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RESEARCH ARTICLE

Diversity of Miscellaneous Crenarchaeotic Group archaea in freshwater karstic lakes and their segregation between planktonic and sediment habitats

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One sentence summary: Widespread subsurface archaea of the Miscellaneous Crenarchaeotic Group are common components of archaeal communities in freshwater karstic lakes where they segregate between planktonic and sediment habitats.

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ABSTRACT

The *Miscellaneous Crenarchaeotic Group* (MCG) is an archaeal lineage whose members are widespread and abundant in marine sediments. MCG archaea have also been consistently found in stratified euxinic lakes. In this work, we have studied archaeal communities in three karstic lakes to reveal potential habitat segregation of MCG subgroups between planktonic and sediment compartments. In the studied lakes, archaeal assemblages were strikingly similar to those of the marine subsurface with predominance of uncultured *Halobacteria* in the plankton and *Thermoplasmata* and MCG in anoxic, organic-rich sediments. Multivariate analyses identified sulphide and dissolved organic carbon as predictor variables of archaeal community composition. Quantification of MCG using a newly designed qPCR primer pair that improves coverage for MCG subgroups prevalent in the studied lakes revealed conspicuous populations in both the plankton and the sediment. Subgroups MCG-5a and -5b appear as planktonic specialists thriving in euxinic bottom waters, while subgroup MCG-6 emerges as a generalist group able to cope with varying reducing conditions. Besides, comparison of DNA- and cDNA-based pyrotag libraries revealed that rare subgroups in DNA libraries, i.e. MCG-15, were prevalent in cDNA-based datasets, suggesting that euxinic, organic-rich sediments of karstic lakes provide optimal niches for the activity of some specialized MCG subgroups.

Keywords: anoxic sediments; euxinia, karstic lakes; Miscellaneous Crenarchaeotic Group; sulphidic redoxclines; uncultured archaea

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INTRODUCTION

The *Miscellaneous Crenarchaeotic Group* (MCG) is an archaeal clade originally described by Inagaki *et al.* (2003) after analyses of 16S rRNA gene sequences recovered from coastal subseafloor sediments. Gene surveys identified MCG rRNA sequences in diverse environments such as terrestrial hot springs (Barns *et al.* 1996), continental shelf sediments (Vetriani *et al.* 1999), terrestrial subsurface sediments (Breuker *et al.* 2011), marine hydrothermal vents (Teske *et al.* 2002), ancient marine sapropels (Coolen *et al.* 2002), and sulphidic springs and caves (Elshahed *et al.* 2003; Chen *et al.* 2009). Despite this widespread distribution, MCG archaea mainly predominate in subseafloor sediments, where they may account from 10 to up to 100% of total archaea (Biddle *et al.* 2006, 2008; Fry *et al.* 2008; Durbin and Teske 2011, 2012; Kubo *et al.* 2012). MCG archaea are thus under the spotlight due to the large amount of carbon accumulated in the subseafloor ($\approx 15 \times 10^{21}$ g C, Fry *et al.* 2008) and the impact that subsurface microbes might have on biogeochemical cycles (Parkes, Cragg and Wellsbury 2000; Parkes *et al.* 2005; Webster *et al.* 2006; Lipp *et al.* 2008; Roussel *et al.* 2008; Orcutt *et al.* 2013). Studies using molecular and isotopic signatures suggested that MCG were heterotrophic anaerobes feeding on buried organic carbon (Biddle *et al.* 2006). This assumption has been supported by results from incubation of estuarine sediments slurries under heterotrophic conditions (Webster *et al.* 2010, 2011; Gagen *et al.* 2013; Seyler, McGuinness and Kerkhof 2014) and by the identification of genes coding for enzymes involved in the degradation of both protein and aromatic compounds in genomic fragments of MCG archaea (Lloyd *et al.* 2013; Meng *et al.* 2014). Although these findings constitute an important step forward the understanding of the potential metabolism of MCG archaea in the subseafloor, the complex intragroup phylogeny of this lineage, composed of 17 subgroups (Kubo *et al.* 2012), hampers any general inference regarding the metabolic capabilities of the whole lineage. Besides, the fact that most subgroups contain sequences retrieved from marine, limnic and terrestrial habitats poses an extra difficulty to infer direct relationships between MCG subgroups and ecological conditions.

Little is known, however, about the diversity and ecology of MCG archaea in habitats other than marine sediments although several studies reported the presence of MCG in anoxic, sulphide-rich water layers of stratified lakes (Lehours *et al.* 2007; Jiang *et al.* 2008; Llirós, Casamayor and Borrego 2008; Llirós *et al.* 2010, 2011; Buckles *et al.* 2013). MCG have also been detected in sediments from freshwater and subsaline lakes (Jiang *et al.* 2008; Ferrer *et al.* 2011; Bhattarai *et al.* 2012; Borrel *et al.* 2012; Buckles *et al.* 2013), as well as in wetlands with different physicochemical conditions (Dorador *et al.* 2013; Llirós *et al.* 2013). The presence of other archaeal groups typical from the deep marine subsurface such as the *Marine Benthic Groups B* and *D* (MBG-B, MBG-D) or the *Deep Hydrothermal Vent Euryarchaeota Group* (DHVEG) in the water column and sediments of mesotrophic lakes is also well documented (Lehours *et al.* 2007; Jiang *et al.* 2008; Bhattarai *et al.* 2012; Borrel *et al.* 2012; Gies *et al.* 2014) and contrasts with the prevalence of ammonia-oxidizing thaumarchaeota in oligotrophic systems (Pouliot *et al.* 2009; Auguet *et al.* 2011, 2012). While the ecological role and significance of the latter group is currently widely accepted (Nicol and Schleper 2006; Prosser and Nicol 2008; Pester, Schleper and Wagner 2011; Biller *et al.* 2012; Bouskill *et al.* 2012), a definitive understanding of same issues for the above-mentioned uncultured groups is still missing due to the lack of cultured representatives from which to infer their metabolism and physiological requirements.

The differences between subsurface environments and euxinic waters and sediments of stratified lakes in both physicochemical conditions and availability of energy and carbon sources pose intriguing questions regarding the diversity and ecological adaptations of uncultured archaeal groups to these habitats. This is especially evident considering the high diversity and complex phylogeny of archaeal lineages such as the *Thermoplasmata* (Barberan *et al.* 2011) and, specially, the MCG (Kubo *et al.* 2012). In this regard, many key questions remain unanswered, such as: Do different habitats share the same (or distinct) MCG subgroups? Which physicochemical conditions favour the growth and activity of certain MCG subgroups and no others? Very recently, Lazar *et al.* (2015) analysed the MCG subgroup distribution at different depth layers in estuarine sediments trying to decipher which environmental conditions influence their distribution and ecology. Although authors provided some clues to better understand habitat preferences of predominant MCG subgroups in estuarine and marine sediments, extrapolation to other habitats (e.g. freshwater plankton) is speculative. In the current work, we have extended these questions to planktonic and sediment compartments of three freshwater karstic lakes with different limnological (from holo- to meromixis) and trophic conditions (from oligotrophy to eutrophy) where MCG archaea have consistently been found. Composition of archaeal communities and abundance and diversity of MCG have been analysed by massive parallel sequencing and quantitative PCR (qPCR) using a newly designed primer set that provides deep phylogenetic resolution of the MCG. Besides, comparison of DNA and cDNA pyrotag libraries revealed that MCG subgroups predominant in DNA fractions were not always among the active members of the community. Results are discussed on the light of recent advances on MCG phylogeny and potential activity in relation to nutrient cycles and ecological preferences.

MATERIAL AND METHODS

Study area

The three studied lakes (lakes Vilar, Cisó and basin C-III) are located in the karstic system of Banyoles (Girona, NE Spain, 42°07′ N, 2°45′ E). All these lakes become thermally stratified in summer and maintain a permanent chemical stratification of the water column due to inflows of water by bottom springs (Moreno-Amich and Garcia-Berthou 1989). This phreatic water contains a high concentration of dissolved sulphate (~10 mM, Guerrero *et al.* 1985) that favours the accumulation of sulphide in bottom water layers as a result of bacterial sulphate reduction. As in other karstic systems, sulphur cycle dominates the chemistry of all lakes and lagoons in the area.

The three studied lakes were selected on the basis of their different nutrient status and limnological characteristics. Basin C-III is a circular, regular-shaped basin of 32 m depth located in the northern lobe of Lake Banyoles. C-III is a meromictic basin that maintains a euxinic monimolimnion below 20 m depth. Lake Banyoles has traditionally been classified as oligotrophic according to their low nutrient concentration and phytoplankton biomass, especially in their epilimnetic waters. Lake Vilar is one of the largest water bodies in the lacustrine system of Banyoles. The lake has a total area of 10 766 m^2 and a volume of 51 453 m^3. The water column usually mixes completely during winter causing a complete oxygenation of the lake. Vilar has been classified as eutrophic according to high nutrient inputs by run-off streams and high epilimnetic

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concentrations of Chl-*a* (25 mg m^{-3}) (Bañeras and Garcia-Gil 1996). Lake Cisó is a small holomictic lake (650 m^2) with a maximum depth of 6.5 m. Physicochemical properties and dynamics of bacterial populations have been studied in detail and can be found elsewhere (Guerrero *et al.* 1985; Gasol *et al.* 1991; Casamayor *et al.* 2000). Lake Cisó thermally stratifies during summer maintaining an anoxic and sulphide-rich hypolimnion below 2 m depth until winter mixing, which extends anoxia to the whole water column.

Sample collection and chemical analysis

In each environment, depth profiles for water temperature, conductivity, pH, redox potential (EH) and oxygen concentration were determined *in situ* with a multiparametric probe OTT-HydrolabMS5 (Hatch Hydromet, Loveland, CO, USA). Water samples were collected at different depths along the vertical physicochemical profile with special emphasis in the oxic-anoxic transition zone at the metalimnion and the anoxic, sulphide-rich hypolimnion. Water samples were collected using a special device consisting of a weighted double cone connected to a battery-driven pump which allowed a laminar water sampling and a minimal disruption of microstratification (Jorgensen, Kuenen and Cohen 1979). On boat, water samples were kept on ice and protected from light in a portable icebox until further analysis within 24 h. For sulphide analysis, 10 mL of water was collected in sterile screw-capped glass tubes and ffixed *in situ* by adding zinc acetate (0.1 M ffinal concentration) under alkaline conditions (NaOH, 0.1 M ffinal concentration). Sulphide concentrations were determined from fixed subsamples (see above) according to Brock *et al.* (1971). Samples for the determination of ammonia were collected in sterile 50 mL Falcon tubes after filtration through 0.22 μm pore-size Millipore membrane filters and acidified with HCl. For nitrate, nitrite and sulphate determinations, 10 mL subsamples were filtered through 0.22 μm pore-size Millipore membrane filters and kept frozen at −30°C until analysis. Sulphate, ammonia, nitrate and nitrite were measured by ionic chromatography (DIONEX, Model IC5000) using IonPac AS18 anion-exchange column (4 × 250 mm) with the AG Guard column (4 × 50 mm), using 30 mM MSA for the production of the mobile phase for cations and 22–40 mM KOH for anions. For both cases (anions and cations), the injection volume was 25 μL with an eluent flow rate of 1 mL min^{-1}. For dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) determinations, water subsamples of 50 mL were filtered through Millex-HA, MF 0.45 μm pore-size, 25 mm diameter Nylon filters and kept at 4°C until measurement. DOC and DIC were oxidized through combustion and analysed in a total organic carbon analyser (TOC-V CSH, Shimadzu). Total phosphorous (TP) was measured according to UNE-EN ISO 6878.

Sediment samples were collected in lakes Vilar and Cisó using a sediment corer (Uwitec Sediment Corer). On board, sediment corers were placed into dry ice and stored at −80°C upon arrival at the laboratory within 2 h after collection. In the laboratory, frozen corers were sliced at 1-cm intervals using sterile instruments and the resulting fractions—16 and 12 for lakes Vilar and Cisó sediments, respectively—were placed in sterile Petri dishes. Two grams of each sediment fraction was stored at −80°C until nucleic acid extraction. The remaining material was used for nutrient analysis as follows: the sediment material was collected into a sterile polypropylene Sorvall Tube (Cat. N° 03147), thawed and centrifuged at 11 000 rpm for 15 min in a Centrifuge 5804R (Eppendorf). Recovered pore water was filtered and analysed for nutrients as described above.

Nucleic acids extraction and processing

Water samples (200 mL) for DNA extraction were filtered through 0.22-μm pore-size, 47-mm diameter polycarbonate ffilters (ISOPORE, Millipore, MA, USA) and kept frozen at −80°C until processing. Total DNA from the microbial community was extracted from filters as previously described (Llirós *et al.* 2010). Dry DNA pellets were finally rehydrated in 50 μL of 10 mM Tris-HCl buffer (pH 7.4). For sediment samples, 2 g of each sediment fraction was used for both RNA and DNA extraction using RNA PowerSoil Total RNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) in combination with RNA PowerSoil DNA Elution Accessory Kit (MoBio Laboratories, Solana Beach, CA, USA) following manufacturer instructions. DNA and RNA were stored at −80°C until processing. The concentration and purity of both DNA and RNA extracts obtained from each sediment layer were determined fluorimetrically using QUBIT2.0 Fluorometer (Invitrogen Molecular probes Inc., Oslo, Norway). RNA extracts were treated with RTS DNase™ kit (MOBIO) to remove traces of contaminant DNA. RNA was then retrotranscribed to cDNA using random hexamer primers and SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

PCR and cloning

DNA from sediment and planktonic samples from lakes Vilar and Cisó was amplified using universal archaeal 16S RNA gene primers Arch21F-Arch958R (DeLong 1992). Amplicons were then used to construct clone libraries aimed to obtain partial length archaeal 16S rRNA gene sequences for primer design. Amplification reactions were carried out using 1 μL of DNA template, 1 μL of each primer, 5 μL of buffer ($MgCl_2$ 15 mM), 1 μL dNTP mix, 1 μL of $MgCl_2$ 25 mM, 2 μL of BSA, 0.25 μL of *Taq* polymerase (QIAGEN, Manchester, UK) and molecular biology grade water to reach a final volume of 50 μL. The PCR programme started with 2 min initial denaturalization at 94°C, followed by 35 cycles of 94°C for 60 s, 56°C for 60 s and 72°C for 2 min, and a final 15 min elongation at 72°C. All PCR were run in a GeneAmp 2700 thermal cycler (Applied Biosystems, Perkin-Elmer, CA, USA). PCR and qPCR products (see below) were purified using QIAQuick PCR Purification kit (QIAGEN, Manchester, UK) and cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. In each library, clones were randomly picked and checked for the presence of correct size inserts. Valid clones (i.e. those showing the correct size insert) were further purified and amplified using the M13 primers provided by the TOPO TA Cloning Kit and sequenced by an external company (Macrogen Inc., Seoul, Korea). Clone sequences were checked for quality using Sequence Scanner Software v1.0 (v1.0, Applied Biosystems, Carlsbad, CA, USA) and manually curated in BioEdit (Hall 1999). Chimera checking and alignment was carried out in MOTHUR (http://www.mothur.org, Schloss *et al.* 2009) using the Greengenes database (DeSantis *et al.* 2006) as reference alignment.

Primer design

A sequence dataset consisting of 305 16S rRNA gene sequences of MCG archaea was obtained after curation and alignment of sequences recovered in clone libraries obtained from planktonic and sediment DNA samples of the studied lakes (see above). Alignment and primer design was done in Geneious Pro software (version 5, Biomatters Ltd). Six primer combinations were

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initially obtained and then checked *in silico* for specificity using the TestProbe tool in ARB (http://www.arb-home.de/, Ludwig *et al.* 2004) loaded with the 16S rRNA gene ARB-compatible database SSU r108 RefNR (September 2011). Coverage of the best primer pair combination (MCG-242F/MCG-678R) was checked against the reference database. Maximum coverage within the MCG lineage was obtained after degeneration of two bases of the forward primer (MCG-242dF, Supporting Information, Table S1), which allowed a good match against representative sequences of all MCG subgroups already described (Kubo *et al.* 2012).

Quantification by qPCR

Copy numbers of bacterial, archaeal and MCG 16S rRNA genes were determined from DNA extracts by qPCR using primers and conditions compiled in Table S2 (Supporting Information). Quantifications were performed in an Mx3005P system (Agilent Technologies) using SYBR Green detection chemistry and qPCR 96-well plates (Agilent Technologies, Cat. N° 401334) and Mx3000P Optical Strip Caps (Agilent Technologies, Cat. N° 401425). Every reaction was prepared for a final volume of 30 μL containing 15 μL of 2× Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies), 1.2 μL of each forward and reverse primer (final concentration of 0.4 μM), 1 μL of template DNA (or cDNA) and molecular biology grade water up to 30 μL volume. Standards for bacterial and archaeal 16S rRNA gene quantification were made using genomic DNA of *Escherichia coli* DSM30083 and *Sulfolobus solfataricus* DSM1616, respectively. A plasmid containing an insert of an environmental MCG 16S rRNA gene isolated from clone libraries (see above) was used as MCG standard for quantification. Data analyses were carried out using the MxPro QPCR Software for Mx3005P QPCR System (Agilent Technologies). Primer sequences, reaction temperatures, R^2 values and amplification efficiencies for each qPCR reaction are compiled in Table S2 (Supporting Information). All qPCR analyses carried out followed the MIQE rules for qPCR analyses (Bustin *et al.* 2009) and all essential information has been included in this section. Several qPCR products obtained on the course of experimentation using the newly designed primer pair MCG-242dF/MCG-678R and pair MCG-528F/MCG-732R (Kubo *et al.* 2012) were cloned (see above) to assess and compare their specificity (see below). Sequences of these cloned products were deposited in GenBank under accession numbers KP702951-KP702976, KP703028–KP703078 (pair MCG-242dF/MCG-678R) and KP702977-KP703027, KP703079-KP703165 (pair MCG-528F/MCG-732R).

Phylogenetic analyses of community composition

DNA and cDNA extracts from selected samples were analysed through tag-encoded FLX-Titanium amplicon pyrosequencing at the Research and Testing Laboratory (Lubbock, TX, USA). Briefly, genomic DNA and cDNA from planktonic and sedimentary communities was used as a template in PCR reactions using archaeal primers PARCH340f (Øvreas *et al.* 1997) and ARCH958R (DeLong 1992) targeting the V3–V5 hypervariable regions of the archaeal 16S rRNA gene complemented with 454-adapters and sample-specific barcodes. Raw sequence dataset was pre-processed at RTL facilities to reduce noise and sequencing artefacts as previously described (Dowd *et al.* 2008). Demultiplexing according to sample barcodes, sequence quality assessments, chimera detection and downstream phylogenetic analyses were conducted in QIIME (Caporaso *et al.* 2010). Details on the phylogenetic analysis pipeline used in QIIME are provided as supporting information. Pyrosequencing data of this study have been deposited in the NCBI database via the Biosample Submission Portal (http://www.ncbi.nlm.nih.gov/biosample/) under accession number PRJNA268143.

Affiliation of MCG sequences to different MCG subgroups was carried out in ARB (Ludwig *et al.* 2004) after importing representative sequences of OTUs affiliated to the MCG lineage into a previously built phylogenetic tree composed of reference sequences of the 17 MCG subgroups known so far (Kubo *et al.* 2012), which were used as phylogenetic anchors. Affiliation of both pyrotags and clone sequences to a given subgroup was done at a similarity cut-off of ≥85%. All the phylogenetic trees in this paper had been edited and modified with metadata with the online tool Interactive Tree of Live (iTOL) (Letunic and Bork 2007).

Statistical analyses

For community composition analyses, we constructed a similarity matrix using the Bray–Curtis distance (Legendre and Legendre 1998) based on the relative abundance (square root transformed) of each archaeal class. Similarity matrices were constructed using the relative abundance of different MCG subgroups and clades within the classes *Halobacteria* and *Thermoplasmata*. Ordination of samples according to Bray–Curtis distance matrices was done using non-metric multidimensional scaling (NMDS) plots. Analysis of similarity (ANOSIM) between communities based on their taxonomic composition was done after grouping samples by habitat (planktonic vs sediment), by system (basin C-III, lakes Vilar and Cisó) or by library (DNA vs RNA). These categories were used as factors for the ANOSIM analysis. The contribution of each taxon to the Bray–Curtis distance between samples grouped by above-mentioned factors was calculated using the analysis of similarity percentages (SIMPER). To identify which combination of environmental variables better explained the composition of archaeal communities, we used a stepwise distance-based linear model permutation test (DistLM, McArdle and Anderson 2001). The stepwise routine was run using 999 permutations and the AIC_c (Akaike's information criterion corrected, Akaike 1978) as selection criterion (Anderson, Gorley and Clarke 2008). The environmental data matrix included ammonia, sulphate, sulphide, TP, DOC and DIC concentrations, which were logarithmically transformed and then standardized by subtracting its mean and dividing by its standard deviation. Results were visualized using distance-based redundancy analysis (dbRDA, Legendre and Andersson 1999). All analyses were carried out in PRIMER 6 statistical package with the PERMANOVA+ add-on (PRIMER-E, Plymouth Marine Laboratory, UK).

RESULTS

Physicochemical characteristics of the studied environments

The three studied lakes (basin C-III in Lake Banyoles and lakes Vilar and Cisó) are characterized by the presence of stable redoxclines and an active sulphur cycle that maintains euxinic bottom water layers throughout the year. Our study includes both planktonic and sediment samples with physicochemical conditions ranging from suboxic (upper hypolimnion) to true anoxic (hypolimnion of lakes Vilar and Cisó) water layers, low (basin C-III) to high (lakes Vilar and Cisó) sulphide concentrations and from oligotrophic (basin C-III) to meso-eutrophic conditions (Vilar and Cisó).

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The water column of the three studied lakes was thermally stratified at the day of sampling, with thermoclines spanning along several meters in depth (Fig. S1, Supporting Information). Lakes Vilar and basin C-III also showed a clear chemical stratification, with sharp gradients located at 5 and 20.5 m depth, respectively. In clear contrast, Lake Cisó resembled the hypolimnetic compartments of basin C-III and Lake Vilar according to the overall physicochemistry of the system (Fig. S1 and Table S3, Supporting Information). Despite this similarity, summer stratification slightly oxygenated the surficial water layer of Lake Cisó generating a narrow, warmer and oxygen-deficient epilimnion (Fig. S1, Supporting Information). In turn, the epilimnion of Lake Vilar was homogenously oxygenated from surface to 4 m depth, where the oxygen began to decrease. In basin C-III, the oxygen concentration showed a maximum at 8 m depth that steadily decreased downwards to complete anoxia at 20 m depth. In lakes Vilar and basin C-III, the sharp chemocline coincided with the oxic-anoxic interphase. Sulphate and sulphide concentrations were higher in the bottom water layers of sampled lakes than in the oxic and suboxic water layers (Table S3, Supporting Information). Ammonia concentrations increased at anoxic water layers of all systems, being higher in the hypolimnia of lakes Vilar and Cisó (9.9 and 9.8 mg L^{-1}, respectively) than in basin C-III (2.4 mg L^{-1}), agreeing with their mesotrophic (Vilar) and eutrophic (Cisó) status (Miracle, Vicente and Pedrós-Alió 1992). Concentrations of nitrite and nitrate were below detection limit in most of the samples analysed.

Nutrient concentrations measured along a centimetre (cm) scale depth in sediments corers collected at lakes Vilar and Cisó were barely constant at each system but showed clear differences among them (Table S4, Supporting Information). As a general trend, ammonia concentrations were higher in Lake Vilar than in Lake Cisó (average concentration of 35.6 $\pm$ 12 and 1.81 $\pm$ 0.30 mg L^{-1}, respectively) whereas sulphate concentrations were one order of magnitude higher in Lake Cisó than in Vilar (average concentration of 44.8 $\pm$ 58 and 257 $\pm$ 16.3 mg L^{-1}, respectively) (Table S4, Supporting Information). In both lakes, concentrations of oxidized species of nitrogen (NO_2^- and NO_3^-) were below the limit of quantification of the technique (0.003 and 0.002 mg L^{-1}, respectively) at most layers. Concentrations of DOC in sediment pore water were lower in Lake Vilar (average concentration 99.3 $\pm$ 31.6 mg C L^{-1}) than in Cisó (average 178 $\pm$ 210 mg C L^{-1}) where a clear maximum was measured at 2-cm depth (878.5 mg C L^{-1}). Concentrations of DIC were, in turn, higher in Lake Vilar (average of 52.7 $\pm$ 2.8 mg C L^{-1}) than in Cisó (average 198 $\pm$ 29 mg C L^{-1}) (Table S4, Supporting Information). TOC measurements carried out in sediments collected in Lake Vilar during a later sampling campaign confirm its eutrophic condition (average TOC of 3.5 $\pm$ 1.2%, unpublished results). Although no TOC data are available for Lake Cisó, both the high DIC concentrations measured for this lake (198 $\pm$ 29 mg C L^{-1} on average, Table S3 and S4, Supporting Information) and the high contribution of organic carbon (77% of total C) in this lake also pointed to organic-rich sediments. Altogether, these results indicated similar eutrophic conditions in both habitats but a major relevance of the N cycle in Lake Vilar sediments and of the S cycle in Lake Cisó. The unavailability to measure sulphide concentrations along sediment profiles hindered a better understanding of active sulphate-reduction zones along sediment depth. Nevertheless, the high sulphide concentrations measured in bottom water layers of Lake Cisó (46.2 $\pm$ 11 mg L^{-1}) pointed to an active sulphate reduction activity fuelled by the high sulphate concentration occurring in this lake (average concentrations of 298.9 $\pm$ 10 and 257 $\pm$ 16.3 mg L^{-1} in the water column and the sediment, respectively; Tables S3 and S4, Supporting Information).

Phylogenetic composition of archaeal communities

As a general trend, archaeal communities in the three studied lakes were mainly composed of uncultured groups of both the *Euryarchaeota* and the *Thaumarchaeota*. Planktonic samples from basin C-III showed a major contribution of *Halobacteria* at all sampling depths (average 54% of total sequences), being the DHVEG-6 and the *Miscellaneous Euryarchaeota Group* (MEG) the predominant clades within this archaeal class. Members of the *Thermoplasmata* such as the MBG-D and the DHVEG-I were mainly identified at the oxic-anoxic interphase (20.25 m) but readily decreased in its relative abundance at euxinic water layers, where MCG archaea increased (Fig. 1). In lake Vilar, planktonic archaeal communities were mainly composed of the same lineages identified in basin C-III with a higher contribution of methanogenic archaea (i.e. *Methanomicrobia* and *Methanobacteria*) at the anoxic water layer just above the sediment (9.15 m depth). In Vilar, ammonia oxidizers of the *Marine Group I* (MG-I) and the *Soil Crenarchaeotic Group* (SCG) were identified just above the oxic-anoxic transition (4.75 m depth) and at the lake bottom, respectively. The contribution of MCG archaea increased with depth, from about 9% at the oxic-anoxic transition (4.75 m depth) to almost 16% at 8 m depth, but decreased again at the water–sediment interface (8.8% at 9.15 m depth). The same archaeal clades were identified in Lake Cisó. In this lake, the planktonic communities showed a higher contribution of *Halobacteria* (especially DHVEG-6 at 2 m depth) and the *Thermoplasmata* (MBG-D and DHVEG-I at 6 m depth). MCG accounted between 20 and 35% of the total archaeal sequences at 2 and 6 m depth, respectively. Similar to Lake Vilar, methanogenic archaea increased their relative abundance in depth, reaching a 17% of total archaeal sequences at the lake bottom.

Archaeal communities in sediments from lakes Vilar and Cisó were composed of the same uncultured clades found in the planktonic compartment. The surficial sediment layers of both lakes harboured similar communities mainly composed of *Thermoplasmata*, MCG and ammonia-oxidizing thaumarchaeota (SCG and MG-I in Vilar and Cisó, respectively) (Fig. 1). Deeper sediment layers showed a higher contribution of methanogens (55% of total sequences) in Lake Vilar and *Thermoplasmata* in Lake Cisó (42% of total sequences), whereas MCG contributed equally (19 and 22% in Vilar and Cisó, respectively) to archaeal diversity at these layers. Remarkably, contrasting differences were observed between DNA- and cDNA-based pyrotag libraries. Archaeal clades showing high relative abundances in DNA libraries barely contributed in the corresponding cDNA-based library. These discrepancies were, however, depth dependent, mainly affecting MG-I and SCG clades at surface layers and methanogenic groups at deeper sediment layers. Despite these differences, planktonic and benthic archaeal communities showed no significative differences in none of the alpha-diversity indices measured (Chao1, Shannon and Faith phylogenetic diversity; Faith 1992) (Fig. S2, Supporting Information).

Phylogenetic microdiversity of MCG communities

The complex intragroup phylogeny of the MCG lineage adds an extra difficulty to unambiguously resolve the affiliation of the recovered MCG sequences to the different known subgroups. In our study, 41 667 out of 132 264 sequences were affiliated with the MCG lineage (31%). These sequences clustered into 116 OTUs

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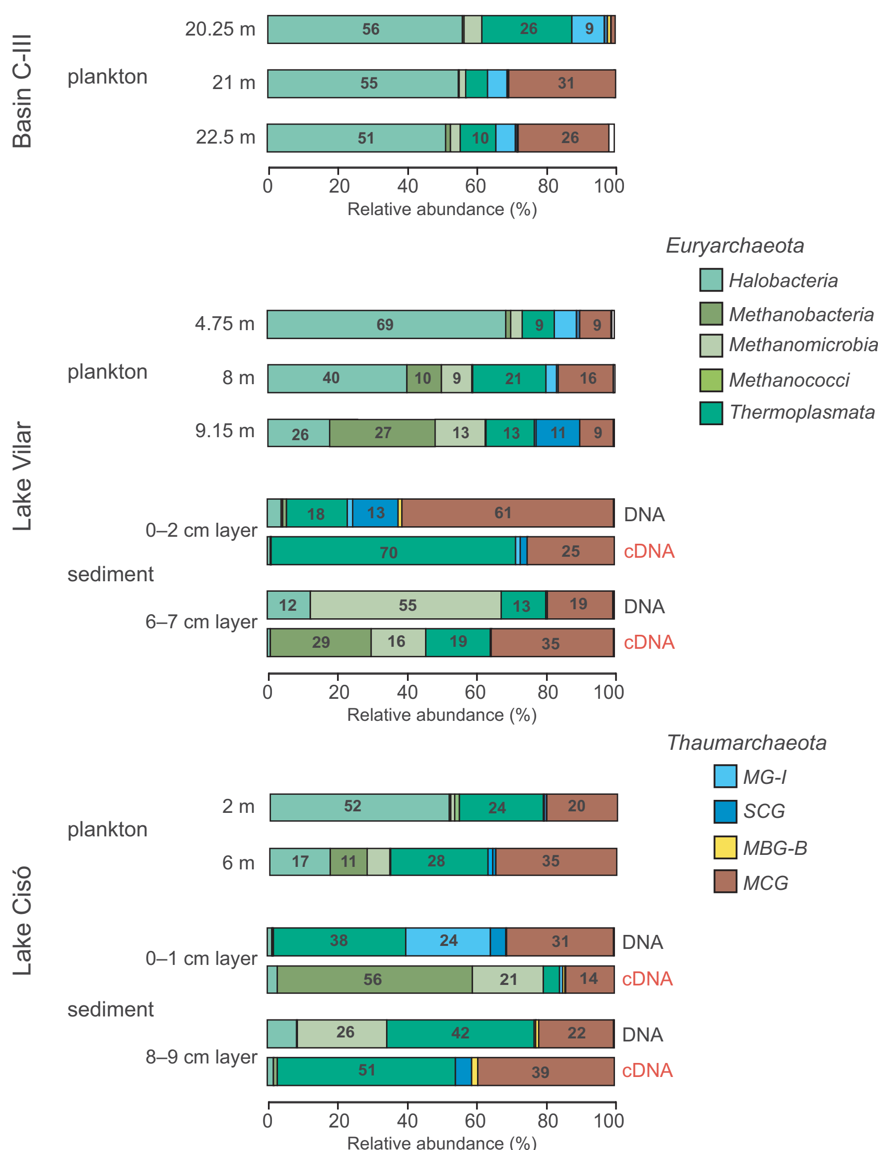

Figure 1. Taxonomic composition of planktonic and sedimentary archaeal communities (relative abundance at Class level) in pyrotag libraries from the studied lakes.

(7% of total OTUs, 97% cut-off) that were further affiliated into subgroups using representative sequences of the 17 MCG subgroups known so far (Kubo *et al.* 2012) as phylogenetic anchors and at a sequence similarity ≥85% to assign sequences to a given subgroup. In basin C-III, the MCG community showed clear variations in depth regarding subgroup composition (Fig. 2). At suboxic waters (20.25 m), the most abundant subgroup was MCG-6 (38% of total MCG sequences) followed by subgroups MCG-15 (also known as Group C3, Inagaki *et al.* 2006), MCG-5b and MCG-11. In turn, the MCG community in deeper, euxinic waters (21 and 22.5 m) was mainly composed of subgroups MCG-5a and -5b (38 and 49% on average, respectively). Planktonic MCG communities in lakes Vilar and Cisó were dominated by subgroup MCG-6, which showed average abundances of 54%±18% and 94%±1%, respectively (Fig. 2). While in Cisó the MCG community was almost entirely composed of MCG-6, in Lake Vilar the community was more diverse, with subgroups MCG-5b and MCG-11 contributing the most.

Benthic MCG communities were fairly similar among lakes but showed important variations when comparing sediment layers and DNA/cDNA libraries. In Vilar, MCG-11 showed a high abundance at the sediment surface (59% of total MCG sequences) whereas MCG-15 sequences were predominant in cDNA-based libraries from both sediment depths (average relative abundance of 63% ± 1.4%) (Fig. 2). MCG sequences affiliated to MCG-6 contributed equally in DNA and cDNA-based libraries from the surficial sediment layer (0–2 cm) whereas a large discrepancy was found between both libraries at the 6–7 cm layer. In Lake Cisó, subgroups MCG-6 and MCG-15 were prevalent in DNA- and cDNA-based libraries, respectively, obtained from both sediment depths.

To allow a better comparison of distribution of the 116 MCG OTUs across samples, a bar chart showing the planktonic/sedimentary distribution of different OTUs was combined with their phylogenetic tree (Fig. 3). Whereas some of the MCG-6 and MCG-15 OTUs were present in both the planktonic and the

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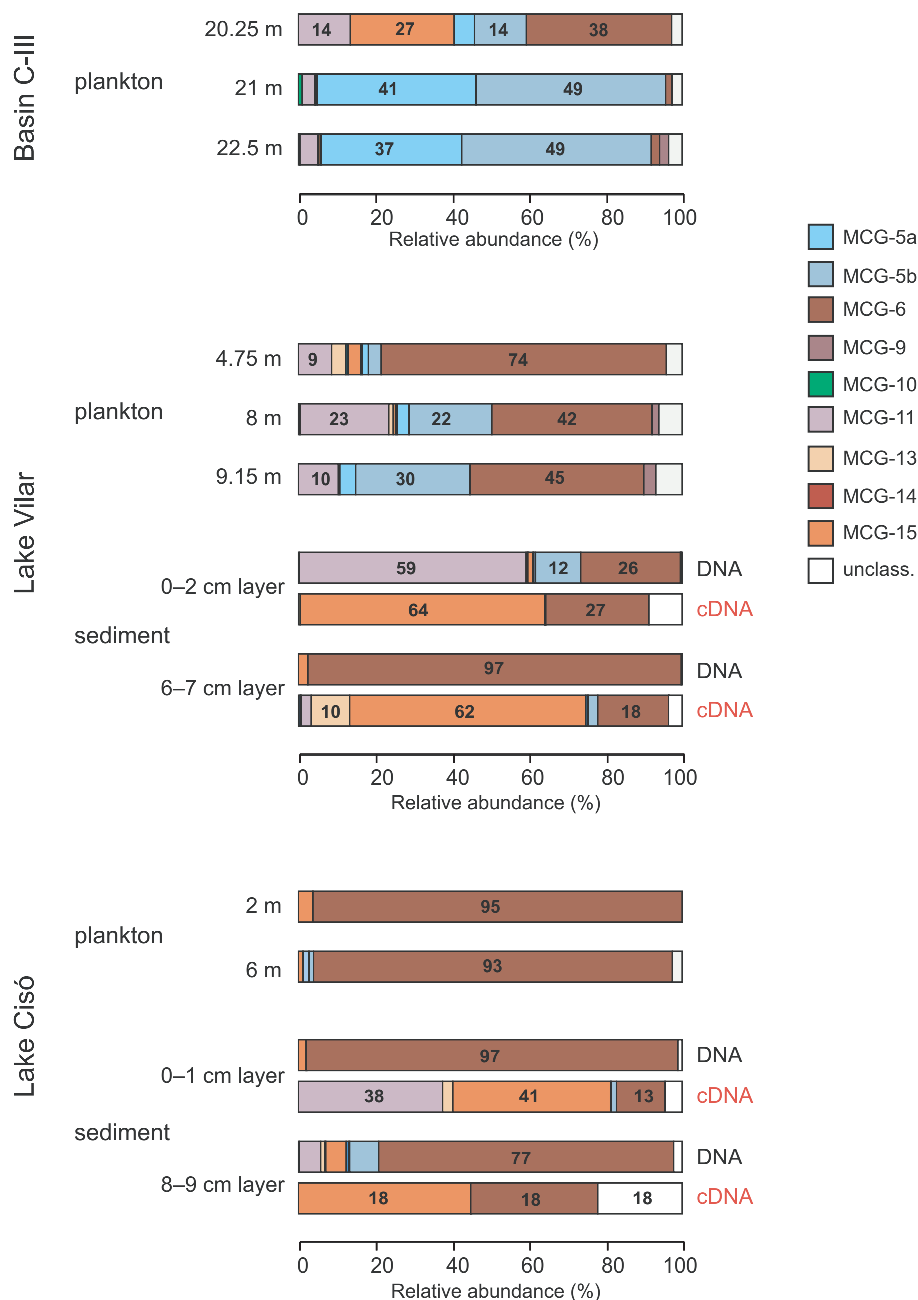

Figure 2. Relative abundance of MCG subgroups of planktonic and benthic MCG communities in pyrotag libraries from the studied lakes. Percentages refer to total sequences affiliated to MCG lineage.

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sediment compartment, some of them only occurred in either one of these habitats (e.g. MCG-5a and -5b in the plankton).

Beta-diversity and influence of environmental variables on archaeal community composition

Although planktonic and benthic communities of the three lakes were mainly composed of the same groups of uncultured archaea, differences in their relative composition were large enough to segregate communities from both habitats in a 2D space with low stress (0.12, Fig. 4A). ANOSIM between planktonic and sediment archaeal communities confirmed this habitat segregation pattern ($R = 0.56$, $P < 0.0002$; Table S5, Supporting Information). Analysis of the percentage contribution of individual archaeal classes to the Bray–Curtis dissimilarity between samples grouped according the above-mentioned factors (SIMPER) showed that *Halobacteria* and *Thermoplasmata*-MCG contributed the most on the segregation of samples according to habitat (planktonic vs sediment) (Table S6, Supporting Information). Remarkably, the same distribution was obtained when archaeal communities were compared using measures of phylogenetic relatedness such as UniFrac distances (Lozupone and Knight 2005) (Fig. S3, Supporting Information). Using unweighted UniFrac, planktonic samples grouped together according to sample origin (Fig. S3A, Supporting Information) but this clustering was not visible using a weighted-based distance ordination (Fig. S3B, Supporting Information). Using weighted UniFrac, PCoA separated planktonic from sediment samples

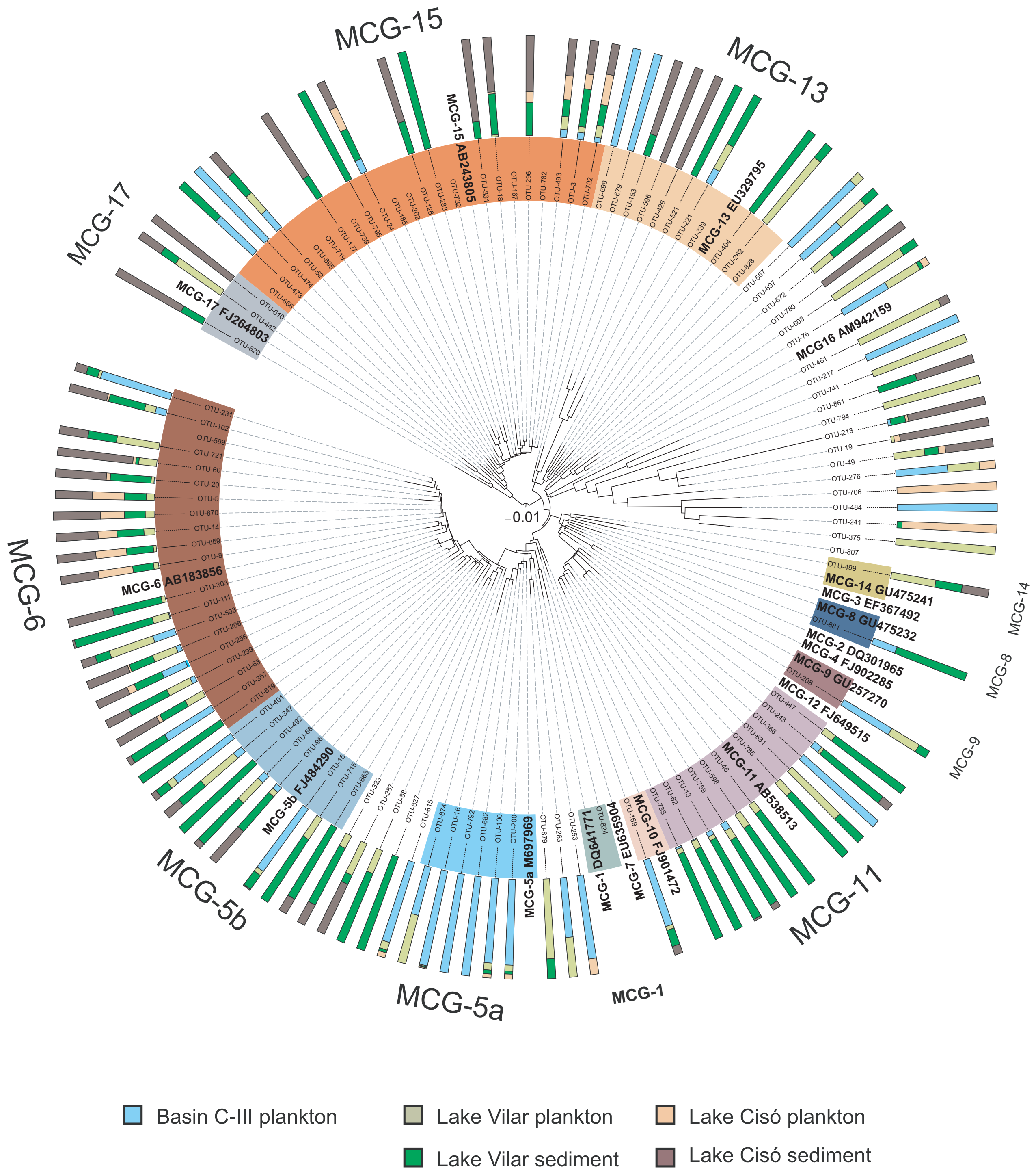

Figure 3. Neighbour-joining (NJ) phylogenetic tree based on MCG SSU rRNA gene sequences obtained from pyrotag libraries. Representative sequences for each MCG subgroup are used as phylogenetic anchors and identified according to subgroup numbering (Kubo *et al.* 2012) and its accession number. Uncoloured leaves indicate MCG sequences unassigned to any subgroup. Outer bar charts indicate the relative contribution of each OTU to a given sample (see legend). OTUs with no bar charts refer to cDNA samples. NJ tree was constructed in ARB (Ludwig *et al.* 2004) and edited in the iTOL tool (Letunic and Bork 2007).

along the first, horizontal axis (27.8% of total variation) whereas sediment samples were mainly distributed along the second, vertical axis (25% of total variation).

The composition of archaeal communities was also analysed at a deeper phylogenetic resolution for the three main archaeal classes identified (e.g. the MCG, the *Halobacteria* and the *Thermoplasmata*). Ordination of samples according to phylogenetic MCG intragroup composition using NMDS showed again a clear segregation of benthic communities (mainly dominated by MCG-6) from planktonic samples with more diverse MCG assemblages (Fig. 4B). All samples from cDNA-based libraries grouped together as a result of the high prevalence of sequences affiliated

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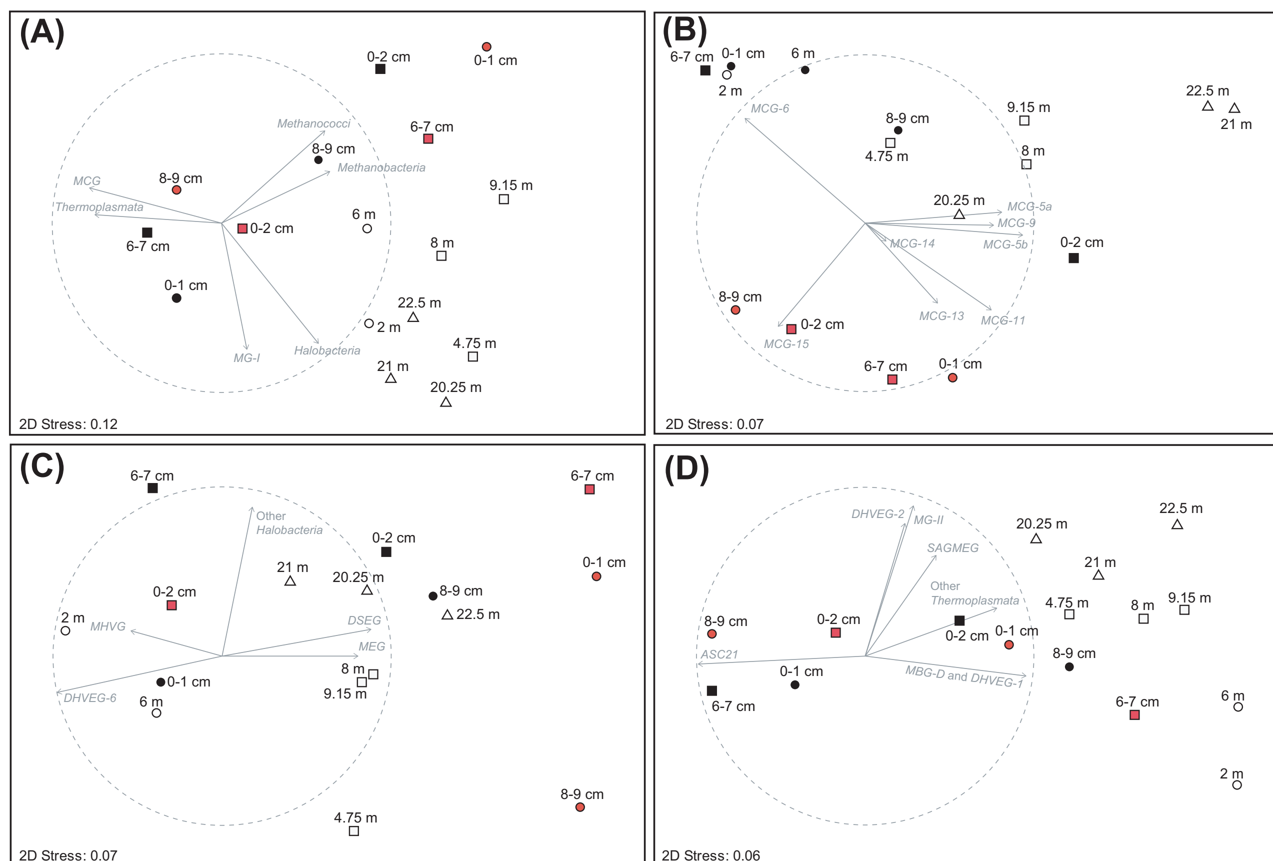

Figure 4. NMDS plot based on Bray–Curtis distance of the relative abundance of archaeal classes (**A**), MCG subgroups (**B**), and different clades within the *Halobacteria* (**C**) and the *Thermoplasmata* (**D**). Overlay vectors show the direction and strength of variables (i.e. archaeal taxa) in the ordination plot (Spearman correlation coefficient >0.5) within a circle of radius 1. Basin C-III (triangles), Lake Cisó (circles) and Lake Vilar (squares). Planktonic and sediment samples are indicated by open and filled symbols, respectively. cDNA samples are highlighted in red. Sample labels indicate sampling depth.

to subgroup MCG-15. In turn, ordination of samples according to the relative composition of different clades within the *Halobacteria* and the *Thermoplasmata* showed no clear trends (Figs 4C and D). ANOSIM revealed significative differences according to habitat (planktonic vs benthic) for the *Thermoplasmata*, to system (i.e. lake) for the MCG subgroups and to library (DNA vs RNA) for the MCG subgroups and the *Halobacteria* (Table S5, Supporting Information). The dissimilarities observed between DNA and cDNA libraries were mainly caused by two *Halobacteria* clades (DHVEG-6 and MEG) and subgroup MCG-15 (Table S6, Supporting Information).

To further understand the influence of C, S and N availability on the taxonomic composition of planktonic and sedimentary archaeal communities, we carried out a stepwise DistLM to identify which set of variables better explained variations on the relative abundance of archaeal classes. The best distance-based linear model indicated that sulphide and DOC concentrations were the main predictor variables, explaining 55% (axis 1: 42%, axis 2: 14%) of the total variation in archaeal composition (Fig. 5 and Table S7, Supporting Information). The first axis of the distance-based redundancy analysis plot (explaining 75.2% of the fitted variation) separated planktonic from sediment samples whereas the second axis, explaining 24.8% of fitted variation, mainly separated suboxic from anoxic, sulphide-rich samples. When the model was run using the relative abundance of both the MCG subgroups and the different clades within the *Thermoplasmata* and the *Halobacteria* as biological data, none of the environmental variables included in the analysis significantly contributed to explain the variations in community composition (data not shown).

Primer design, coverage and specificity

Recently, Kubo and co-workers published both CARD-FISH probes and qPCR primers that allowed the specific detection and quantification of MCG archaea in a large variety of marines sediments collected worldwide (Kubo *et al.* 2012; Lloyd *et al.* 2013). The application of this primer pair (MCG-528F/MCG-732R) for the quantification of MCG in freshwater sediments was problematic despite the high relative abundance of sequences affiliated to this group identified in pyrotag libraries (data not shown). Assuming that these problems probably arose by the high intragroup diversity of the MCG lineage and the fact that the primer used was originally designed using 16S rRNA gene sequences from marine MCG representatives, we designed a new primer pair using a collection of MCG sequences from freshwater sediments. The newly designed forward primer MCG-242F was further modified (two bases degeneration) to improve its coverage across all MCG subgroups (Table S1, Supporting Information). Coverage of degenerate forward primer was adequate for most subgroups (average of 58 and 88% allowing 0 and 1 mismatch, respectively) except for MCG-10 and -17 subgroups, which showed very low coverage values when mismatches were not allowed (4.7 and 0.9%, respectively) (Table 1). These values largely

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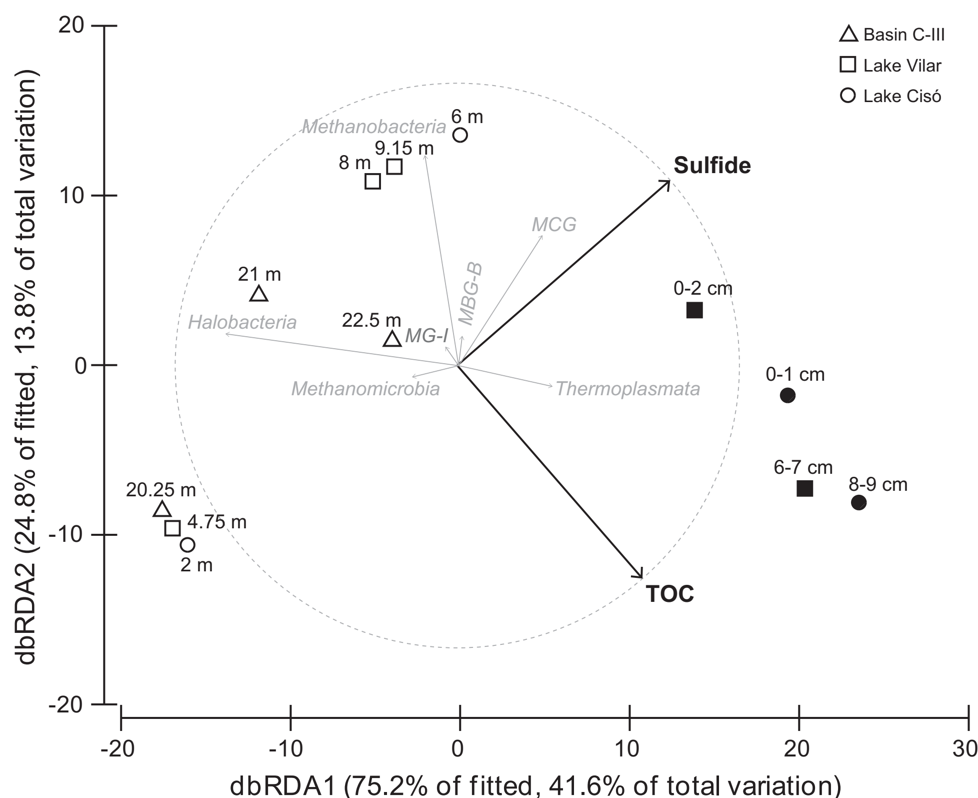

Figure 5. dbRDA plot of the DistLM based on environmental variables (ammonia, sulphide, sulphate, DIC, DOC and TP concentrations) fitted to the taxonomic composition of archaeal communities (at Class level). Only significant explanatory variables [sulphide ($p < 0.001$) and DOC ($p < 0.033$) concentrations] are indicated (black vectors). Grey vectors show the direction and strength of multiple partial correlations (>0.3) between each archaeal class and the dbRDA axis scores. Basin C-III (triangles), Lake Cisó (circles) and Lake Vilar (squares). Planktonic and sediment samples are indicated by open and filled symbols, respectively. Sample labels indicate sampling depth.

increased to 79.1% (MCG-10) and to 88.7% (MCG-17) with only 1 mismatch. Coverage within subgroup MCG-15 was also below the average (26.7% with 0 mismatches). Remarkably, subgroup MCG-15 (or C3) clusters together with MCG-17 and both branch apart from the rest of subgroups (Fig. 3), suggesting a higher phylogenetic distance with the rest of subgroups. Reverse primer MCG-678R showed reduced coverage to most subgroups with 0 mismatches but substantially increased with only 1 mismatch. Besides, the longer fragment produced by pair MCG-242dF/MCG-678R (430 bp) provides 2-fold higher phylogenetic information than that obtained with pair MCG-528F/MCG-732R (204 bp) thus improving taxonomical affiliation of sequences into MCG phylogeny. Although this is probably not very useful in quantitative analysis using qPCR, it is of great advantage for comparative studies of a large number of samples using fingerprinting techniques such as DGGE (unpublished results).

To assess the suitability of the newly designed primer pair MCG-242dF/MCG-678R for the quantification of MCG in environmental samples, primer specificity and coverage were determined using cloned and sequenced amplicons obtained from sediment samples collected in lakes Vilar and Cisó. Besides, qPCR products from North Sea sediments were also included in the analysis to assess the reliability of the new primer pair to quantify MCG in marine samples. Same samples were processed using primer pair MCG-528F/MCG-732R (Kubo *et al.* 2012) to allow comparison of MCG subgroup recovery. All qPCR products obtained using both primer pairs yielded sequences unequivocally affiliated to MCG lineage. A detailed phylogenetic analysis of clone sequences revealed that pair MCG-242dF/MCG-678R mostly recovered MCG-6 sequences (53% of total) from lakes Vilar and Cisó (Fig. S4A, Supporting Information) and sequences affiliated to subgroups MCG-1, -5b, -8, -12, -13, -14 in North Sea sediment samples. Remarkably, no sequences affiliated to subgroup MCG-15 were recovered from any of the cDNA samples analysed despite the high relative abundance of this subgroup in pyrotag libraries obtained from cDNA samples (Fig. 2). This result agreed with the low coverage of the new primer pair against subgroup MCG-15 (Table 1) and evidenced the need of specific primers targeting these highly diverse subgroups (e.g. MCG-15 and MCG-17). Using pair MCG-242dF/MCG-678R, only five sequences remained unclassified (6.2% of total clones). Similar results on the distribution of amplicon sequences across different MCG subgroups were obtained using pair 528F-732R (Fig. S4B, Supporting Information). Again, sequences from lakes Vilar and Cisó mainly affiliated to subgroup MCG-6 (33.6% of total) whereas amplicons obtained from the North Sea sediment grouped into MCG-1, -8, -10, -13 and -14. No sequences affiliated to MCG-15 were obtained from cDNA samples also due to the low coverage of this primer against MCG-15 (Kubo *et al.* 2012). Using this primer pair, 58.6% of sequences showed less than 85% similarity to any of the subgroups and remained unassigned.

Abundance of MCG archaea in the studied systems

SSU rRNA gene copy numbers of bacterial, archaeal and MCG measured in planktonic samples were similar between lakes. As a general trend, archaeal 16S rRNA gene copy numbers were always one order of magnitude lower than bacterial 16S rRNA gene

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Table 1. Intragroup MCG coverage and specificity of primers MCG-242dF and MCG-678R.

	MCG-242dF Coverage (%)			MCG-678R Coverage (%)				Intragroup
Subgroup	0 mismatch	1 mismatch	2 mismatch	0 mismatch	1 mismatch	2 mismatch	Nº Seqs.[a]	% similarity
MCG-1	82.3	92.4	93.7	32.9	79.7	93.7	79	89
MCG-2	85.7	90.5	95.2	0.0	0.0	95.2	21	89
MCG-3	23.1	80.8	84.6	20.0	90.0	90.0	40	91
MCG-4	50.0	70.0	70.0	0.0	76.9	96.2	26	94
MCG-5a	91.2	91.2	91.2	20.0	30.0	80.0	10	93
MCG-5b	86.5	91.3	91.3	76.5	97.1	100	34	91
MCG-6	86.5	91.3	91.3	77.0	88.1	92.1	126	89
MCG-7	40.0	80.0	80.0	0.0	100	100	5	96
MCG-8	82.6	91.3	91.3	1.4	81.9	92.0	138	88
MCG-9	82.1	89.7	94.9	0.0	25.6	35.9	39	88
MCG-10	4.7	79.1	83.7	0.0	7.0	65.1	43	91
MCG-11	65.7	85.7	85.7	5.7	14.3	80.0	35	93
MCG-12	93.8	93.8	93.8	6.3	100	100	16	96
MCG-13	60.7	82.0	88.5	31.1	88.5	95.1	61	85
MCG-14	43.8	91.7	95.8	0.0	77.1	89.6	48	91
MCG-15	26.8	83.9	92.7	4.00	6.3	41.0	205	83
MCG-16	41.7	91.7	91.7	0.0	58.3	91.7	12	92
MCG-17	0.9	88.7	94.8	2.3	18.3	90.6	213	82
Unclassified. (Nº seqs)	80	137	145	28	102	147		
Non-target hits								
Archaea	12	151	n.d.	277	1444	n.d.	18,797	
Thermoplasmata	5	n.d.	n.d.	0	n.d.	n.d.		
Halobacteria	1	n.d.	n.d.	0	n.d.	n.d.		
MG-I and SCG	5	n.d.	n.d.	0	n.d.	n.d.		
MBG-B	1	n.d.	n.d.	2	n.d.	n.d.		
THSG	6	n.d.	n.d.	35	n.d.	n.d.		
Thermoprotei	1	n.d.	n.d.	206	n.d.	n.d.		
Other archaea	0	n.d.	n.d.	0	n.d.	n.d.		
Bacteria	0	4	n.d.	0	0	n.d.	464 618	

[a]Number of sequences of each subgroup (SILVA 111 reference dataset).

copies (Table 2). In Lake Cisó and basin C-III, copies of MCG 16S rRNA gene increased at greater depths, agreeing with the prevalence of pyrotags affiliated to this lineage at bottom water layers (Fig. 1). Planktonic samples from Lake Vilar had lower copy numbers of MCG 16S rRNA genes when compared to basin C-III and lake Cisó. In Lake Vilar, the highest copy number of MCG 16S rRNA genes was measured at 8 m depth coinciding with the highest percentage of reads affiliated to MCG in pyrotag libraries (Fig. 1). In sediment samples, SSU rRNA gene copies of all targeted groups were higher than those from the planktonic habitat, exceeding them by three and four orders of magnitude depending on the target group (Table 2 and Table S8, Supporting Information). No peaks in abundance of either Archaea or MCG were detected along the sediment profile indicating the homogeneous distribution of both groups along depth.

DISCUSSION

A pioneering study carried out in Lake Vilar identified that rich archaeal planktonic communities mainly composed of *Thermoplasmata* and mesophilic crenarchaeota that showed seasonal variations in abundance (Casamayor, Muyzer and Pedrós-Alió 2001). MCG archaea were later identified as the major crenarchaeotal lineage persisting throughout different year cycles in euxinic waters of Lake Vilar, whereas euryarchaeotal clades such as the DHVEG and the *Terrestrial Euryarchaeota Group* were also identified as minor components of the planktonic archaeal community (Llirós, Casamayor and Borrego 2008). In the current study, we have shown that this community structure is shared in suboxic and anoxic water compartments of oligotrophic basin C-III and meso-eutrophic Lake Cisó, where planktonic archaeal communities were characterized by a large contribution of *Halobacteria* while benthic archaeal assemblages were mainly composed of MCG and *Thermoplasmata*. Most of the halobacterial clades identified in the plankton (e.g. the DHVEG-6, the *Deep Sea Euryarchaeota Group* and the MEG) were initially described in gene surveys carried out in hydrothermal vent ecosystems and hot springs (Takai and Sako 1999; Takai *et al.* 2001, 2004; Inagaki *et al.* 2003) and they are considered common inhabitants of such extreme environments (Teske and Sørensen 2008). Their presence in the water column of the studied lakes is then surprising although both the availability of reduced inorganic sulphur compounds (i.e. H_2S, S^0 and thiosulphate) and the slightly brackish waters of the Banyoles Karstic System (conductivity of hypolimnetic waters ≈2000–2500 μS cm^{-1}, Table S3, Supporting Information) might provide a plausible explanation. Further research is needed to elucidate if these planktonic *Halobacteria* clades might obtain energy from the oxidation of reduced sulphur compounds as many of their relatives do in deep sea vents and sulphur springs (Kletzin *et al.* 2004; Sievert *et al.* 2008).

In sediments, the *Thermoplasmata* and the MCG contribute the most to the structure of the benthic archaeal assemblage. Both groups are well-known inhabitants of marine and freshwater sediments worldwide, especially those characterized by

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Table 2. Quantification of Bacteria, Archaea and MCG by qPCR in planktonic and sediment samples of the studied karstic lakes.

	16S rRNA gene copies ml^{-1} or g^{-1}		
Sample	Bacteria	Archaea	MCG
Basin C-III			
20.25 m	2.1×10^7	3.0×10^6	3.2×10^4
21 m	4.9×10^7	7.9×10^6	1.0×10^5
22.5 m	5.3×10^7	6.1×10^6	3.9×10^5
Lake Vilar			
4.75 m	2.4×10^7	1.9×10^6	4.1×10^4
8 m	3.0×10^7	3.9×10^6	7.1×10^4
9.15 m	1.3×10^7	1.0×10^6	1.5×10^4
Sediment			
0–2 cm	3.0×10^{10}	8.2×10^9	1.8×10^8
6–7 cm	5.3×10^{10}	2.1×10^{10}	4.1×10^8
Lake Cisó			
2 m	9.6×10^7	5.5×10^6	5.1×10^4
6 m	3.3×10^7	3.4×10^6	1.4×10^5
Sediment			
0–1 cm	1.2×10^{10}	2.8×10^9	1.1×10^8
8–9 cm	1.5×10^{10}	4.7×10^9	1.7×10^8

anoxic and eutrophic conditions (Biddle *et al.* 2006; Ferrer *et al.* 2011; Schubert *et al.* 2011; Durbin and Teske 2012; Lloyd *et al.* 2013). This distribution agrees with both the high organic content of sediments from lakes Vilar and Cisó (Table S4, Supporting Information) and the identification of sulphide and DOC concentrations as main predictor variables for archaeal community composition, especially considering the direction and strength of correlation vectors for these variables and those associated to classes MCG and *Thermoplasmata* (Fig. 5). The MBG-D, the DHVEG-1, the *South African Gold Mine Euryarchaeota Group* and the *Rice Cluster-III* have consistently been found in marine and freshwater sediments in relation to either the methane cycle (Paul *et al.* 2012; Borrel *et al.* 2013; Iino *et al.* 2013) or to the degradation of complex organic compounds (Borrel *et al.* 2012; Jorgensen *et al.* 2012; Vigneron *et al.* 2014). Although methanogenic archaea were identified in pyrotag libraries from bottom waters and deep sediment layers of lakes Vilar and Cisó, methanogenesis is probably not relevant according to the high sulphate concentrations measured in these systems (Tables S3 and S4, Supporting Information) and the low activity of methanogens in the presence of active sulphate reduction (Senior *et al.* 1982; Lovley and Klug 1983). In the studied sediments, benthic *Thermoplasmata* mostly affiliated to clade ASC21, a rare group recovered from marine and terrestrial mud volcanoes (Omoregie *et al.* 2008; Wrede *et al.* 2012). To our knowledge, this is the first report about the presence of ASC21 in freshwater sediments, opening several questions regarding the environmental factors behind this distribution. The prevalence of an active S cycle in sediments from mud volcanoes (Omoregie *et al.* 2008; Wrede *et al.* 2012) provides an interesting analogy with karstic lakes that deserves further investigation.

The MCG archaea are widespread in the marine subsurface where they may account to up to 100% of total archaea in sediments from different oceanic regions (Kubo *et al.* 2012; Lloyd *et al.* 2013). In the current work, SSU rRNA gene copy numbers of MCG were in the same range than those obtained using a different primer pair in marine sediments worldwide (Kubo *et al.* 2012; Lloyd *et al.* 2013; Vigneron *et al.* 2014). Calculation of the relative abundance of MCG using qPCR data is, however, problematic due to the use of specific primer pairs for the different target groups (i.e. Bacteria, Archaea and MCG) and the sensitivity of the technique to biases caused by different primer specificities (Engelbrektson *et al.* 2010). Direct cross-comparison of relative abundances obtained by qPCR and high-throughput sequencing technologies might probably only be possible using universal primers with similar efficiencies and maximum coverage for target groups in both techniques (Jorgensen *et al.* 2012). This solution is, albeit desirable, not always feasible especially when the study is aimed to resolve the specific contribution and abundance of uncultured microbial groups with complex phylogenies (e.g. MCG, MBG-D, among others). Accordingly, the relative abundance of MCG obtained from archaeal pyrotag libraries was used as indicative of MCG contribution in the archaeal communities under investigation.

After the throughout analyses of the phylogeny of the MCG lineage carried out by Kubo *et al.* (2012) and the discrimination of 17 subgroups sharing low-sequence similarity (76%), questions regarding potential ecological preferences and habitat segregation of members of the different subgroups rapidly emerged. Since then, several authors have tried to unveil hidden links between the distribution of certain MCG subgroups and the environmental conditions in their habitats. SSU rRNA gene sequences of subgroups MCG-6 and MCG-8 have consistently been identified in marine and brackish sediments (Meng *et al.* 2009, 2014; Breuker, Stadler and Schippers 2013; Gagen *et al.* 2013; Lloyd *et al.* 2013; Lazar *et al.* 2015; Seyler, McGuinness and Kerkhof 2014) whereas sequences affiliated to subgroups MCG-3, MCG-12 and MCG-15 were rarely recovered (Breuker, Stadler and Schippers 2013). Interestingly, none of these studies reported the identification of sequences affiliated to subgroups MCG-5a and -5b. Both subgroups were initially described to be composed of sequences retrieved from inland waters (lakes, ponds, aquifers and hot springs) (Kubo *et al.* 2012). SSU rRNA gene sequences affiliated to subgroups MCG-5a and -5b were previously identified in anoxic water compartments of Lake Vilar (Llirós, Casamayor and Borrego 2008), in a karst phreatic system (Sahl *et al.* 2010) and in anoxic waters of a cave system (Chaudhary *et al.* 2009; Kubo *et al.* 2012) but neither from marine or estuarine samples. Our current results agree with this habitat segregation, suggesting that members of subgroups MCG-5a and -5b are planktonic specialists thriving in sulphidic redoxclines and euxinic water compartments.

Subgroups MCG-6 and MCG-11 were, in turn, mainly recovered in both the plankton and the sediment of lakes Vilar and Cisó showing affinity to anoxic, sulphide-rich, eutrophic conditions. The almost exclusively dominance of MCG-6 in the water column of both lakes (with average sulphide concentrations of 0.5 mM (Vilar) and 1 mM (Cisó)) contrasts with the recent hypothesis of Lazar *et al.* (2015) on the specialization of this subgroup towards suboxic sediment layers with minimal or no free sulphide. According to our results, MCG-6 emerges as a generalist group of MCG archaea able to cope with both mild and harsh reducing conditions in both planktonic and benthic habitats. Despite its prevalence in DNA pyrotag libraries, the contribution of subgroup MCG-6 in cDNA libraries points to a low activity in the sediment. In clear contrast, members of subgroup MCG-15, which were sparsely detected in DNA libraries, were predominant in cDNA libraries from all sediment layers. Recently, the analysis of a single-cell genome from a member of subgroup MCG-15 revealed the presence of genes encoding for extracellular peptidases targeting D-aminoacids, di- and tripeptide transporters and intracellular amino-acid degradation pathways (Lloyd *et al.* 2013). According to these findings, MCG

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archaea—or at least members of the MCG-15 subgroup—might probably feed on proteinaceous material accumulated in the sediment as a result of organic matter inputs of either internal sources (i.e. sedimentation of both algal and bacterial biomass) or terrestrial origin (i.e. plant material). In this regard, isotopic analysis of sedimentary biomarkers of bacterial and terrestrial organic matter showed that most of the organic matter accumulated in sediments from Lake Cisó derive from terrestrial sources, with a high contribution of lignin derivatives (Hartgers *et al.* 1997, 2000). This is congruent with the recent identification of genes-encoding enzymes involved in the decarboxylation of protocatechuate, an intermediate in the lignin degradation pathway, in genomic fragments of MCG archaea recovered from estuarine sediments (Meng *et al.* 2014). The sediment of lake Cisó thus provides a suitable niche for growth of MCG archaea either degrading proteins from settled bacterial and algal biomass or feeding on aromatic compounds derived from plant material. Nevertheless, this hypothesis must be taken with caution considering the lack of experimental evidences, the scarce information available on potential metabolisms and physiological requirements of different MCG subgroups, and the high phylogenetic diversity of the entire lineage. Besides, the well-known limitations on the use of rRNA as indicator of microbial activity should be also taken into account (Blazewicz *et al.* 2013). Despite these considerations, the disconnection between results from DNA and cDNA samples reinforces the need for carrying out both types of analyses to discern active from latent members of microbial communities.

Concluding remarks

Archaeal communities in stratified karstic lakes are mainly composed of uncultured groups within the classes *Halobacteria*, *Thermoplasmata* and MCG sharing remarkable similarities with those inhabiting the marine subsurface. Our results suggest that the distribution and diversity of archaeal communities in these lakes are mainly driven by sulphide and DOC concentrations, agreeing with the putative heterotrophic metabolism assumed for most of the groups identified. The lack of significative correlations between the composition of archaeal communities at lower taxonomic levels (e.g. MCG subgroups) and the selected environmental variables suggests that the pattern of variation is probably driven by factors not included in the model (e.g. quality of the DOC pool). Concerning the ecological preferences and habitat segregation of MCG subgroups, our study indicates that subgroups MCG-5a and MCG-5b are adapted to the planktonic habitat, especially in anoxic, sulphide-rich water layers where inorganic and organic reduced compounds accumulate. In turn, MCG-6 appears to be a generalist subgroup, adapted to both the planktonic and the sediment habitat and able to thrive in a wide range of sulphidic conditions. Further research is needed to elucidate which conditions and organic compounds stimulate the growth and activity of both prevalent and rare members of the MCG community. In this regard, the accumulation in the sediment of the studied lakes of both settled microbial biomass and plant-derived compounds provides an optimal laboratory to carry out *in situ* investigations aimed to investigate their precise role on the diversity and activity of distinct MCG subgroups at spatial and temporal scales.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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