RESEARCH ARTICLE

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

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Abstract

We surveyed the archaeal assemblage in a stratified sulfurous lake (Lake Vilar, Banyoles, Spain) over 5 consecutive years to detect potential seasonal and interannual trends in the free-living planktonic Archaea composition. The combination of different primer pairs and nested PCR steps revealed an unexpectedly rich archaeal community. Overall, 140 samples were analyzed, yielding 169 different 16S rRNA gene sequences spread over 14 *Crenarchaeota* (109 sequences) and six *Euryarchaeota* phylogenetic clusters. Most of the *Crenarchaeota* (98% of the total crenarchaeotal sequences) affiliated within the Miscellaneous *Crenarchaeota* Group (MCG) and were related to both marine and freshwater phylotypes. *Euryarchaeota* mainly grouped within the Deep Hydrothermal Vent *Euryarchaeota* (DHVE) cluster (80% of the euryarchaeotal sequences) and the remaining 20% distributed into three less abundant taxa, most of them composed of soil and sediment clones. The largest fraction of phylotypes from the two archaeal kingdoms (79% of the *Crenarchaeota* and 54% of the *Euryarchaeota*) was retrieved from the anoxic hypolimnion, indicating that these cold and sulfide-rich waters constitute an unexplored source of archaeal richness. The taxon rank-frequency distribution showed two abundant taxa (MCG and DHVE) that persisted in the water column through seasons, plus several rare ones that were only detected occasionally. Differences in richness distribution and seasonality were observed, but no clear correlations were obtained when multivariate statistical analyses were carried out.

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 environments studied (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 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 monitored seasonally 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 others).

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 detailed research on the diversity, dynamics, and function of anoxygenic photosynthetic sulfur bacteria (Guerrero *et al.*, 1980, 1985; Bañeras & García-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, 2001). 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.*, 2001). 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.*, 2001). 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 harbor a high archaeal richness.

Materials and methods

Study site

Lake Vilar is one of the largest water bodies in the lacustrine system of Banyoles (Spain, 42°8′N, 2°45′E). The lake has a total area of 10 766 m^2 and a volume of 51 453 m^3. It is composed by two separated circular basins of 10 m depth fed with bottom springs that provide a water inflow with a high sulfate concentration (0.8 $g\,L^{-1}$). The lake has traditionally been considered to be of a meromictic nature because of its permanent chemical stratification, which maintains an anoxic, sulfide-rich monimolimnion (Guerrero *et al.*, 1980, 1985).

Sampling and analytical procedures

Water samples were collected along 20 sampling cruises at the southern basin of the lake at different date intervals for a period of 5 consecutive years (2001–2005). 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, OH). 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 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 was collected in sterile screw-capped glass tubes and fixed by adding zinc acetate (0.1 M final concentration) under alkaline conditions (NaOH, 0.1 M final concentration). Sulfide concentration was measured using the leucomethylene blue method (Brock *et al.*, 1971).

DNA extraction

Water samples (100–250 mL) for DNA extraction were first filtered through 5.0 μm pore size, 47 mm diameter polycarbonate filters (Isopore^{TM}, Millipore, MA) to remove particulate debris as well as large protozoa, which are potential hosts for endosymbiontic archaea (i.e. methanogens, Casamayor *et al.*, 2001). Eluents were then filtered through 0.22 m pore size, 47 mm diameter polycarbonate Isopore filters 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 *et al.* (1994). The cell lysis was accomplished using a two-step protocol consisting of a first incubation step using lysozyme (final concentration 1 $mg\,mL^{-1}$) at 37 °C for 45 min, followed by a second one using proteinase K (final concentration 0.2 $mg\,mL^{-1}$) at 55 °C 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 (final concentration 2% v/v, Sigma-Aldrich, Germany) and polyvinylpyrrolidone (PVP, final concentration 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 °C. 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

stored at −80 °C until use. DNA concentration and purity were further determined using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop, DE).

PCR amplification

Amplification of archaeal 16S rRNA gene fragments was carried out using nested-PCR reactions with the primer pairs and PCR conditions summarized in Table 1. The first PCR amplification round was performed using the universal archaeal primer pair 21f/958r (DeLong, 1992). PCR products (*c.* 900 bp) were later used as templates for nested-PCR reactions using three combinations of internal primers that yielded amplicons suitable for further denaturing gradient gel electrophoresis (DGGE) analysis as follows:

(1) 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, 2001);

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

(3) PAIR-3: the ARC337f/ARC915r set, combining the reverse primer of PAIR-1 with a newly designed forward primer biased towards freshwater *Crenarchaeota* (Table 1). Primer design was performed 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 (accession numbers in GenBank 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 primer ARC337f at the 5′- and 3′ ends, respectively. Despite these differences, CREN334f showed the same unspecificities as ARC337f when applied for the direct amplification of archaea using the same PCR conditions (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 the ARC337f/ARC915r combination after a previous

Table 1. PCR conditions and primers used

			PCR conditions†							
				Denaturation		Annealing		Elongation		
Target	Primer*	Sequence (5′–3′)	Cycles	°C	Time (min)	°C	Time (min)	°C	Time (min)	References
First PCR round										
Universal Archaea	21f	TTCCGGTTGATCCYGCCGGA	30	94	1	56	1	72	2	DeLong (1992)
	958r	YCCGGCGTTGAMTCCAATT								DeLong (1992)
Second PCR round										
General Archaea	PAIR1		10+20	94	1	68‡/60	1	72	1.5	
	ARC344f	ACGGGGCGCAGCAGGCGCGA								Raskin *et al.* (1994)
	ARC915r	GTGCTCCCCCGCCAATTCCT								Stahl & Amann (1991)
Marine *Crenarchaeota*	PAIR2		25	94	1	58	1	72	1.5	
	CREN28f	AATCCGGTTGATCCTGCCGGACC								Schleper *et al.* (1997)
	CREN457r	TTGCCCCCCGCTTATTCSCCC								Schleper *et al.* (1997)
Freshwater *Crenarchaeota*	PAIR3		25	94	1	58	1	72	1.5	
	ARC337f	ATGGGCACTGAGACAAGG								This work
	ARC915r	GTGCTCCCCCGCCAATTCCT								Stahl & Amann (1991)

*A GC-rich clamp was attached to the 5′ end of each forward primer used in nested-PCR for DGGE analysis (Muyzer *et al.*, 1993).
†Before first cycle, the temperature was held at 94° for 4 min and after all cycles the temperature was kept at 72° during 30 min for final template elongation.
‡Program consisted in a touch-down protocol where the initial annealing temperature decreased by 0.5 °C each cycle during the first 16 cycles.
f, forward; r, reverse.

amplification using universal archaeal primers (e.g. 21f/958r) may still offer a good strategy to enhance the detection of freshwater archaea (see Results).

PCR amplification reactions were carried out in a GeneAmp 2700 thermocycler (Applied Biosystems, Perkin-Elmer, CA). PCR mixtures (50 μL) for first-round amplifications contained 5 μL of 10 × PCR buffer containing 15 mM $MgCl_2$ (Qiagen, GmbH, Hilden, 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, New England), 0.2 μM of each primer (21f/958r), 0.025 U of Qiagen-DNA Taq polymerase (Qiagen), 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, Hamburg, Germany). Nested reactions were prepared as described above, except for primer pair used, the final $MgCl_2$ concentration (1.5 mM instead of 2 mM), and no BSA addition. Details (i.e. number of cycles and annealing temperatures) for universal and nested-PCR conditions are compiled in Table 1. Positive PCR products were visualized using electrophoresis on 1.5% (w/v) agarose gels after ethidium bromide staining.

DGGE fingerprinting

PCR products were separated using an INGENY phorU-2 (Ingeny International BV, the Netherlands) DGGE system. Between 500 and 1000 ng of the PCR product was loaded onto 6.0% polyacrylamide gels and run with 1 × TAE buffer using a 20–80% linear gradient of urea and formamide [100% denaturant agent contains 7 M urea and 40% deionized formamide (McCaig *et al.*, 2001)]. This gradient was broad enough to accommodate most of the archaeal phylotypes (Yu *et al.*, 2008). Electrophoreses were run at 60 °C and at a constant voltage of 120 V for 17 h. After electrophoresis, gels were stained for 30 min with 1 × SYBR Gold nucleic acid stain (Molecular Probes Inc.) in 1 × TAE buffer, rinsed, and visualized under UV radiation using a GelPrinter system (TDI, Spain). Discrete bands were excised from the DGGE gel and rehydrated in 30 μL of Tris-HCl 10 mM buffer (pH = 7.4). DNA was eluted after incubation at 65 °C for 3 h and amplified using the same primer pairs (without GC clamp) and PCR conditions as cited above but sizing down the number of PCR cycles up to 20. PCR products were directly sent for sequencing to Macrogen Inc. (Seoul, Korea).

Phylogenetic analysis

All retrieved sequences were compared for the closest relatives to partial 16S rRNA gene sequences in NCBI sequence database (http://www.ncbi.nlm.nih.gov/blast/) using the BLASTN algorithm tool (Altschul *et al.*, 1990). Sequences 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 the ARB software package (http://www.arb-home.de/; Ludwig *et al.*, 2004) using the 16S rRNA gene ARB-compatible database (updated May 2007) available at the Greengenes website. All sequences obtained from DGGE bands (*c.* 400–600 bp), as well as other closely related archaeal sequences (*c.* 500–1300 bp) from databases, were then added to the backbone tree using the parsimony quick add marked tool implemented in ARB, thereby maintaining the overall tree topology provided by default. Clustering, grouping, and naming used in this study were based on the cluster redefinition for *Crenarchaeota* and *Euryarchaeota* proposed by Teske & Sorensen (2008).

Nucleotide sequences accession numbers

All 16S rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers AM697959–AM698009 and EU683310–EU683429.

Results

Physico-chemical characterization of the water column

Lake Vilar has traditionally been considered a meromictic lake due to the continuous incoming flux of sulfate-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 decrease 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 (Fig. 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. 1). Oxygen minimum values were detected at the chemical transition zones (*c.* from 4 to 5 m depth), whereas below 5 m depth the water became anoxic and sulfide accumulated, reaching values up to 1500 μmol L^{-1}. Representative water samples from different depths were collected to compare the planktonic archaeal assemblage thriving in both water compartments.

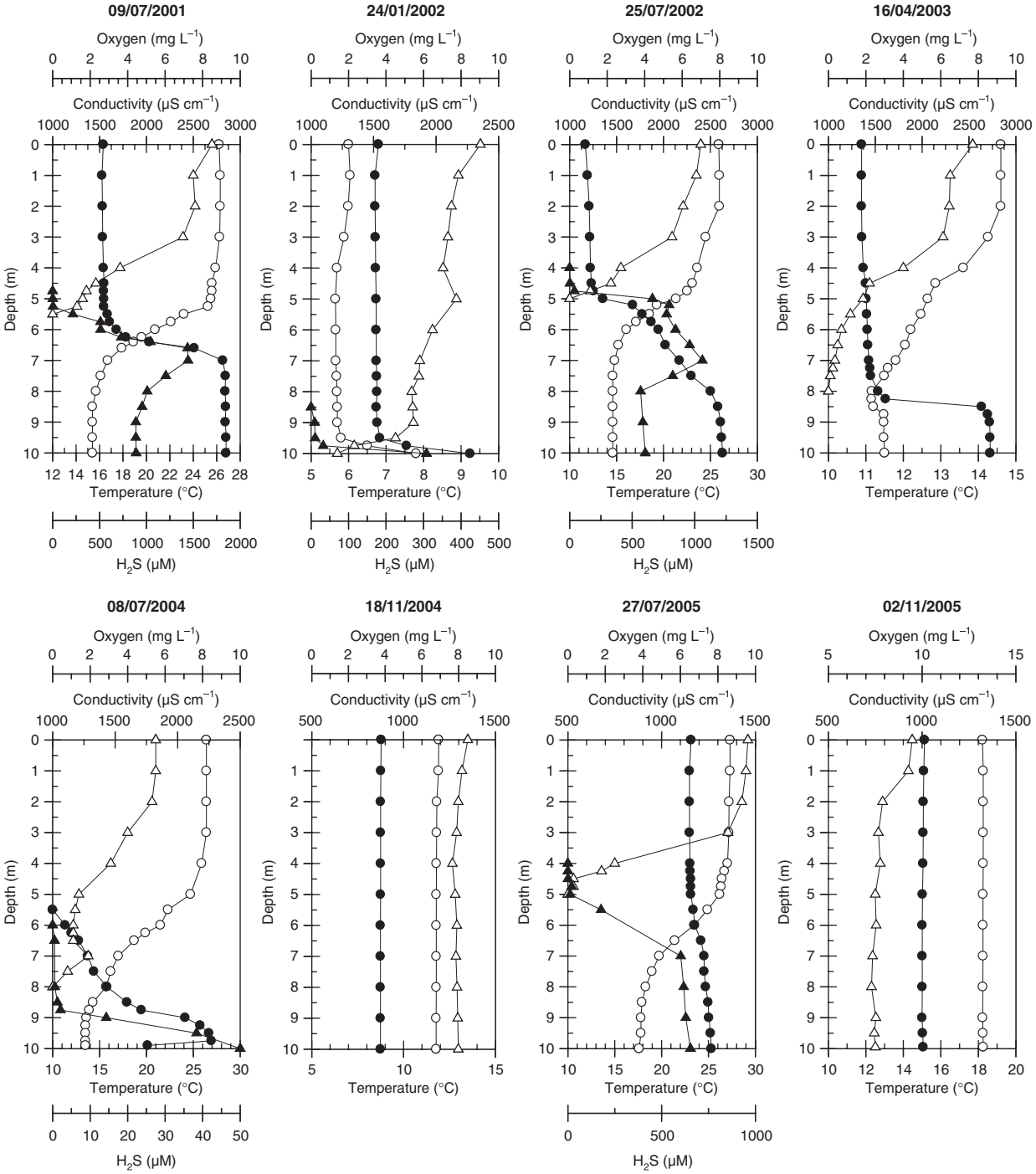

Fig. 1. Physico-chemical depth profiles for temperature (○), conductivity (●), oxygen (△), and sulfide (▲) in the water column of Lake Vilar. Dates have been selected to illustrate periods of summer stratification and winter mixing.

Molecular analysis

We used a PCR-DGGE approach using three primer combinations (Table 1) 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 and 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 smeared 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 2 and Fig. 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 2). Fifty six percent of these sequences (35 out of 63) clustered within Group 1.2 of the Miscellaneous *Crenarchaeota* Group (MCG), a large cluster of environmental crenarchaeotal sequences retrieved from different environments (Inagaki *et al.*, 2003) (Fig. 3, sequences in red). The remaining sequences (44%) were distributed among different subclusters of the MCG (Table 2), 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 2). From this total, 38 and 33 sequences were affiliated within the DHVE-3 and the MCG Group 1.3 clusters, respectively (Figs 2 and 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 2).

Table 2. 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*			Total seq.	% of total	% within kingdom
	PAIR1	PAIR2	PAIR3			
Euryarchaeota						
Hydrothermal 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†						
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	

*For primer pair details and PCR conditions see Table 1 and Materials and methods section.

†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; DMS, deep marine surface; SSM, subsurface marine.

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 2). 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 2). Interestingly, most of the sequences (34 *Crenarchaeota* and 15 *Euryarchaeota*) were recovered from anoxic layers during summer stratification (Fig. 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). Besides, when the number of sequences vs. the taxon was plotted (Fig. 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).

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

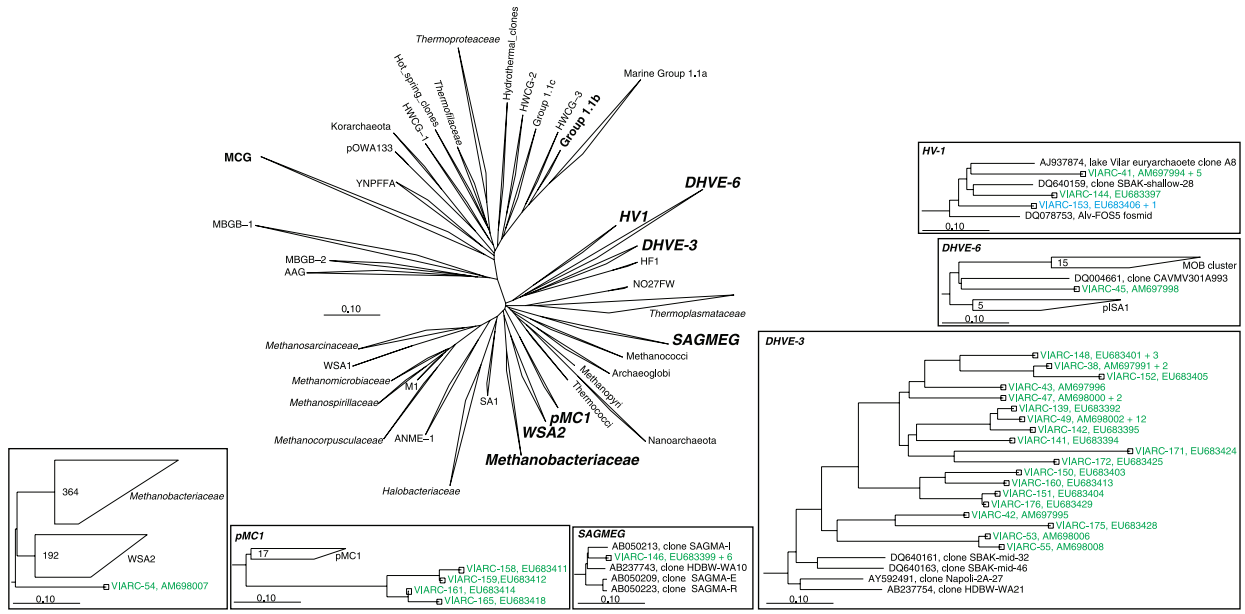

Fig. 2. Phylogenetic tree showing the affiliation of *Euryarchaeota* partial 16S rRNA sequences retrieved from the water column of Lake Vilar during the study period. Sequences recovered using PAIR-1 and PAIR-3 are shown in blue and green color, respectively. Database sequences are shown in black. When possible, only one representative sequence of each phylotype (> 98% identity) is shown together with the number of additional sequences ascribed to the same phylotype. Reference sequences are described by 'clone name and accession number'. The scale bar indicates 10% estimated sequence divergence.

conditions (i.e. temperature, conductivity, and both oxygen and sulfide concentrations) at the date of sampling.

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.*, 2001). Probably, there was lower abundance of archaea during this 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 the 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 Materials 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. PAIR2, 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 2). 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 arises 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,

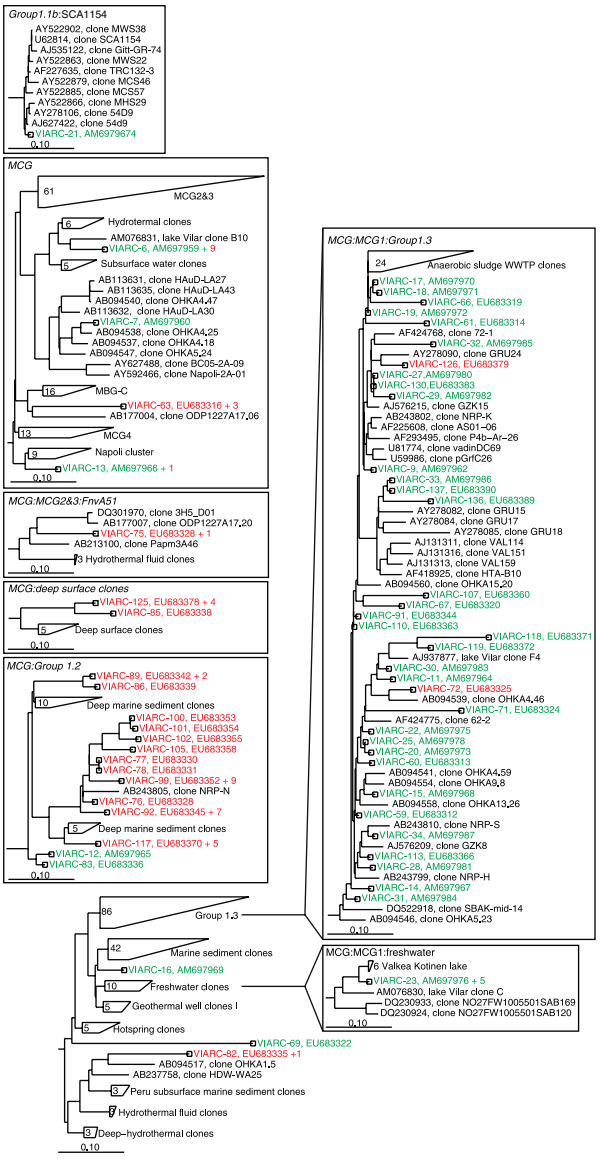

Fig. 3. Same as Fig. 2 but for the kingdom *Crenarchaeota*. Sequences recovered using PAIR-2 and PAIR-3 are shown in red and green color, respectively. When possible, only one representative sequence of each phylotype (> 98% identity) is shown together with the number of additional sequences ascribed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

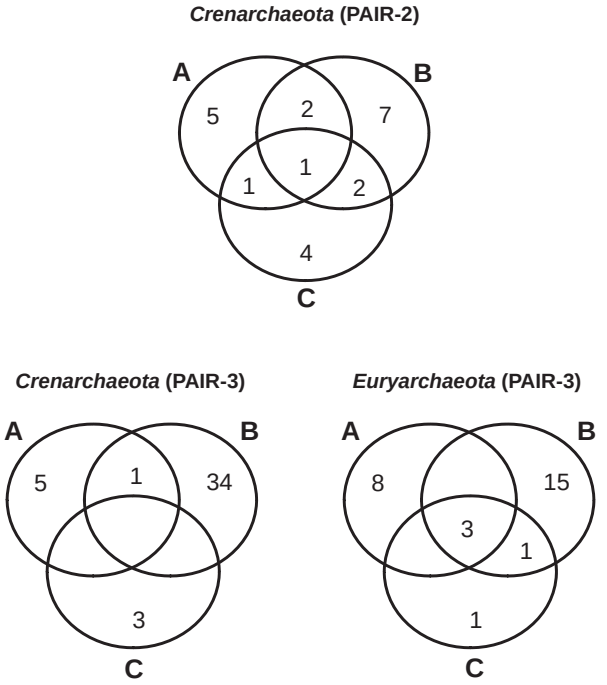

Fig. 4. Venn diagram representation of total phylotypes affiliated to *Crenarchaeota* and *Euryarchaeota* retrieved using different primer combinations from specific water compartments of Lake Vilar during the study period. A, Phylotypes exclusively found in oxic water layers during summer stratification; 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 the oxic, anoxic, and whole water column, respectively.

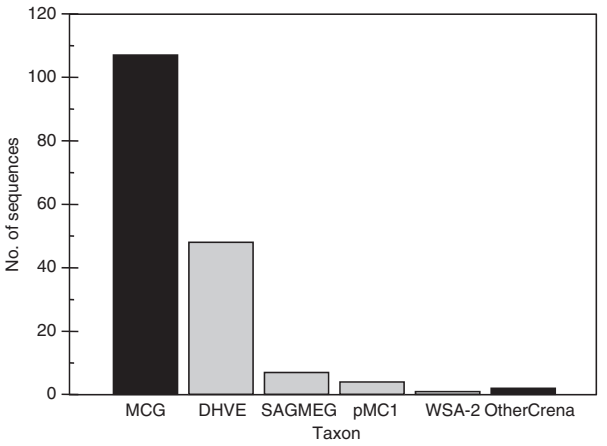

Fig. 5. Distribution of the total number of sequences retrieved from the water column of Lake Vilar vs. Taxon, with taxa ranked according to their respective sequence abundance. *Crenarchaeota* and *Euryarchaeota* clusters are shown in black and gray, respectively. All sequences affiliated to MCG subclusters (see text) and hydrothermal vent clusters (HV-1, DHVE-3, and DHVE-6) have been grouped together.

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 (Bosshard *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.*, 2001). 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, 2001). 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 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 & Fuhrman, 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 (A. Plasencia & C.M. Borrego, 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 & García-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; Varela *et al.*, 2008; Beman *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 for 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 season suggests that they constituted an archaeal seed-bank with low population losses a waiting better conditions for 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 assumed previously, 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.*, 2001). 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 (A. Plasencia & C.M. Borrego, 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.*, 2007), 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 nonextreme environments because only after isolates are studied, will we succeed in the ecophysiological interpretation of environmental 16S rRNA gene data.

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Supporting Information

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

Table S1. Complete list of archaeal 16S rRNA partial gene sequences retrieved from Lake Vilar during the study period and used to construct the phylogenetic trees shown in Figs 2 and 3. Sequences are listed by phylogenetic group, name and accession number.

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