comprehensive picture of the lacustrine habitats, especially those defined as EU.

Altogether, we present global distribution patterns for lacustrine mesophilic archaeal assemblages with two strong driving environmental components such salinity and oxygen concentration. Furthermore, the identification of at least one indicator lineage for each of the analysed lacustrine typologies could contribute to a more understandable and ecological archaeal classification. These results would help to reinforce the archaeal nomenclature adding clarifying information to actual clusters named according to a commonly retreived clone sequence.

8. General Discussion

8. General Discussion

Mesophilic Archaea have been found in the planktonic assemblage of different stratified freshwater lakes worldwide such as Mariager Fjord (Ramsing et al 1996; Teske et al 1996); Lake Saelenvannet (Ovreas et al 1997); Lake Cadagno (Bosshard et al 2000); Crater Lake (Urbach et al 2001, 2007); Mono Lake (Humayoun et al 2003); Lake Pavin (Lehours et al 2005, 2007); distinct karstic lagoons (Casamayor et al 2001b); and high mountain and arctic lakes (Pernthaler et al 1998; Auguet & Casamayor 2008; Pouliot et al 2009). In stratified lakes the different physico-chemical conditions along the water column generate gradients of light, oxygen, sulfide and nutrients which allow microbial populations to stratify in narrow water layers (e.g., van Gemerden & Mas 1995; Casamayor et al 2000) thus seggregating both microbial communities and metabolic processes. In these environments, the archaeal contribution in terms of abundance is highly variable depending on the studied ecosystems with overall lower abundances than those reported for the marine biome (Casamayor & Borrego 2009).

The study of low abundant microbial populations faces methodological problems, specially related with sensitivity and bias. This is especially true for the ecological studies dealing with freshwater Archaea. In the present work nucleic acids were extracted using a combination of enzymatic cell lysis and a modified CTAB extraction protocol (Lodhi et al 1994) to achieve the best cell lysis and subsequent efficient nucleic acids recovery. In all samples analysed, direct 16S rRNA gene PCR amplifications using general archaeal primers failed and nested-PCR reactions were needed to obtain suitable amplicons for further DGGE fingerprinting. Similar results have been recently reported by Vissers and co-workers (2009) when analysing sediment and water samples from different European freshwater lakes. However, it is well-known that nested-PCR has some drawbacks (e.g., priming selectivity and specificity of internal primers; Suzuki et al 1998; Mahmood et al 2006) but it still offers some advantages such as the possibility to recover the maximal microbial diversity (Teske & Sorensen 2008) and the characterisation of non-dominant phylotypes (Benlloch et al 2002; Pedrós-Alió 2006). This is improved when different primer combinations (targeting general or specific groups) are applied. In this regard, it was recently demonstrated that PCR primers miss half of the microbial diversity in any natural sample (Hong et al 2009). These authors suggest that the unique alternative is the combination of molecular techniques (e.g., clone libraries, fingerprinting) and PCR primers to minimize problems and to obtain the most complete picture of the microbial diversity in any environmental sample. Our results evidenced that the design of a new primer (ARC337 used as forward primer) have increased the recovery efficiency for

members of the archaeal *MCG* lineage that otherwise remained undetectable. Moreover, current FISH and CARD-FISH probes and PCR primers targeting large taxonomic groups were designed more than 10 years ago (e.g., EUB338, Amann et al 1990; ARC915, Stahl & Amann 1991) when the rRNA database was less than 10% of its current size. In fact, the general probe for Bacteria (EUB338) was improved by modifying the original probe sequence (Daims et al 1999) to increase its targert coverage (up to 96% of bacterial entries on SILVA's database; Amann & Fuchs 2008). In spite of this, the ARC915 oligonucleotide has received little attention and it still has some flaws that must be addresed to avoid unespecificities (Amann & Fuchs 2008). In fact, ARC915 oligonucleotide might not target the 100% of the archaeal community when used as reverse primer (Amann & Fuchs 2008). In this sense, Teske & Sorensen (2008) found a 34% of missmatched sequences for this primer, fairly agreing with values reported by Amann & Fuchs (2008). Overall, an improvement on ARC915 coverage might be performed in both probe and PCR-primer use. It is then strongly recomended to submit long and high-quality rRNA sequences to public database to allow a better *in silico* design that permits the best target coverage.

Previous analyses on karstic lagoons of the Banyoles lacustrine area using direct-PCR-DGGE analyses with the general primer combination (ARC344f/ARC915r), only retrieved 12 archaeal phylotypes corresponding to methanogens (4), *Thermoplasmatales* (7) and *Crenarchaeota* (1; Casamayor et al 2001b). These findings suggested a low archaeal diversity and richness in these environments. The archaeal planktonic assemblage in Lake Vilar and Coromina lagoon was followed to unveil the hidden archaeal diversity in the planktonic assemblage by using several PCR primer combinations. Overall, up to 186 distinct phylotypes were recovered from the two lagoons. The archaeal planktonic assemblage in both systems was mainly represented by phylotypes affiliated to *MCG* Crenarchaeota (63.3% and 17.6% of total retrieved phylotypes in Lake Vilar and Coromina lagoon, respectively); to *DHVE* (28.4% and 23.5%, respectively); and to methanogenic archaeal clusters (exclusively found in Coromina lagoon, 47.1% of the total phylotypes). It is interesting to note that the major fraction of the *MCG* phylotypes were mainly recovered from suboxic and anoxic water layers agreeing with recent reports suggesting *MCG* as a lineage mainly composed by anaerobic heterotrophs (Biddle et al 2006). Concerning abundances, quantification analyses carried out in both lakes have shown a low contribution of archaeal cells to total prokaryoplankton. In this sense, both lagoons harboured high-rich but low abundant archaeal assemblages. These results further support the necessity of use nested-PCR techniques when performing molecular analyses but also the availability of better molecular probes. Besides, low archaeal abundances also suggested either a poor adaptation to the prevalent environmental conditions or a slow growth rates under these conditions. Enrichment cultures set with water samples from

Lake Vilar also support the idea that freshwater Archaea have very long generation times (Plasencia A, unpublished results). Despite the low numbers measured in both ecosystems, the long-term persistence of the main archaeal lineages (*MCG*-, *DHVE*- and methane-related phylotypes) suggest that the planktonic archaeal assemblage compose a sort of *seed-bank* waiting for better conditions to grow. The statistical analyses carried out with a 16S rRNA gene database for lacustrine environments evidenced that *MCG* can be considered indicator taxa for these stratified and karstic environments. In turn, the archaeal assemblage thriving in the oligotrophic stratified Lake Kivu (either collected from open or closed basins) appeared to be fairly homogeneous in all sampling basins exhibiting a segregation imposed by the oxic-anoxic transition. Phylotypes related to AOA (*Marine I.1a and Soil I.1b Crenarchaeota* groups; Prosser & Nicol 2008) represented the larger fraction of the archaeal assemblage in oxic and suboxic water layers. The phylotypes thriving in the upper anoxic waters were mainly affiliated to *MCG*, *ANME* and methanogenic clusters. Interestingly, the diversity within *Marine Group I.1a* was very low (only one phylotype was retrieved), but in contrast *Soil Group I.1b* included up to 17 distinct phylotypes. A link between the archaeoplankton of Lake Kivu and nitrification processes occuring at some depths (oxic-anoxic transition) could be envisaged on the basis of archaeal abundances found, phylogenetic affiliations and identification of functional markers for archaeal ammonia oxidation (*amoA*). In this regard, this is the first report of AOA in a non-polar freshwater environment and suggests that these microorganisms can contribute to N cycle in all aquatic ecosystems characterised by oligotrophy (Erguder et al 2009).

Concerning activity, evidences of distinct metabolic processes carried out by the archaeoplankton in differnt environments have been previously obtained using indirect apporaches such as single-cell techniques (e.g., MAR-FISH, Teira et al 2004, 2006; Varela et al 2008); isotopic composition of signature molecules (Hoëfs et al 1997; Schouten et al 2000; Kuypers et al 2001; Sinninghe-Damsté et al 2002a, b); or the detection of functional genes involved in either nitrification (the *amoA* gene, Francis et al 2005) or CO_2 fixation pathways (*accC* gene, Auguet et al 2008; Yakimov et al 2009). Furthermore, carbon fixation processes mediated by *Archaea* may cover a wide range of metabolic alternatives involving N and S cycles (Berg et al 2007). Although, dark carbon fixation processes have been traditionally neglected even being quantitatively important in oxic-anoxic interfaces (e.g., Cluver & Brunskill 1969; García-Cantizano et al 2005). Besides, recent studies have demonstrated that mesophilic *Archaea* could use inorganic carbon as a sole carbon source (Kuypers et al 2001; Pearson et al 2001; Wuchter et al 2003; Herndl et al 2005; Ingalls et al 2006). The detection of *amoA* gene signatures in the water column of Lake Kivu suggests that AOA might be involved in cycling N in this lake. Moreover, analyses carried out with the archaeal *amoA* gene sequences retrieved

from Lake Kivu showed a sequence clustering according to habitat, as previously suggested by different authors (Francis et al 2005, 2007; Beman et al 2007, 2008; Pouliot et al 2009). On the other hand, the application of MICRO-CARD-FISH using radiolabeled bicarbonate as tracer revealed low archaeal dark inorganic carbon uptake in the water samples analysed from Coromina lagoon. This low carbon uptake could be related to both the prevalence of putative anaerobic archaeal heterotrophs such the MCG-related phylotypes (Biddle et al 2006) and the high amount of organic matter in this lagoon. Finally, the switching from anoxic to complete oxygenation of the water column together with eutrophic condition and an active sulfur cycle in both karstic lakes are environmental conditions far from those considered as optimal for autotrophic archaea such as AOA (Erguder et al 2009).

Altogether, the present work demonstrated the presence of very rich archaeal communities within the water column of several stratified lakes. Also, it promotes further suppot to evidence the importance of applying different molecular (functional genes analyses) and single-cell approaches both to identify active members of the archaeoplankton and to ascertain their roles in the ecosystem functioning. Recent reports suggested that some central microbial activities can be carried out by a small, but very active subset of community members (Sogin et al 2006; Fry et al 2008). On the contrary, a high abundance of a specific prokaryotic group does not necessarily imply a high contribution to the biogeochemistry of the system. Further work is then needed to better correlate some metabolic processes and microbial players involved, especially in environments with complex microbial communities. In this regard, the isolation of the key players is mandatory to warrant further experimentation that permits to unequivocally link identity and function.

9. Future prospects

9. Future prospects

Microbial ecology research focused on Archaea has produced an impressive amount of data shortly after their recognition as a third domain of life. Nowadays, nearly any described environment on Earth harbours either an archaeal cultured representative or an archaeal retrieved 16S rDNA sequence. In this sense, microbial ecologists have acquired a wide knowledge on the diversity of natural archaeal communities, their dynamics and environmental drivers that affect their distribution and, in recent years, on their activity and impact on the biogeochemical cycles. Moreover, molecular techniques applied to archaeal microbial ecology has produced large amounts of information, far beyond those results provided by just applying classical microbiology approaches.

The identification of different archaeal lineages that prevail in the environments studied in this study has suggested the existence of archaeal indicator taxa whatever the environment sampled. In this sense, members of the MCG (*Miscellaneous Crenarchaoetic Group*) group were mainly retrieved from the anoxic hypolimnion of those karstic, small, stratified and meso- to eutrophic freshwater lakes; as is the case of Lake Vilar and Coromina lagoon. In turn, phylotypes affiliated within the *Marine Crenarchaeota I.1a* group were exclusively found in oligotrophic freshwater lakes (e.g., Lake Kivu) resembling the marine environments where these Archaea were originally found.

Overall, further efforts in some aspects of the ecology and biology of Archaea deserve attention in the future:

Diversity. Microbial diversity has been extremely expanded by distinct molecular approaches producing some important bias in archaeal phylogeny in comparison to information obtained from culture representatives (Schleper et al 2005). Moreover, current molecular tools such as PCR-primer and phylogenetic or functional probes are still not optimal since either miss some members of the target group or have a large number of out-group hits (Amann & Fuchs 2008; Hong et al 2009). In this regard, available molecular databases (e.g., GenBank) and bioinformatic tools (e.g., ARB software package) allows a precise refinement and design of better probes and primers. Accordingly, new primer and probes designed from the database constructed with the environmental 16S rRNA sequences retrieved in the present work might improve FISH and/or CARD-FISH detection and allow the specific monitoring of archaeal populations in these stratified environments through time and space. Special efforts must be focused in targeting *MCG*; *DHVE* and *Marine I.1a* lineages.

Activity. Evidences based on molecular analyses, carbon-isotopic signatures and environmental conditions suggested that MCG group, one of the most commonly retrieved phylogenetic groups in our studies, are mostly anaerobic heterotrophs. Results based on radiolabeled bicarbonate incorporation in a hypereutrophic lagoon revealed low dark carbon incorporation mediated by Archaea (<5% of total community). Accordingly, further experiments to stimulate archaeal growth must be conducted using distinct radiolabeled substrates as simple organic matter (e.g., amino acids, Teira et al 2004, 2006; Varela et al 2008) or other reduced compounds to fuel this putative heterotrophic metabolism of MCG.

Identification of signature functional genes. Distinct molecular results evidenced that Archaea posse functional genes involved in key metabolic processes as their bacterial counterparts (e.g., *accC* or *amoA* genes). Moreover, environmental surveys using these functional gene markers revealed distinct environmental distributions. Due to the suggested behaviour for the archaeal assemblage thriving in stratified karstic lakes and the strong influence of sulfur cycle in these environments, other functional genes might be targeted to establish links between archaeal identity and function in these ecosystems. In this sense, the presence of an active sulfur cycle may provide an optimal environment for sulfur-based metabolisms such as those described in thermophilic archaea (Kletzin et al 2004). Potential gene biomarkers for such studies could be those involved in sulfate reduction to sulfite (*aps* gene; Friedrich et al 2002); in sulfite reduction to sulfide (*dsrAB* gene; Loy et al 2009) or in sulfide oxidation (*sqr* gene; Kletzin et al 2004). Future research in these topics might help to determine to what extent Archaea thriving in temperate sulfur-rich environments have an impact on sulfur cycle.

Cultivation and Isolation. To overcome the low culturability of non-extremophilic Archaea is a topic that must be addressed by microbiologists as soon as possible. Only after new isolates available in public collections for further experimentation, our knowledge on archaeal physiology and ecology will be complete. By now, most of the current knowledge an archaeal metabolism and activity rely on indirect measurements based on genomic data. In this regard, novel isolation techniques and imaginative enrichment strategies must be applied to optimise time and efforts and to yield as many isolates as possible. In this sense, undergoing enrichment cultures in our laboratory are promising (Plasencia A, unpublished results). The application of culture techniques as microdrop (Bruns et al 2003) and/or micromanipulation (Ishoy et al 2006) might help to isolate archaeal cells from highly enriched archaeal cultures. As isolation efforts produce new pure cultures; classical microbiology studies (such those based on physiology, metabolism or biochemistry) will provide a better understanding of archaeal

diversity and activity. The isolation of representatives of rare archaeal lineages, which have solely been detected by molecular techniques, is an exciting topic.

10. Conclussions

10. Conclusions

1. The application of different primer combinations to study planktonic archaeal assemblages in stratified freshwater lakes allowed us to recover a high number of archaeal phylotypes that otherwise had remained undetectable. Despite some limitations, the new primer ARC337f provides a useful bias towards several lineages of lacustrine Crenarchaeota.

2. Stratified karstic lakes ranging from mesotrophy to hypereutrophy usually contain very rich, but low abundant, planktonic archaeal communities, especially in their anoxic, sulfide-rich hypolimnia. These cold and anoxic waters constitute an unexplored source of archaeal richness.

3. In stratified karstic freshwater lakes, the archaeal communities are mainly composed by members of the *Miscellaneous Crenarchaeotic Group* (MCG) and the *Deep Hydrothermal Vent Euryarchaeota* (DHVE) clusters. In spite of it, both groups did not reach high cell densities. Their long-term persistence through seasons suggests that they constitute a sort of archaeal *seed-bank*, with low population losses, waiting for better conditions to growth.

4. The MCG and the DHVE archaeal lineages have very complex phylogenies, including a growing number of phylotypes recovered from very distinct environments. Unfortunately, their metabolic properties and ecological implications are still far from being resolved.

5. In oligotrophic stratified lakes, the archaeal assemblages are mainly composed by ammonia oxidizers (from both *Marine Group I.1a* and *Soil Group I.1b* clusters) at suboxic water layers (redoxcline) whereas MCG Crenarchaeota are prevalent in the anoxic water layers. The identification of AOA in a temperate freshwater lake (Lake Kivu) is a significant contribution to the ecology of Archaea since it is the first time that these lineages have been detected in a non-polar, non-marine environment.

6. The incorporation experiments carried out in a hipereutrophic stratified lagoon (Coromina) showed a low archaeal contribution (up to 10%) in uptaking radiolabeled bicarbonate under dark incubation conditions. However, known unspecificities of ARC915 probe may hinder the proper interpretation of the experimental data. Further refinement of ARC915 probe or the design of a new, better probe is therefore needed.

7. Our results suggest that archaeal lineages prevalent in eutrophic sulfurous freshwater lakes are mainly represented by anaerobic heterotrophs although some phylotypes might be capable to incorporate inorganic carbon.

8. Unexpectedly, bacteria traditionally considered heterotrophic such as *Cytophaga* appeared to be able to uptake bicarbonate both in the oxic epilimnion and in the anoxic bottom water layers. This observation deserves further investigation.

9. Statistical analyses recognized *MCG* and *Marine Group I.1a* as indicator archaeal taxa in stratified eutrophic and oligotrophic freshwater lakes, respectively.

10. Oxygen and salinity appear as the main environmental drivers affecting the global distributional patterns of mesophilic Archaea in lacustrine environments.

11. References

11. References

Abella CA & Garcia-Gil LJ (1998) Diel migrations as a mechanism for enrichment of natural populations of branching species of Pelodictyon. In: Green Photosynthetic Bacteria; Olson JM, Ormerod JG, Amesz J, Stackembrandt E & Trüper HG (Eds.) Plenum Press, pp 269–285.

Achtman M & Wagner M (2008) Microbial diversity and the genetic nature of microbial species. Nature Reviews Microbiology 6 (6), 431–440.

Agogué H, Brink M, Dinasque J & Herndl GJ (2008) Major gradients in putatively nitrifying and non–nitrifying Archaea in the deep North Atlantic. Nature Letters 456 (7223), 788–791.

Alcántara-Hernández RJ, Valenzuela–Encinas C, Zavala–Díaz de la Serna FJ, Rodriguez–Revilla J, Dendooven L & Marsch R (2009) Haloarchaeal assimilatory nitrate–reducing communities froma saline alkaline soil. FEMS Microbiology Letters 298 (1), 56–66.

Allers T & Mevarech M (2005) Archaeal genetics – the third way. Nature Reviews Genetics 6, 58–73.

Alonso C & Pernthaler J (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. Applied and Environmental Microbiology 71 (4), 1709–1716.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215, 403–410.

Amann R, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA–targeted oligonucleotid probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology 56 (6), 1919–1925.

Amann R & Fuchs BM (2008) Single–cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Reviews Microbiology 6, 339–349.

Amann R & Ludwig W (2000) Ribosomal RNA–targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiology Reviews 24, 555–565.

Amend JP & Shock EL (2001) Energetics of overall metabolic reactions o thermophilic and hyperthermophilic Archaea and Bacteria. FEMS Microbiology Reviews 25, 175–243.

American Public Health Association (1998) Standard Methods for the Examination of Water and Wastewater, 20th Ed. APHA, Washington DC.

Auguet JC & Casamayor EO (2008) A hotspot for cold crenarchaeota in the neuston of high mountain lakes. Environmental Microbiology 10 (4), 1080–1086.

Auguet JC, Borrego CM, Bañeras L & Casamayor EO (2008) Fingerprinting the genetic diversity of the biotin carboxylase gene (accC) in aquatic ecosystems as a potential marker for studies of carbon dioxide assimilation in the dark. Environmental Microbiology 10 (10), 2527–2536.

Auguet JC, Barberan A & Casamayor EO (2009) Global ecological patterns in uncultured Archaea. The ISME Journal, *in press*.

Balch WE, Magrum LJ, Fox GE, Wolfe RS & Woese CR (1977) An ancient divergence among the bacteria. Journal of Molecular Evolution 9, 305–311.

Bañeras Ll & García-Gil LJ (1996) Role of photosynthetic microbial populations in the phosphorous exchange through the oxic–anoxic boundary in a meromictic eutrophic lake. Archieves of Hydrobiology/Special Issue Advances in Limnology 48, 171–181.

Bañeras Ll, Rodriguez-Gonzalez J & García-Gil LJ (1999) Contribution of photosynthetic sulfur bacteria to the alkaline phosphatase activity in anoxic aquatic ecosystems. Aquatic Microbial Ecology 18, 15–22.

Barberan A & Casamayor EO (2009) Phylogenetic Beta–diversity patterns of surface bacterioplankton communities. Submitted.

Barns SM, Delwiche CF, Palmer JD & Pace NR (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proceedings of National Academy of Sciences USA 93, 9189–9193.

Baroos JA (1995) Isolation, growth, and maintenance of hyperthermophiles. In: Archaea, a laboratory manual, Thermophiles. Robb FT & Place AR (Eds.), Cold Spring Harbor, New York, pp 15–23.

Bass-Becking LGM (1934) Geobiologie of inleideing tot de milieukunde. van Stockum WP & Zoon NV (Eds.) den Haag, The Netherlands.

Béjà O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN & DeLong EF (2000) Bacterial rhodopsin, evidence for a new type of phototrophy in the sea. Science 289, 1902–1906.

Béjà O, Spudich EN, Spudich JL, Leclerc M & DeLong EF (2001) Proteorhodopsin phototrophy in the ocean. Nature 411, 786–789.

Beman JM, Roberts KJ, Wegley L, Rohwer F & Francis CA (2007) Distribution and diversity of archaeal ammonia monooxygenase genes associated with corals. Applied and Environmental Microbiology 73 (17), 5642–5647.

Beman JM, Popp BN & Francis CA (2008) Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California. The ISME Journal 2, 429–441.

Benlloch S, López–López A, Casamayor EO, Ovreas L; Goddard V, Daae FL, Smerdom G, Massana R, Joint I, Thingstad F, Pedrós–Alió C & Rodríguez–Valera F (2002) Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern. Environmental Microbiology 4 (6), 349–360.

Berg IA, Kockelkom D, Buckel W & Fuchs G (2007) A 3–hydroxypropionate/4–hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. Science 318, 1782–1786.

Bernander R (2000) Chromosome replication, nucleoid segregation and cell division in archaea. Trends in Microbiology 8, 278–283.

Biddle JF, Lipp JS, Lever MA, Lloyd KG Sorensen KB, Anderson R, Fredericks HF, Elvert M, Kelly TJ, Schrag DP, Sogin ML, Brenchley JE, Teske A, House CH, & Hinrichs KU (2006) Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. Proceedings of National Academic of Sciences USA 103 (10), 3846–3851.

Bintrim SB, Donohue TJ, Handelsman J, Roberts GP & Goodman RM (1997) Molecular phylogeny of Archaea from soil. Proceedings of National Academy of Sciences USA 94, 277–282.

Böck A & Kandler O (1985) Antibiotic sensitivity of archaebacteria. In, The Bacteria. A treatise on structure and function, Archaebacteria, Vol. VIII. Woese CR & Wolfe RS (Eds.) New York, Academic Press, pp. 525–544.

Boetius A, Ravenschlag K, Schubert CJ, Rickert D, Widdel F, Gieseke A, Amann R, Jorgensen BB, Witte U & Pfannkuche (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407, 623–626.

Borrego CM & García-Gil LJ (1994a) Caracterización limnológica de la Coromina, una laguna hipereutrófica del sistema del sistema lacustre de Banyoles. Limnética 10 (1), 43–51. (in spanish).

Borrego CM & García-Gil LJ (1994b) Separation of bacteriochlorophyll homologues from green photosynthetic sulfur bacteria by reversed–phase HPLC. Photosynthesis Research 41, 157–163.

Borsheim KY, Kuenen JG, Gottschal KJ & Dundas I (1985) Microbial activities and chemical gradients in the chemocline of a meromictic lake in realtion to the precision of the sampling procedure. FEMS Microbiology Ecology 31, 337–341.

Bosshard PP, Santini Y, Grüter D, Stettler R & Bachofen R (2000) Bacterial diversity and community composition in the chemocline of the meromictic alpine Lake Cadagno as revealed by 16S rDNA analysis. FEMS Microbiology Ecology 31, 173–182.

Boucher D, Jardillier L & Debroas D (2006) Succession of bacterial community composition over two consecutive years in two aquatic systems, a natural lake and a lake–reservoir. FEMS Microbiology Ecology 55, 79–97.

Boucher Y (2007) Lipids: Biosynthesis, function and evolution. In: Archaea, Molecular and Cellular Biology. Cavicchioli R (Ed.) Washington, ASM Press; pp, 341–354.

Bräuer SL; Yavitt JB & Zinder SH (2004) Methanogenesis in McLean Bog, an acidic peat bog in upstate New York: stimulation by H_2/CO_2 in the presence of Rifampicin, or by low concentration of actetate. Geomicrobiology Journal 21, 433–443.

Bräuer SL, Yashiro R, Ueno NG, Yavitt JB & Zinder SH (2006) Characterization of acid–tolerant H_2/CO_2–utilizing methanogeneic enrichment cultures from an acidic peat bog in New York State. FEMS Microbiology Ecology 57 (2), 206–216.

Briée C, Moreira D & López-García P (2007) Archaeal and Bacterial community composition of sediment and plankton from a suboxic freshwater pond. Research in Microbiology 158, 213–227.

Brochier-Armanet C, Boussau B, Gribaldo S & Forterre P (2008) Mesophilic crenarchaeota, proposal for a third archaeal phylum, the Thaumarchaeota. Nature Reviews Microbiology 6, 245–252.

Brock TD, Brock ML, Bott TL & Edwards MR (1971) Microbial life at 90ºC, the Sulfur Bacteria of boulder spring. Journal of Bacteriology 107, 303–314.

Brown JR (1999) Universal tree of life. In, Encyclopedia of Life Sciences, http://www.els.net; London, Nature Publishing Group.

Bruns A, Cypionka H & Overmann J (2002) Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. Applied and Environmental Microbiology 68 (8), 3978–3987.

Bruns A, Hoffelner H & Overmann J (2003) A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. FEMS Microbiology Ecology 45, 161–171.

Buckley DH, Graber JR & Schmidt TM (1998) Phylogenetic analysis of nonthermophilic members of the Kingdom Crenarchaeota and their diversity and abundance in soils. Applied and Environmental Microbiology 64 (11), 4333–4339.

Bull AT (2004) Microbial Prospecting ASM Press, American Society for Microbiology, Washington, DC.

Burggraf S, Huber H & Stetter KO (1997) Reclassification of the new archaeal orders and families in accordance with 16S rRNA sequence data. International Journal of Systematic Bacteriology 47, 657–660.

Burns DG, Camakaris HM, Janssen PH & Dyall-Smith ML (2004) Cultivation of Walsby's square haloarchaeon. FEMS Microbiology Letters 238, 469–473.

Camacho A, Erez J, Chicote A, Florín M, Squires MM, Lehmann C & Bachofen R (2001) Microbial microstratification, inorganic carbón photoassimilation and dark carbon fixation at the chemocline of the meromictic Lake Cadagno (Switzerland) and its relevance to the food web. Aquatic Sciences 63, 91–106.

Cann IKO (2008) Cell sorting protein homologs reveal an unusual diversity in archaeal cell division. Proceedings of National Academy of Sciences USA 105 (48), 18653–18654.

Casamayor EO, Schäffer H, Bañeras L, Pedrós-Alió C & Muyzer G (2000) Identification of and spatio–temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 66 (2), 499–508.

Casamayor EO, García-Cantizano J, Mas J & Pedrós-Alió C (2001a) Primary production in estuarine oxic/anoxic interfaces: contribution of microbial dark CO_2 fixation in the Ebro River salt wedge estuary. Marine Ecology Progress Series 215, 49–56.

Casamayor EO, Muyzer G & Pedrós-Alió C (2001b) Composition and temporal dynamics of planktonic archaeal assemblages from anaerobic sulfurous environments studied by 16S rDNA denaturing gradient gel electrophoresis and sequencing. Aquatic Microbial Ecology 25, 237–246.

Casamayor EO, Pedrós-Alió C, Muyzer G & Amann R (2002) Microheterogeneity in 16S rDNA-defined bacterial populations from a stratified planktonic environment is realted to temporal succesion and to ecological adaptations. Applied and Environmental Microbiology 68 (4), 1706–1714.

Casamayor EO, García-Cantizano J & Pedrós-Alió C (2008) Carbon dioxide fixation in the dark by photosynthetic bacteria in sulfide-rich stratified lakes with oxic–anoxic interfaces. Limnology and Oceanography 53 (4), 1193–1203.

Casamayor EO & Borrego CM (2009) Archaea in inland waters. In: Enclyclopedia of Inland Waters. Likens G (Ed.), Elsevier Academic Press, Oxford, pp 167–181.

Cavicchioli R (2007) Archaea: Molecular and Cellular Biology. Washington, ASM Press.

Chaban B, Ng SYM & Jarrell KF (2006) Archaeal habitats – from the extreme to the ordinary. Canadian Journal of Microbiology 52, 73–116.

Cloern JE, Cole BE & Oremland RS (1983) Autotrophic processes in meromictic Big Soda Lake, Nevad. Limnology and Oceanography 28, 1049–1061.

Connon SA & Giovannoni SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new many isolates. Applied and Environmental Microbiology 68 (8), 3878–3885.

Coolen MJL, Abbas B, van Blejiswijk J, Hopmans EC, Kuypers MMM, Wakeham SG & Sinninghe–Damsté JS (2007) Putative ammonia-oxidizing Crenarchaeota in suboxic waters of the Black Sea, a basin-wide ecological study using 16S ribosomal and

functional genes and membrane lipids. Environmental Microbiology 9 (4), 1001–1016.

Culver DA & Brunskill GJ (1969) Fayetteville Green Lake, New York. V. Studies of primary production and zooplankton in a meromictic marl lake. Limnology and Oceanography 14, 862–873.

Daims H, Bruhl A, Amann R, Schleifer KH, & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology 15, 593–600.

Daubin V, Gouy M & Perrière G (2001) Bacterial molecular phylogeny using supertree approach. Genome Informatics 12, 155–164.

Davis KER, Joseph SJ & Janssen PH (2005) Effects of growth, inoculum size, and incubation time on culturability and isolation of soil bacteria. Applied and Environmental Microbiology 71 (2), 826–834.

de Iongh H, Spliethoff P & Frank V (1983) Feeding habits of the clupeid Limnothrissa miodon (Boulenger), in Lake Kivu. Hydrobiologia 102 (2), 113–122.

de la Torre JR, Walker CB, Ingalls AE, Könnek M & Stahl DA (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. Environmental Microbiology 10 (1), 810–818.

de Rosa M, Gambacorta A, Tricone A, Basso A, Zillig W & Holz I (1987) Lipids of Thermococcus celer, a sulfur–reducing Archaebacterium: Structure and biosynthesis. System Applied Microbiology 9, 1–5.

de Rosa M & Gambacorta A (1988) The lipids of archaebacteria. Progress in Lipid Research 27 (3), 153–175.

Degens E, Herzen RP, Wong HK, Deuser WG & Jannasch HW (1973) Lake Kivu, structure, chemistry and biology of an East African Rift Lake. Geologische Rundschau 62, 245–277.

DeLong EF (1992) Archaea in coastal marine environments. Proceedings of National Academy of Sciences USA 89, 5685–5689.

DeLong EF (1998) Everything in moderation, Archaea as "non-extremophiles". Current Opinion in Genetics and Development 8, 649–654.

DeLong EF (2003) Oceans of Archaea. ASM News 69 (10), 503–511.

DeLong EF, Taylor LT, Marsh TL & Preston CM (1999) Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and Fluorescent in situ Hybridization. Applied and Environmental Microbiology 65 (12), 5554–5563.

DeSantis TZ, Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R & Andersen GL (2006a) NAST, a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Research 34, 394–399.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P & Andersen GL (2006b) Greengenes, a chimera–checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72, 5069–5072.

Deuser WG, Degens ET & Harvey GR (1973) Methane in Lake Kivu, New data bearing its origin. Science 181, 51–54.

Donachie SP, Foster JS & Brown MV (2008) Culture clash, challenging the dogma of microbial diversity. The ISME Journal 1, 97–102.

Dufrene M & Legendre P (1997) Species assemblages and indicator species, The need for a flexible asymmetrical approach. Ecological Monographs 67, 345–366.

Durisch–Kaiser E, Klauser L, Wehrli B & Schubert C (2005) Evidence of intense archaeal and bacterial methanotrophic activity in the Black Sea water column. Applied and Environmental Microbiology 71, 8099–810.

Edgell DR & Doolittle WF (1997) Archaea and the origin(s) of DNA replication proteins. Cell 89, 995–998.

Elferinck MG, de Wit JG, Driessen AJ & Konings WN (1994) Stability and proton–permeability of liposomes composed of archaeal tetraether lipids. Biochimica et Biophysica Acta 1193, 247–254.

Elsaied HE & Maruyama A (2008) Biodiversity of archaea in Manzala lake, Egypt, based on 16S rRNA gene. Egyptian Journal of Genetics and Cytology 37, 57–72.

Erguder TH, Boon N, Wittebolle L, Marzorati M & Verstraette W (2009) Environmental factors shapping the ecological niches of ammonia–oxidizing archaea. FEMS Microbiology Reviews 33, 855–869.

Faguy DM & Doolittle WF (1999) Lessons from the Aeropyrum pernix genome. Current Biology 9, R883–R886.

Faith DP (1992) Conservation evaluation and phylogenetic diversity. Biological Conservation 61, 1–10.

Field D, Garrity G, Gray T, Morrison N, Selengut J, Sterk P, Tatusova T, Thomson N, Allen MJ, Angiuoli SV, Ashburner M, Axelrod N, Baldauf S, Ballard S, Boore J, Cochrane G, Cole J, Dawyndt P, de Vos P, dePamphilis C, Edwards R, Faruque N, Feldman R, Gilbert J, Gilna P, Glöckner FO, Goldstein P, Guralnick R, Haft D, Hancock D, Hermjakob H, Hertz-Fowler C, Hugenholtz P, Joint I, Kagan L, Kane M, Kennedy J, Kowalchuk G, Kottmann R, Kolker E, Kravitz S, Kyrpides N, Leebens-Mack J, Lewis SE, Li K, Lister AL, Lord P, Maltsev N, Markowitz V, Martiny J, Methe B, Mizrachi I, Moxon R, Nelson K, Parkhill J, Proctor L, White O, Sansone SA, Spiers A, Stevens R, Swift P, Taylor C, Tateno Y, Tett A, Turner S, Ussery D, Vaughan B, Ward N, Whetzel T, San Gil I, Wilson G & Wipat A (2008) The minimum information about a genome sequence (MIGS) specification. Nature Biotechonlogy 26, 541–547.

Fischer C, Wiggli M, Schanz F, Hanselmann KW & Bachofen R (1996) Light environment and synthesis of bacteriochlorophyll by populations of Chromatium okenii under natural environmental conditions. FEMS Microbiology Ecology 21, 1–9.

Fitz-Gibbon ST & House CH (1999) Whole genome-based phylogenetic analysis of free-living microorganisms. Nucleic Acids Research 27, 4218–4222.

Forterre P (1997) Archaea: what can we learn from their sequences?. Current Opinion in Genetics & Development 7, 764–770.

Forterre P (1999) Displacement of cellular proteins by functional analogues from plasmids or viruses could explain puzzling phylogenies of many DNA informational proteins. Molecular Microbiology 33, 457–465.

Forterre P & Philippe H (1999) Where is the root of the universal tree of life? BioEssays 21, 871–879.

Forterre P, Bouthier de la Tour C, Philippe H & Duguet M (2000) Reverse gyrase from hyperthermophiles, probable transfer of a thermoadaptation trait from archaea to bacteria. Trends in Genetics 16 (4), 152–154.

Forterre P, Brochier C & Philippe H (2002) Evolution of the Archaea. Theoretical Population Biology 61, 409–422.

Fox GE, Pechman KR & Woese CR (1977) Comparative cataloging of 16S ribosomal RNA, molecular approach to procaryotic systematics. International Journal of Systematic Bacteriology 27, 44–57.

Francis CA, Roberts KJ, Beman JM, Santoro AE & Oakley BB (2005) Ubiquity and diversity of ammonia–oxidizing archaea in water columns and sediments of the ocean. Proceedings of National Academy of Sciences USA 102 (41), 14683–14688.

Francis CA, Beman JM & Kuypers MMM (2007) New processes and players in the nitrogen cycle, the microbial ecology of anaerobic and archaeal ammonia oxidation. The ISME Journal 1, 19–27.

Friedrich MW (2002) Phylogenetic analysis reveals multiple lateral trransfers of adenosine-5'-phosphosulfate reductase genes among sulfate-reducing microorganisms. Journal of Bacteriology 184, 278-289.

Fry JC, Parkes RJ, Cragg BA, Weightman AJ & Webster A (2008) Prokaryotic biodiversity and activity in the deep subseafloor biosphere. FEMS Microbiology Ecology 66, 181–196.

Fuhrman JA, McCallum K & Davids AA (1992) Novel major archaebacterial group from marine plankton. Nature 356, 148–149.

Galand PE, Fritze H & Yrjälä K (2003) Microsite–dependent changes in methanogenic populations in a boreal oligotrophic fen. Environmental Microbiology 5 (11), 1133–1143.

Galand PE, Loveloy C & Pouliot J (2008) Microbial community diversity and heterotrophic production in a coastal Arctic ecosystem, a stamukhi lake and its source waters. Limnology and Oceanography 53 (2), 813–823.

Galand PE, Lovejoy C, Hamilton AK, Grant Ingram R, Pedneault E & Carmack EC (2009) Archaeal diversity and a gene for ammonia oxidation are coupled to oceanic circulation. Environmental Microbiology 11, 971–980.

García-Cantizano J, Casamayor EO, Gasol JM, Guerrero R & Pedrós-Alió C (2005) Partitioning of CO_2 incorporation among planktonic microbial guilds and estimation of in situ specific growth rates. Microbial Ecology 50, 230–241.

García-Gil LJ, Vicente E, Camacho A, Borrego CM, Vila X, Cristina XP & Rodriguez-Gonzalez J (1999) Vertical distribution of photosynthetic sulphur bacteria linked to saline gradients in Lake "El Tobar" (Cuenca, Spain). Aquatic Microbial Ecology 20, 299–303.

Garret RA & Klenk HP (2007) Archaea: Evolution, Physiology and Molecular Biology. Blackwell Publishing Ltd.

Gasol JM, Casamayor EO, Joint I, Garde K, Gustavson K, Benlloch S, Díez, B, Schauer M, Massana R & Pedrós-Alió C (2004) Control of heterotrophic prokaryotic abundance and growth rate in hypersaline planktonic environments. Aquatic Microbial Ecology 34, 193–206.

Gich FB, Borrego CM, Martinez-Planells A, Steensgaard DB, Garcia-Gil LJ, & Holzwarth AR (2001) Variability of the photosynthetic antenna of a Pelodictyon clathratiforme population from a freshwater holomictic pond. FEMS Microbiology Ecology 37, 11–19.

Gich FB, Schubert K, Bruns A, Hoffelner H & Overmann J (2005) Specific detection, isolation, and characterization of selected, previously uncultured members of the freshwater bacterioplankton community. Applied and Environmental Microbiology 71 (10), 5908–5919.

Giovannoni S & Stingl U (2007) The importance of culturing bacterioplankton in the 'omics' age. Nature Reviews in Microbiology 5, 820–826.

Golyshina OV, Pivovarova TA, Karavaiko GI, Kondrat'eva TF, Moore ERB, Abraham WR, Lünsdorf H, Timmis KN, Yakimov MM & Golyshin PN (2000) Ferroplasma acidiphilum gen. nov., sp. nov., an acidophilic autotrophic, ferrous–iron–oxidizing, cell–wall–lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea. International Journal of Systematic and Evolutionary Microbiology 50, 997–1006.

Gribaldo S, Lumia V, Creti R, de Macario EC, Sanangelantoni A & Cammarano P (1999) Discontinuous occurrence of the hsp70 (dnaK) gene among Archaea and sequence features of HSP70 suggest a novel outlook on phylogenies inferred from this protein. Journal of Bacteriology 181, 434–443.

Guerrero R, Montesinos E, Esteve I & Abella CA (1980) Physiological adaptation and growth of purple and green sulfur bacteria in a meromictic lake (Vila) as compared to a holomictic lake (Siso). Shallow Lakes. Contribution to their limnology.

Developments in Hydrobiology. Dokulil M, Metz H, & Jewson D (Eds.), Dr. W. Junk, The Hague, pp. 161–171.

Guerrero R, Montesinos E, Pedrós–Alió C, Esteve I, Mas J, van Gemerden H, Hofman PAG & Bakker J (1985) Phototrophic sulfur bacteria in two Spanish lakes, vertical distribution and limiting factors. Limnology and Oceanography 30, 919–931.

Hadas O, Malinski–Rushanski N, Pinkas R, Halicz & Erez J (2000) High chemoautotrophic primary production across a transect in Lake Kinneret, Israel. Archieves in Hydrobiology Special Issues Advances in Limnology 55, 413–420.

Hallam SJ, Mincer TJ, Schleper C, Preston CM, Roberts K, Richardson PM & Delong EF (2006) Pathways of Carbon assimilation and Ammonia Oxidation suggested by environmental genomic analysis of marine Crenarchaeota. PLOS Biology 4 (4), 520–536.

Hansel CM, Fendorf S, Jardine PM & Francis CA (2008) Changes in bacterial and archaeal community structure and functional diversity along a geochemically variable soil profile. Applied and Environmental Microbiology 74 (5), 1620–1633.

Hatzenpichler R, Lebedeva EV, Spieck E, Stoecker K, Richter A, Daims H & Wagner M (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. Proceedings of National Academy of Sciences USA 105 (6), 2134–2139.

He JZ, Shen JP, Zhang LM, Zhu YG, Zheng YM, Xu MG & Di H (2007) Quantitative analyses of the abundance and composition of ammonia–oxidizing bacteria and ammonia–oxidizing archaea of a Chinese upland red soil under long–term fertilization practices. Environmental Microbiology 9 (9), 2364–2374.

Helmus MR, Bland TJ, Williams CK & Ives AR (2007) Phylogenetic Measures of Biodiversity. The American Naturalist 169 (3), E68–E83.

Henneberger R, Moissl C, Amann T, Rudolph C & Huber R (2006) New insights into the lifestyle of the cold-loving SM1 Euryarchaeon, natural growth as a monospecies biofilm in the subsurface. Applied and Environmental Microbiology 72 (1), 192–199.

Herfort L, Schouten S, Abbas B, Veldhuis MJW, Coolen MJL, Wuchter C, Boon JP, Herndl GJ & Sinninghe-Damsté JS (2007) Variations in spatial and temportal distribution of Archaea in the North Sea in relation to environmental variables. FEMS Microbiology Ecology 62, 242–257.

Herndl GJ, Reinthaler T, Teira E, van Aken H, Veth C, Pernthaler A & Pernthaler J (2005) Contribution of *Archea* to total prokaryotic production in the deep atlantic ocean. Applied and Environmental Microbiology 71 (5), 2303–2309.

Hirasawa JS, Sarti A, Saavedra, Del Aguila NK & Varesche MBA (2008) Application of molecular techniques to evaluate the methanogenic archaea and anaerobic bacteria in the presence of oxygen with different COD, Sulfate ratios in a UASB reactor. Anaerobe 14, 209–218.

Hixon WG & Searcy DG (1993) Cytoskeleton in the archaebacterium Thermoplasma acidophilum? viscosity increase in soluble extracts. BioSystems 29, 151–160.

Hoëfs MJL, Schouten S, de Leeuw JW, King LL, Wakeham SG & sinninghe–Damsté JS (1997) Ether lipids of plantkonic Archaea in the marine water column. Applied and Environmental Microbiology 63 (8), 3090–3095.

Hong S, Bunge J, Leslin C, Jeon S & Epstein SS (2009) Polymerase chain reaction primers miss half of rRNA microbial diversity. The ISME Journal, Epub.

Hopmans EC, Weijers JWH, Schefuss E, Herfort L, Sinninghe-Damsté JS & Schouten S (2004) A novel proxy for terrestrial organic matter in sediments based on branched and isoprenoid tetraether lipids. Earth and Planetary Science Letters 224 (1–2), 107–116.

Huber R, Huber H & Stetter KO (2000) Towards the ecology of hyperthermophiles, biotopes, new isolation strategies and novel metabolic properties. FEMS Microbiology Reviews 24, 615–623.

Huber T, Faulkner G & Hugenholtz P (2004) Bellerophon, a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20, 2317–2319.

Huet J, Schnabel R, Sentenac A & Zillig W (1983) Archaebacteria and eukaryotes posses DNA-dependent RNA polymerases of a comon type. EMBO Journal 2 (8), 1291–1294.

Humayoun SB, Bano N & Hollibaugh JT (2003) Depth distribution of microbial diversity in Mono Lake, a meromictic soda lake in California. Applied and Environmental Microbiology 69 (2), 1030–1042.

Hügler M, Huber H, Stetter KO & Fuchs G (2003) Autotrophic CO_2 pathways in Archaea (Crenarchaeota). Archieves of Microbiology 179, 160–173.

Inagaki F, Suzuki M, Takai K, Oida H, Sakamoto T, Aoki K, Nealson KH & Horikoshi K (2003) Microbial communities associated with geological horizons in coastal subseafloor sediments from the sea of Okhotsk. Applied and Environmental Microbiology 69 (12), 7224–7235.

Ingalls AE, Shah SR, Hansman RL, Aluwihare LI, Santos GM, Druffel ERM & Pearson A (2006) Quantifying archaeal community autotrophy in the mesopelagic ocean using natural radiocarbon. Proceedings of National Academic of Sciences USA 103 (17), 6442–6447.

Ishoy T, Kvist T, Westermann P & Ahring BK (2006) An improved method for single-cell isolation of prokaryotes from meso-, thermo- and hypereutophic environments using micromanipulation. Applied and Environmental Microbiology 69, 510–514.

Isumbisho M, Kaningini M, Descy JP & Baras E (2004) Seasonal and diel variations in diet of the Young stages of Limnothrissa miodon in lake Kivu, Eastern Africa. Journal of Tropical Ecology 20, 73–83.

Isumbisho M, Sarmento H, Kaningini B, Micha JC & Descy JP (2006) Zooplankton of Lake Kivu, East Africa, half a century after the Tanganyika sardine introduction. Journal of Planktonic Research 28, 971–989.

Jahn U, Huber H, Eisenreich W, Hügler M & Fuchs G (2007) Insights into the autotrophic CO_2 fixation pathway of the archaeon Igniococcus hospitalis, comprehensive analysis of the central Carbon metabolisms. Journal of Bacteriology 189 (11), 4108–4119.

Jain R, Rivera MC & Lake JA (1999) Horizontal gene transfer among genomes, The complexity hypothesis. Proceedings of National Academy of Sciences USA 96, 3801–3806.

Jannasch HW (1975) Methane oxidation in Lake Kivu (Central Africa). Limnology and Oceanography 80, 860–864

Jarrell KF, Ng SYM & Chaban B (2007) Flagelation and chemotaxis In, Archaea: Molecular and Cellular Biology. Cavicchioli R (Ed.) Washington, ASM Press; pp, 385–410.

Jones MN (1984) Nitrate reduction by shaking with cadmium alternative to cadmium columns. Water Research 18, 643–646.

Jorgensen BB, Kuenen JG & Cohen Y (1979) Microbial transformation of sulfur compounds in a stratified lake (Solar Lake, Sinai). Limnology and Oceanography 24 (5), 799–822.

Juottonen H, Galand PE, Tuittila ES, Laine J, Fritze H & Yrjälä K (2005) Methanogen communities and bacteria along an ecohydrological gradient in a northern raised bog complex. Environmental Microbiology 7 (10), 1547–1557.

Jurgens G, Lindstrom K & Saano A (1997) Novel group within the Kingdom Crenarchaeota from boreal forest soil. Applied and Environmental Microbiology 63 (2), 803–805.

Jurgens G, Glöckner FO, Amman R, Saano A, Montonen L, Likolammi M & Münster U (2000) Identification of novel Archaea in bacterioplankton of a boreal forest lake by phylogenetic analysis and Fluorescent in situ Hybridization. FEMS Microbiology Ecology 34, 45–56.

Kaningini B, Isumbisho M, Ndayike N & Micha JC (2003) Etude du zooplancton du lac Kivu, composition, variations d'abondance et distribution. Bulletin des Séances, Académie Royale des Sciences d'Outre–Mer, 49 (2), 145–160.

Karner MB, Delong EF & Karl DM (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 409, 507–510.

Kashefi K & Lovley DR (2003) Extending the upper temperature limit for Life. Science 301, 934.

Kates M (1993) Membrane lipids of Archaea. In, The Biochemistry of Archaea (Archaebacteria). Kates M, Kushnen DJ & Matheson AT (Eds.) Amsterdam, Elsevier; pp, 261–295.

Kembel S, Ackerly D, Blomberg S, Cowan P, Helmus M & Webb C (2008) Picante, Tools for integrating phylogenies and ecology. Version 0.4–0. http://picante.r-forge.r-project.org/.

Keough BP, Schmidt TM & Hicks RE (2003) Archaeal nucleic acids in picoplankton from great lakes from three continents. Microbial Ecology 46, 238–248.

Kilham P, Kilham SS & Hecky RE (1986) Hypothesized resource relationships among African planktonic diatoms. Limnology and Oceanography 31, 1169–1181.

Kirchman DL (2002) The ecology of Cytophaga–Flavobacteria in aquatic environments. FEMS Microbiology Ecology 29, 91–100.

Kletzin A, Urich T, Müller F, Bandeiras TM & Gomes CM (2004) Dissimilatory oxidation and reduction of elemental sulfur in thermophilic Archaea. Journal of Bioenergetics and Biomembranes 36 (1), 77–91.

Kletzin A (2007) General characteristics and important model organisms In: Archaea, Molecular and Cellular Biology. Cavicchioli R (Ed.) Washington, ASM Press; pp, 14–92.

Koch M, Rudolph C, Moissl C & Huber R (2006) A cold-loving crenarchaeon is a substantial part of a novel microbial community in cold sulphidic marsh water. FEMS Microbial Ecology 57, 55–66.

Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB & Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature Letters 437 (22), 543–546.

Koonin EV, Mushegian AR, Galperin MY & Walker DR (1997) Comparison of archaeal and bacterial genomes, Computer analysis of protein sequences predicts novel functions and suggests a chimeric origin for the archaea. Molecular Microbiology 25, 619–637.

Kozubal M, Macur RE, Korf S, Taylor WP, Ackerman GG, Nagy A & Inskeep WP (2008) Isolation and distribution of a novel iron-oxidizing crenarchaeon from acidic geothermal springs in Yellowstone National Park. Applied and Environmental Microbiology 74 (4), 942–949.

Krisktjansson JK, Schönheit P & Thauer RK (1982) Different K_s values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: An explanation for the apparent inhibition of methanogenesis by sulfate. Archieves of Microbiology 131, 278–282.

Kuuppo–Leinikki P & Salonen K (1992) Bacterioplankton in small polyhumic lake with an anoxic hypolimnion. Hydrobiologia 229, 159–168.

Kuypers MMM, Blokker P, Erbacher J, Kinkel H, Pancost RD, Schouten S & Sinninghe-Damsté JS (2001) Massive expansion of marine Archaea during a Mid-Cretaceus ocean anoxic event. Science 293, 92–94.

Labrenz M, Jost G & Jürgens K (2007) Distribution of abundant prokaryotic organisms in the water column of the central Baltic Sea with an oxic-anoxic interface. Aquatic Microbial Ecology 46, 177–190.

Lam P, Jensen MM, Lavik G, McGinnis DF, Müller B, Schubert CJ, Amann R, Thamdrup B & Kuypers MMM (2007) Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. Proceedings of National Academic of Sciences USA 104, 7104–7109.

Lanzotti V, Trincone A, Nicolaus B, Zillig W, de Rosa M & Gambacorta A (1989) Complex lipids of Pyrococcus and AN1, Thermophilic members of Archaebacteria belonging to Thermococcales. Biochimica et Biophysica Acta 1004, 44–48.

Lehours AC, Bardot C, Thenot A, Debroas D & Fonty G (2005) Anaerobic microbial communities in lake Pavin, a unique meromictic lake in France. Applied and Environmental Microbiology 71 (11), 7389–7400.

Lehours AC, Evans P, Bardot C, Joblin K & Gérard F (2007) Phylogenetic diversity of Archaea and Bacteria in the anoxic zone of a meromictic lake (Lake Pavin, France). Applied and Environmental Microbiology 73 (6), 2016–2019.

Leipe DD, Aravind L & Koonin EV (1999) Did DNA replication evolve twice independently?. Nucleic Acids Research 27, 3389–3401.

Leininger S, Urich T, Schloter M, Schwatk L, Qi J, Nicol GW, Prosser JI, Schuster SC & Schleper C (2006) Archaea predominate among amonia-oxidizing prokaryotes in soils. Nature Letters 442, 806–809.

Lin X, Wakeham SG, Putnam If, Astor YM, Scranton MI, Chistoserdov AY & Taylor GL (2006) Comparison of vertical distribution of prokaryotic assemblages in the anoxic Cariaco Basin and Black Sea by use of Fluorescence in situ Hybridization. Applied and Environmental Microbiology 72 (4), 2679–2690.

Lindström ES (1998) Bacterioplankton community composition in a boreal forest lake. FEMS Microbiology Ecology 27, 163–174.

Lipp JS, Morono Y, Inagaki F & Hinrichs KU (2008) Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature 454, 991–994.

Llirós M, Casamayor EO & Borrego CM (2008) High archaeal richness in the water column of a freshwater sulfurous karstic lake along an interannual study. FEMS Microbiology Ecology 66, 331–342.

Llirós M, Gich F, Plasencia A, Darchambeau F, Casamayor EO, Descy JP & Borrego C (2009) Segregation of archaeal populations along the epipelagic waters of the stratified oligotrophic freshwater Lake Kivu (East Africa). Submitted.

Lodhi MA, Ye GN, Reisch BI & Weeden NF (1994) A simple and efficient method for DNA extraction from grapevine cultivars and Vitis species. Plant Molecular Biology Reporter 12, 6–13.

Lovley DR, Dwyer DF & Klug MJ (1982) Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Applied and Environmental Microbiology 43, 1373–1379.

Lovley DR & Klug MJ (1983) Sulfate reducers can outcompete methanogens at freshwater sulfate concentrations. Applied and Environmental Microbiology 45, 187–192.

Loy A, Duller S, Baranyi C, MuBmann M, Ott J, Sharon I, Béjà O, Le Paslier D, Dahl C & Wagner M (2009) Reverse dissimilatory sulfide reductase as phylogenetic marker for a subgroup of sulfur-oxidising prokaryotes. Environmental Microbiology 11 (2), 289-299.

Lozupone C, Hamady M & Knight R (2006) Unifrac – An online tool for comparing microbial communitydiversity in a phylogenetic context. BMC Bioinformatics 7, 371–385.

Lozupene C & Knight R (2007) Global patterns in bacterial diversity. Proceedings of National Academy of Sciences USA 104 (27), 11436–11440.

Lozupone C & Knight R (2008) Species divergence and the measurement of microbial diversity. FEMS Microbiology Reviews 32 (4), 557-578.

Ludwig W, Rosello–Mora R, Aznar R, Klugbauer S, Spring S, Reetz K, Beimfohr C, Brockmann E, Kirchhof G, Dorn S, Bachleitner M, Klugbauer N, Springer N, Lane D, Nietupsky R, Weizenegger M & Schleifer KH (1995) Comparative analysis of 23S ribosomal RNA from Proteobacteria. Systematic and Applied Microbiology 18, 164–188.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Föster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lübmann R, May M, Nonhoff B, Reichel B, Strenhlow R, Stamakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A &

Schleifer KH (2004) ARB, a software environment for sequence data. Nucleic Acids Research 32 (4), 1363–1371.

MacGregor BJ, Moser DP, Alm EW, Nealson KH & Stahl DA (1997) Crenarchaeota in Lake Michigan sediment. Applied and Environmental Microbiology 63 (3), 1178–1181.

Magurran AE & Henderson PA (2003) Explaining the excess of rare species in natural species abundance distributions. Nature 422, 714–716.

Mahmood S, Freitag TE & Prosser JI (2006) Comparison of PCR primer–based strategies for characterization of ammonia oxidizer communities in environmental samples. FEMS Microbiology Ecology 56, 482–493.

Makarova KS, Aravind L, Galperin MY, Grishin NV, Tatusov RL, Wolf YI & Koonin EV (1999) Comparative genomics of the Archaea (Euryarchaeota), Evolution of conserved protein families, the stable core, and the variable shell. Genome Research 9, 608–628.

Makarova KS & Koonin EV (2003) Comparative genomics of Archaea, How much have we learned in six years, and what's next?. Genome Biology 4, 115.1–115.17.

Manz W, Amann R, Ludwig W, Vancanneyt M & Schleifer KH (1996) Application of a suite of 16S rRNA specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga–Flavobacter–Bacteroidetes in the natural environment. Microbiology 142, 1097–1106.

Marshall BE (1991) Seasonal and annual variations in the abundance of the clupeid Limnothrissa miodon in Lake Kivu. Journal of Fish Biology 39, 641–648.

Massana R, Murray AE, Preston CM & Delong EF (1997) Vertical distribution and phylogenetic characterization of marine planktonic archaea in the Santa Barbara channel. Applied and Environmental Microbiology 63 (1), 50–56.

Massana R, DeLong EF & Pedrós-Alió C (2000) A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. Applied and Environmental Microbiology 66 (5), 1777–1787.

McArdle B (2001) Fitting multivariate models to community data, A comment on distance-based redundancy analysis. Ecology 82, 290–297.

McCaig AE, Glover LA, & Prosser JI (2001) Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Applied and Environmental Microbiology 67 (10), 695–700.

Menendez C, Bauer Z, Huber H, Gad'On N, Stetter KO & Fuchs G (1999) Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic Crenarchaeota and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. Journal of Bacteriology 181 (4), 1088–1098.

Mesbah NM, Abou-El-Ela SH & Wiegel J (2007) Novel and unexpected prokaryotic diversity in water and sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. Microbial Ecology 54, 598–617.

Moissl C, Rachel R, Briegel A, Engelhardt H & Huber R (2005) The unique structure of archaeal "hami", highly complex cell appendages with nano-grappling hooks. Molecular Microbiology 56 (2), 361–370.

Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction–amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59 (3), 695–700.

Muyzer G & Ramsing NB (1995) Molecular methods to study the organization of microbial communities. Water Science and Technology 32 (8), 1–9.

Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeeuwenhoek 73, 127–141.

Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology 2, 317–322.

Myllykallio H & Forterre P (2000) Mapping of a chromosome replication origin in an archaeon, Response. TRENDS in Microbiology 8, 537–539.

Mylvaganam S & Dennis PP (1992) Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium Haloarcula marismortui. Genetics 130, 399–410.

Navarro JB, Moser DP, Flores A, Ross C, Rosen MR, Dong H, Zhang G & Hedlund BP (2009) Bacterial succession within an ephemeral hypereutrophic mojave desert palya lake. Microbial Ecology 57, 307–320.

Nesbo CL, L'Haridon S, Stetter KO & Doolittle WF (2001) Phylogenetic analyses of two ''archaeal'' genes in thermotoga maritima reveal multiple transfers between archaea and bacteria. Molecular Biology and Evolution 18, 362–375.

Ng SYM, Chaban B & Jarrell KF (2006) Archaeal flagella and type IV pili, a comparison of genes and posttranscriptional modification. Journal of Molecular Microbiology and Biotechnology 11, 167–191.

Ng SYM, Zolghadr B, Driessen AJM, Alberts SV & Jarrell KF (2008) Cell surface structures of Archaea. Journal of Bacteriology 190 (18), 6039–6047.

Nichols D (2007) Cultivation gives context to the microbial ecologist. FEMS Microbiology Ecology 60, 351–357.

Nickell S, Hegerl R, Baumeister W & Rachel R (2003) Pyrodictium cannulae enter the periplasmic space but do not enter the cytoplasm, as revealed by cryo–electron tomography. Journal of Structural Biology 141, 34–42.

Nicol GW, Tscherko D, Embley TM & Prosser JI (2005) Primary succession of soil Crenarchaeota across a receding glacier foreland. Environmental Microbiology 7 (3), 337–347.

Nicol GW & Schleper C (2006) Ammonia–oxidising Crenarchaeota, important players in the nitrogen cycle? TRENDS in Microbiology 14 (5), 207–212.

Ochsenreiter T, Selezi D, Quaiser A, Bonch–Osmolovskaya L & Schleper C (2003) Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. Environmental Microbiology 5 (9), 787–797. 2003.

Ogunsseitan O (2005) Microbial Diversity Blackwell Publishing, Maden, MA.

Oksanen J, Kindt R, Legendre P, O'hara B, Simpson GL & Stevens MHH (2008) Vegan, Community Ecology Package. version 1.11–4. http://vegan.r-forge.r-project.org.

Olsen GJ & Woese CR (1997) Archaeal genomics, An overview. Cell 89, 991–994.

Orphan VJ, House CH, Hinrichs KU, McKeegan KD & Delong EF (2001) Methane–consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. Science 293, 484–487.

Ouverney C & Fuhrman JA (1999) Combined microautoradiography–16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Applied and Environmental Microbiology 65 (4), 1746–1752.

Ouverney C & Fuhrman JA (2000) Marine planktonic Archaea take up amino acids. Applied and Environmental Microbiology 66 (11), 4829–4833.

Ovreas L, Fourney L, Daae FL & Torsvik V (1997) Distribution of bacterioplankton in meromictic lake Sælenvannent, as determined by Denaturing Gradient Gel Electrophoresis of PCR–amplified gene fragments coding for 16S rRNA. Applied and Environmental Microbiology 63 (9), 3367–3373.

Pace NR (2006) Time for a change. Nature 441, 289.

Pace NR (2009) Problems with “Procaryote”. Journal of Bacteriology 191 (7), 2008–2010.

Patel GB & Sprott GD (1999) Archaeabacterial ether lipid liposomes (archaeosomes) as novel vaccine and drug delivery systems. Critical Reviews in Biotechnology 19, 317–357.

Pearson A, McNichol AP, Benitez–Nelson BC, Hayes JM & Eglinton TI (2001) Origins of lipid biomarkers in Santa Monica Basin surface sediment: a case study using compound–specific $\delta^{14}C$–analyses. Geochimica et Cosmochimica Acta 65 (18), 3123–3137.

Pedrós-Alió C & Guerrero R (1991) Abundance and activity of bacterioplankton in warm lakes. Verh. Internat. Verein. Limnol. 24, 1212–1219.

Pedrós-Alió C, García-Cantizano J & Calderon-Paz JI (1993) Bacterial production in anaerobic water columns. In: Current Methods in Aquatic Microbial Ecology; Kemp PF, Sherr BF, Sherr EB & Cole JJ (Eds.) Lewis Publishers, Boca Raton, Florida, pp 519–530.

Pedrós-Alió C (2006) Marine microbial diversity, can it be determined?. TRENDS in Microbiology 14, 257–263.

Pedrós-Alió C (2007) Dipping into the Rare Biosphere. Science 315, 192–193.

Pernthaler J, Glöckner FO, Unterholzner S, Alfreider A, Psenner R & Amann R (1998) Seasonal community and population dynamics of pelagic bacteria and archaea in a high mountain lake. Applied and Environmental Microbiology 64 (11), 4299–4306.

Pernthaler A, Pernthaler J & Amann R (2004) Sensitive multi–color fluorescence in situ hybridization for the identification of environmental microorganisms. In: Molecular Microbial Ecology Manual, Second Edition; Akkermans ADL, van Elsas JD & de Brujin FJ (Eds.) Kluwer Academic Publishers, The Netherlands, pp 711–726.

Pommier T, Canbäck B, Rienmann L, Boström KH, Simu K, Lundberg P, Lundber P, Tunlid A & Hagström A (2007) Global patterns of diversity and community structure in marine bacterioplankton. Molecular Ecology 16, 867–880.

Pouliot J, Galand PE, Loveloy C & Vincent WF (2009) Vertical structure of archaeal communities and the distribution of ammonia monooxygenase A gene variants in two meromictic high arctic lakes. Environmental Microbiology 11 (3), 687–699.

Powers LA, Werne JP, Johnson TC, Hopmans EC, Sinninghe–Damsté JS & Schouten S (2004) Crenarchaeotal membrane lipids in lake sediments: A new paleotemperature proxy for continental paleoclimate reconstruction? Geology 32 (7), 613–616.

Prat C, Ruiz-Rueda O, Trias R, Anticó E, Capone D, Sefton M & Bañeras L (2009) Molecular fingerprinting by PCR-Denaturing Gradient Gel Electrophoresis reveals differences in the levels of microbial diversity for musty-earthy tainted corks. Applied and Environmental Microbiology 75, 1922–1931.

Prosser JI & Nicol GW (2008) Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. Environmental Microbiology 10, 2931–2941.

Purdy KJ, Nedwell DB & Embley TM (2003a) Analysis of the sulfate-reducing bacterial and methanogenic archaeal populations in contrasting antarctic sediments. Applied and Environmental Microbiology 69 (6), 3181–3191.

Purdy KJ, Munson MA, Cresswell-Maynard T, Nedwell DB & Embley TM (2003b) Use of 16S rRNA–targeted oligonucleotide probes to investigate function and phylogeny of sulphate–reducing bacteria and methanogenic archaea in a UK estuary. FEMS Microbiology Ecology 44, 361–371.

Quaiser A, Ochsenreiter T, Klenk HP, Kletzin A, Treusch AH, Meuer G, Eck J, Sensen CW & Schleper C (2002) First insight into the genome of an uncultivated crenarchaeote from soil. Environmental Microbiology 4 (10), 603–611.

Ramette A & Tiedje JM (2007) Biogeography, An emerging cornerstone for understanding prokaryotic diversity, ecology, and evolution. Microbial Ecology 53, 197–207.

Ramsing NB, Fossing H, Ferdelman TG, Andersen F & Thamdrup B (1996) Distribution of bacterial populations in a stratified fjord (Mariager Fjord, Denmark) quantified by in situ hybridisation and related to chemical gradients in the water column. Applied and Environmental Microbiology 62 (4), 1391–1404.

Rappé MS, Connon SA, Vergin KL & Giovannoni SJ (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418, 630–633.

Raskin L, Stromley JM, Ritmann BE & Stahl DA (1994) Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Applied and Environmental Microbiology 60 (4), 1232–1240.

Rees HC, Grant WD, Jones BE & Heaphy S (2004) Diversity of Kenyan soda lake alkaliphiles assessed by molecular methods. Extremophiles 8, 63–71.

Reiter WD, Palm P & Zillig W (1988) Analysis of transcription in the archaebacterium Sulfolobus indicates that archaebacterial promoters are homologous to eukaryotic pol II promoters. Nucleic Acids Research 16, 1–19.

Roberts E, Sethi A, Montoya J, Woese CR & Luthy-Schulten Z (2008) Molecular signatures of ribosomal evolution Proceedings of National Academy of Sciences USA 105, 13953–13958.

Robertson CE, Harris JK, Spear JR & Pace NR (2005) Phylogenetic diversity and ecology of environmental Archaea. Current Opinion in Microbiology 8, 638–642.

Rothschild LJ & Mancinelli RL (2001) Life in extrem environments. Nature 409, 1092–1101.

Rudolph C, Wanner G & Huber R (2001) Natural communities of novel Archaea and Bacteria Growing in cold sulfurous springs with a string-of-pearls-like morphology. Applied and Environmental Microbiology 67 (5), 2336–2344.

Ruepp A, Graml W, Santos-Martinez ML, Koretke KK, Volker C, Mewes HW, Frishman D, Stocker S, Lupas AN & Baumeister W (2000) The genome sequence of the thermoacidophilic scavenger Thermoplasma acidophilum. Nature 407, 508–513.

Saitou N & Nei M (1987) The neighbour–joining method, A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406–425.

Sandaa RA, Enger O & Torsvik V (1999) Abundance and diversity of Archaea in heavy–metal–contaminated soils. Applied and Environmental Microbiology 65 (8), 3293–3297.

Sarmento H, Isumbisho M & Descy JP (2006) Phytoplankton ecology of Lake Kivu. Journal of Plankton Research 28, 815–829.

Sarmento H, Unrein F, Isumbisho M, Stenuite S, Gasol JM & Descy JP (2008) Abundance and distribution of picoplankton in tropical, oligotrophic Lake Kivu, eastern Africa. Freshwater Biology 53, 756–771.

Schleper C, Puehler G, Holz I, Gambacorta A, Janekovic D, Santarius U, Klenk HP & Zillig W (1995) Picrophilus gen. nov., fam. nov.: a novel aerobic, heterotrophic, thermoacidophilic genus and family comprising archaea capable of growth around pH 0. Journal of Bacteriology 177 (24), 7050–7059.

Schleper C, Holben W & Klenk HP (1997) Recovery of Crenarchaeotal ribosomal DNA sequences from freshwater-lake sediments. Applied and Environmental Microbiology 63 (1), 321–323.

Schleper C, Jurgens G & Jonuscheit M (2005) Genomic studies of uncultivated archaea. Nature Reviews Microbiology 3, 479–489.

Schleper C (2007) Diversity of uncultivated Archaea, perspectives from microbial ecology and metagenomics. In: Archaea: Evolution, Physiology and Molecular Biology. Garret RA & Klenk HP (Eds.) Blackwell Publishing, pp. 39–53.

Schleper C (2008) Metabolism of the deep. Nature Letters 456, 712–714.

Schmid M, Tietze K, Halbwachs M, Lorke A, McGinnis D & Wüest A (2002) How hazardous is the gas accumulation in Lake Kivu? Arguments for a risk assessment in light of the Nyiragongo volcano eruption of 2002. Acta Vulcanologica 14(1–2), 15(1–3); 115–122.

Schmid M, Halbwachs M, Wehrli B & Wüest A (2005) Weak mixing in Lake Kivu, New insights indicate increasing risk of uncontrolled gas eruption. Geochemistry, Geophysics, Geosystems 6, 1–11.

Schöell M, Tietze K & Schoberth SM (1988) Origin of methane in Lake Kivu (east-central Africa). Chemical Geology 71, 257–265.

Schouten S, Hopmans EC, Pancost RD & Sinnighe-Damsté JS (2000) Widespread occurence of structurally diverse tetraether membrane lipids: Evidence for the ubiquitous presence of low-temperature relatives of hyperthermophiles. Proceedings of National Academic of Sciences USA 97, 14421–14426.

Schouten S, Hopmans EC, Schefuss E & Sinnighe-Damsté JS (2002) Distributional variations in marine creanrchaeotal membrane lipids: a new tool for reconstructing ancient sea water temperatures? Earth and Planetary Science Letters 204 (1–2), 265–274.

Searcy DG & Hixon WG (1991) Cytoskeletal origins in sulfur–metabolizing archaebacteria. BioSystems 25, 1–11.

Selig U, Hubener T, Heerkloss R & Schubert H (2004) Vertical gradient of nutrients in two dimictic lakes – influence of phototrophic sulfur bacteria on nutrient balance. Aquatic Sciences 66, 247–256.

Shade A, Jones SE & McMahon KD (2008) The influence of habitat heterogeneity on freshwater bacterial community composition and dynamics. Environmental Microbiology 10 (4), 1057–1067.

Shaw AK, Halpern AL, Beeson K, Tran B, Venter CJ & Martiny JBH (2008) It's all relative, ranking the diversity of aquatic bacterial communities. Environmental Microbiology 10 (9), 2200–2210.

Shively JM & Barton LL (1991) Variations in autotrophic life. Academic Press, London.

Simon HM, Jahn CE, Bergerud T, Sliwinski MK, Weimer PJ, Willis DW & Goodman RM (2005) Cultivation of mesophilic soil Crenarchaeotes in enrichment cultures from plant roots. Applied and Environmental Microbiology 71 (8), 4751–4760.

Sinninghe-Damsté JS, Schouten S, Hopmans EC, van Duin ACT & Geenevasen JAJ (2002a) Crenarchaeol: the characteristic core glycerol dibiohytanyl glycarol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota. Journal of Lipid Research 43, 1641–1651.

Sinninghe-Damsté JS, Rijpstra WIC, Hopmans EC, Prashl F, Wakeham SG & Schouten S (2002b) Distribution of membrane lipids of planktonic Crenarchaeota in the Arabian Sea. Applied and Environmental Microbiology 68 (6), 2997–3002.

Snel B, Bork P & Huynen MA (1999) Genome phylogeny based on gene content. Nature Genetics 21, 108–110.

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM & Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored ‘‘rare biosphere’’. Proceedings of National Academic of Sciences USA 103, 12115–12120.

Stahl DA & Amann R (1991) Development and application of nucleic acid probes. In: Nucleic Acid Techniques in Bacterial Systematics; Stackebrandt E & Goodfellow M (Eds.) John Wiley, Chischester, pp 205–248.

Standing Committee of Analysts (1981) Methods for the Examination of Waters and Associated Materials. Ammonia in waters. H.M.S.O., London, 42 pp.

Stanley JT & Konopka A (1985) Measurements of in situ activities of heterotrophic microorganisms in aquatic and terrestrial habitats. Annual Review of Microbiology 62, 625–630.

Sugai A, Masuchi Y, Uda I, Itoh T & Itoh YH (2000) Core lipids of hyperthermophilic archaeon, Pyrococcus horikoshii OT3. Journal of Japanese Oil Chemical Society 49, 695–700.

Sugai A, Uda I, Itoh YH & Itoh T (2004) The core lipid composition of the 17 strains of hyperthermophilic Archaea, Thermococcales. Journal of Oleo Science 53 (1), 41–44.

Suzuki M, Rappé MS & Giovannoni SJ (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small–subunit rRNA gene PCR amplicon length heterogeneity. Applied and Environmental Microbiology 64 (11), 4522–4529.

Takai K & Horikoshi H (1999) Genetic diversity of Archaea in deep–sea hydrothermal vent environments. Genetics 152, 1285–1297.

Takai K, Komatsu T, Inagaki F & Horikoshi K (2001a) Distribution of Archaea in black smoker chimney structure. Applied and Environmental Microbiology 67 (8), 3618–3629.

Takai K, Moser DP, deFlaun M, Onstott TC & Fredrickson JK (2001b) Archaeal diversity in waters from Deep South African gold mines. Applied and Environmental Microbiology 67 (12), 5750–5760.

Tamura K & Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzes. Molecular Biology and Evolution 10, 512–526.

Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4, Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Tassi F, Vaselli O, Tedesco D, Montegrossi G, Darrah T, Cuoco E, Mapendano MY, Poreda R & Delgado Huertas A (2009) Water and gas chemistry at Lake Kivu (DRC), Geochemical evidence of vertical and horizontal heterogeneities in a multibasin structure. Geochemistry, Geophysics, Geosystems 10, Q02005, doi:10.1029/2008GC002191.

Teira E, Reinthaler T, Pernthaler A, Pernthaler J & Herndl GJ (2004) Combining catalyzed reporter deposition–fluorescence in situ hybridization and microautoradiography to detect substrate utilization by *Bacteria* and *Archaea* in the deep ocean. Applied and Environmental Microbiology 70 (7), 4411–4414.

Teira E, van Aken H, Veth C & Herndl GJ (2006) Archaeal uptake of enantimeric amino acids in the meso- and bathypelagic waters of the North Atlantic. Limnology and Oceanography 51 (1), 60–69.

Tekaia F, Lazcano A & Dujon B (1999) The genomic tree as revealed from whole proteome comparisons. Genome Research 9, 550–557.

Teske A, Wawer C, Muyzer G & Ramsing NB (1996) Distribution of sulfate–reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by Most–Probable–Number counts and denaturing gradient gel electrophoresis of PCR–amplified ribosomal DNA fragments. Applied and Environmental Microbiology 62, 1405–1415.

Teske A & Sorensen KB (2008) Uncultured archaea in deep marine subsurface sediments, have we caught them all? The ISME Journal 1 (8), 1–16.

Tietze K, Geyh M, Müller H, Schröder L, Stahl W & Wehner H (1980) The genesis of methane in lake Kivu (central Africa). Geologische Rundschau 69, 452–472.

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk HP & Schleper C (2005) Novel genes for nitrite reductase and Amo–related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environmental Microbiology 7 (12), 1985–1995.

Uda I, Sugai A, Itoh YH & Itoh T (2001) Variation in molecular species of polar lipids from Thermoplasma acidophilum depends on growth temperature. Lipids 36, 103–105.

Urbach E, Vergin KL, Young L & Morse A (2001) Unusual bacterioplankton community structure in ulra-oligotrophic Crater Lake. Limnology and Oceanography 46 (3), 557–572.

Urbach E, Vergin KL, Larson GL & Giovannoni SJ (2007) Bacterioplankton communities of Crater Lake, OR, dynamic changes with euphotic zone food web structure and stable deep water populations. Hydrobiologia, 574, 161–177.

Valentine DL (2002) Biogeochemistry and microbial ecology of methane oxidation in anoxic environments, a review. Antonie van Leeuwenhoek 81, 271–282.

van de Wielen PWJJ, Bolhuis H, Borin S, Daffonchio D, Corselli C, Giuliano L, D'Auria G, de Lange GJ, Huebner A, Varnavas SP, Thomson J, Tamburini C, Marty D, McGenity TJ & Timmis KN (2005) The enigma of Prokaryotic life in deep hypersaline anoxic basins. Science 307, 121–123.

van Gemerden H & Mas J (1995) Ecology of phototrophic sulphur bacteria. In: Anoxygenic photosynthetic bacteria. Advances in Photosynthesis, Vol. 11; Blankenship RE, Madigan MT & Bauer CE (Eds.) Dordrecht, Kluwer Academic Publishers, pp 49–85.

Varela MM, van Aken HM, Sintes E & Herndl GJ (2008) Latitudinal trends of Crenarchaeota and Bacteria in the meso- and bathypelagic water masses of the Eastern North Atlantic. Environmental Microbiology 10 (1), 110–124.

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson w, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH & Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304, 66–74.

Vetriani C, Tran HV & Kerkhof LJ (2003) Fingerprinting microbial assemblages from the Oxic/Anoxic chemocline of the Black Sea. Applied and Environmental Microbiology 69 (11), 6481–6488.

Vila X & Abella C (1994) Effects of light quality on the physiology and the ecology of planktonic green sulfur bacteria in lakes. Photosynthesis Research 41, 53–65.

Visser EW, Bodelier PLE, Muyzer G & Laanbroek HJ (2009) A nester PCR approach for improved recovery of archaeal 16S rRNA gene fragments from freshwater samples. FEMS Microbiology Letters 298, 193–198.

Wakeham SG, Lewis CM, Hopmans EC, Schouten S & Sinninghe–Damsté JS (2003) Archaea mediate anaerobic oxidation of methane in deep euxinic waters of the Black Sea. Geochimica et Cosmochimica Acta 67, 1359–1374.

Whitby C, Earl J, Lanyon C, Gray S, Robinson J, Meadows J & Edwards C (2004) The molecular diversity of the methanogenic community in a hypereutrophic freshwater lake determined by PCR–RFLP. Journal of Applied Microbiology 97, 973–984.

Whitman WB, Coleman DC & Wiebe WJ (1998) Prokaryotes, the unseen majority. Proceedings of National Academy of Sciences USA 95, 6578–6583.

Winfrey MR & Ward DM (1983) Substrates for sulfate reduction and methane production in intertidal sediments. Applied and Environmental Microbiology 45, 193–199.

Woese CR & Fox GE (1977) Phylogenetic structure of the prokaryotic domain, The primary kingdoms. Proceedings of National Academy of Sciences USA 74, 5088–5090.

Woese CR (1987) Bacterial evolution. Microbiology Reviews 51, 221–271.

Woese CR, Kandler O & Wheelis ML (1990) Towards a natural system of organisms, Proposal for the domains Archaea, Bacteria, and Eucarya. Proceedings of National Academy of Sciences USA 87, 4576–4579.

Woese CR (2000) Interpreting the universal phylogenetic tree. Proceedings of National Academy of Sciences USA 97 (15), 8392–8396.

Wolf YI, Rogozin IB, Grishin NV, Tatusov RL & Koonin EV (2001) Genome trees constructed using five different approaches suggest new major bacterial clades. BMC Evolutionary Biology 1, 8.

Wuchter C, Schouten S, Boschker HTS & Sinninghe–Damsté JS (2003) Bicarbonate uptake by marine Crenarchaeota. FEMS Microbiology Letters 219, 203–207.

Wuchter C, Schouten S, Coolen NJL & Sinninghe-Damsté JS (2004) Temperature–dependent variation in the distribution of tetraether membrane lipids of marine Crenarchaeota: implications for TEX_{86} paleothermometry. Paleoceanography 19, PA4028, doi:10.1029/2004PA001041.

Wuchter C, Schouten S, Wakeham SG & Sinninghe-Damsté JS (2005) Temporal and spatial variation in tetraether membrane lipids of marine Crenarchaeota in particulate organic matter: implications for TEX_{86} paleothermometry. Paleoceanography 20, PA3013, doi:10.1029/2004PA001110.

Wuchter C (2006a) Ecology and membrane lipid distribution of marine Crenarchaeota, Implications of TEX86 paleothermometry. PhD dissertation, University of Utrecht.

Wuchter C, Abbas B, Coolen MJL, Herfort L, Van Blejiswijk J, Timmers P, Strous M, Teira E, Herndl GJ, Middelburg JJ, Schouten S & Sinninghe-Damsté JS (2006b) Archaeal nitrification in the ocean. Proceedings of National Academy of Sciences USA 103 (33), 12317–12322.

Xia X & Xie Z (2001) DAMBE, Data analysis in molecular biology and evolution. Journal of Heredity 92, 371–373.

Yakimov MM, La Cono V & Denaro R (2009) A first insight into the occurence and expression of functional amoA and accA genes of autotrophic and ammonia-oxidizing bathypelagic Crenarchaeota of Tyrrhenian Sea. Deep-Sea Research II 56, 748–754.

Yu Z, García-González R, Schanbacher FL & Morrison M (2008) Evaluations of different hypervariable regions of archaeal 16S rRNA genes in profiling of methanogens by Archaea–specific PCR and Denaturing Gradient Gel Electrophoresis. Applied and Environmental Microbiology 74, 889–893.

Zillig W (1991) Comparative biochemistry of Archaea and Bacteria. Current Opinion in Genetics & Development 1, 544–551.

Zolghadr B, Weber S, Szabó Z, Driessen AJM & Albers SV (2007) Identification of a system required for the functional surface localization of sugar binding proteins with class III signal peptides in Sulfolobus solfataricus. Molecular Microbiology 64 (3), 795–806.

Zweifel UL & Hagström A (1995) Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (Ghosts). Applied and Environmental Microbiology 61, 2180–2185.