FACTORS AFFECTING THE DISTRIBUTION, ABUNDANCE AND DIVERSITY OF UNCULTURED ARCHAEAL GROUPS IN FRESHWATER SEDIMENTS

Sergi Compte Port

Doctoral thesis

Factors affecting the distribution, abundance and diversity of uncultured archaeal groups in freshwater sediments

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The present thesis contains one annex containing the supplementary material from the chapters at the end of the document.

This thesis is submitted in fulfilment of the requirements for the degree of Doctor from the University of Girona.

Hereby, Dr Carles Borrego Moré, associate professor of Microbiology at the University of Girona and research professor at the Catalan Institute for Water Research (ICRA),

D E C L A R E S

That the doctoral thesis entitled "*Factors affecting the distribution, abundance and diversity of uncultured archaeal groups in freshwater sediments*" submitted by Sergi Compte Port to obtain the doctoral degree from the Universitat de Girona has been done under my supervision and meets all the requirements to opt for the *International Doctor* mention.

In witness whereof and for such purposes as may arise, I signed this statement in Girona on May 23h 2018.

Dr. Carles Borrego Moré

“Quan camines, el camí deixa d’existir”

Proverbi zen

“Tothom vol arribar lluny, però es queixa quan ha arribat”

Ciceró

Dedicatòria

Es ben cert que tal i com diu el refrany "Sol vas mes de pressa, però acompanyat arribes molt més lluny". De ben segur que no hagués estat possible complir aquest compromís sense l'ajuda de moltes persones que directe o indirectament han contribuït a que el projecte arribés a bona fi. I entre tots ells la principal font d'ajuda encara que subtil han estat els meus pares. Aquest treball s'ha fet, en part, mitjançant la quantificació de gens o l'anàlisi multivariant (entre d'altres), però el principal factor ha estat la determinació i les ganes de tirar endavant volent aprendre sempre un mica més. Per aconseguir-ho, es imprescindible haver tingut un bon suport i una motivació contínua al llarg de tota la trajectòria acadèmica. I tot i que a vegades trigui molts anys a donar fruit, la perseverança per millorar és un tret que es manté per sempre. Per a ells va dedicada aquesta tesi.

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List of abbreviations

Abbreviation	Description
amoA	Ammonia monooxygenase subunit A gene
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ASGARD	Archaeal superphylum composed of Lokiarchaeota,Thorarchaeota,Odinarchaeota and Heimdallarchaeota
BLAST	Basic local alignment search tool
BrdU	Bromodesoxiuridine
cDNA	Complementary deoxyribonucleic acid
CV	Coefficient of variation
DAPI	4′,6-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DHVEG-6	Deep Hydrothermal Vent Euryarchaeotic Group 6
DPANN	Archaeal superphylum composed of Diapherotrites, Parvarchaeota, Aenigmarchaeota and Nanoarchaeota
DSAG	Deep Sea Archaeal Group
dsDNA	Double stranded deoxyribonucleic acid
DSEG	Deep Sea Euryarchaeotal Group
dsrA	Dissimilatory sulfite reductase subunit A gene
FISH	Fluorescence in-situ hybridization
HTS	High-throughput sequencing
HWCG	Hot Water Crenarchaeotic Group
iTOL	Interactive tree of life
MBG-B	Marine Benthic Group B
MBG-D	Marine Benthic Group D
MCG	Miscellaneous Crenarchaeotic Group
mcrA	Methyl coenzyme M reductase subunit A gene
MEG	Miscellaneous Euryarchaeotic Group

MG-II	Marine group II
MIQE	Minimum information for publication of qPCR experiments
NANO-SIMS	Nano-scale ion mass spectrometry
NMDS	Nonmetric multidimensional scaling
nosZ	Nitrous oxide reductase gene
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PD	Phylogenetic distance
PERMANOVA	Permutational analysis of variance
pRDA	Partial redundancy analysis
qPCR	Quantitative polymerase chain reaction
RDA	Redundancy analysis
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAGMCG-1	South African Gold Mine Crenarchaeotic group 1
SAGMEG	South African Gold Mine Euryarchaeotic group
SCG	Single cell genomics
SIMPER	Similarity percentages
SIP	Stable isotope probing
SQG	Sediment quality guidelines
SRA	Small read archive
SSU	Small subunit
TACK	Archaeal superphylum composed of Thaumarchaeota, Aigarchaeota, Crenarchaeota, Korarchaeota, Bathyarchaeota and Geoarchaeota
TCD	Taxonomic compositional diversity
TMEG	Terrestrial Miscellaneous Crenarchaeotic Group
TSD	Taxonomic structural diversity

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Chapter 2. *Contrasting Effect of Metal Contaminations on Various Components of Archaeal Diversity and Biomass in Remote Alpine Lakes.*

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SUMMARY

Members of the domain Archaea are widespread in sediments worldwide. Despite their ubiquity, most sediment-dwelling archaea are reluctant to cultivation and their study relies in gene-based approaches. Current advances in (meta)genomics recently unveiled a vast metabolic potential of sedimentary archaea, placing them into a functional and ecological framework. However, most studies have been focused on marine sediments and less information is available for uncultured archaeal lineages inhabiting freshwater sediments.

First, we studied the abundance and composition of archaeal communities thriving in freshwater sediments from systems characterized by a wide spectrum of environmental variables (e.g. trophic state, stratification regime and typology). The main fraction of these communities was composed by methanogenic lineages, however, members of two prevalent uncultured lineages in freshwater sediments: the phylum Bathyarchaeota and the class *Thermoplasmata* were especially abundant in stratified karstic systems. In the case of the former, *Bathyarchaeota-6* was the most represented subgroup, and some Thermoplasmata lineages like *Terrestrial Miscellaneous Euryarchaeota Group* or *Methanomassiliicoccales* were also abundant in several systems characterized by their anthropogenic nature or shallowness. The correlation (in terms of abundance and diversity) found between Bathyarchaeota and Thermoplasmata, suggests some kind of trophic linkage between them.

Second, we studied the effects of metal pollution on the distribution and abundance of archaeal groups in a set of high-mountain lakes polluted by varying levels of metal depositions. In these lakes, we found same uncultured lineages than in the previous low-land lake survey, but in this case, just a minor fraction of reads affiliated with methanogens. Even so, the abundance of methanogenesis biomarkers highlighted production of methane as an outstanding process in the studied high-mountain systems. As hypothesized, metal concentrations were the main environmental factor shaping the abundance and diversity of archaeal communities, being Arsenic and Tin the ones explaining the main part of this variation. Likewise, lead was an important inhibitor of methanogenesis. In addition, the presence of metals (regardless of their source of origin) had a negative effect on the abundances of all studied sedimentary lineages, with a toxic effect especially pronounced in the case of Cadmium.

Third, we were also interested in decipher which organic compounds are able stimulate the activity of the members of Bathyarchaeota and *Thermoplasmata* that inhabit anoxic sediments. Hence, we realized controlled experiments using laboratory

microcosms in order to evaluate the effects of different sources of organic carbon (different aminoacids, aromatic compounds and complex polysaccharides) over the archaeal composition and activity of archaea inhabiting sediment and biofilms attached to decaying leaves. All carbon supplementations (D- and L-Arginine, Tryptophan, Protocatechuate, humic acids and Pectin) stimulated the members of Bathyarchaeota and Thermoplasmata along the 30 days of the experiment. Some of their lineages were the main contributors to the large differences observed in relation to the studied fraction (DNA or RNA), substrate (sediment/biofilm) or incubation time (7 or 30 days). The response to the supplementations was different for biofilm and sediment communities. The biofilm communities showed strong responses in the short term (7 days), with acute effects of humic acids. The sediment communities showed a clear response in the long term (30 days) under the addition of Tryptophan. In addition, the active fraction of several uncultured taxa showed variations in their microdiversity at OTU level when the carbon supplementations of proteic (aminoacids) and vegetal origin (Pectin and humic acids) were compared.

The results summarized here will provide clues for further studies aimed to better understand the metabolic preferences and ecological role of archaea in sedimentary compartments of freshwater systems.

RESUM

Els arqueus són ubics en sediments d'arreu del món. La seva baixa cultivabilitat, però, fa que la majoria dels estudis es basin en tècniques moleculars centrades en la identificació i recompte de gens específics pels aquests microorganismes. Estudis recents basats en metagenòmica ha revelat que els arqueus disposen d'un ampli potencial metabòlic i, per tan, d'un repertori funcional inimaginable fins fa poc. Tot i així, la majoria dels estudis s'han centrat en sediments marins i hi ha menys informació sobre els sediments d'aigua dolça.

En aquesta tesi hem estudiat en primer lloc l'abundància i la composició de les comunitat d'arqueus de sediments d'aigua dolça en sistemes caracteritzats per un ampli ventall de variables ambientals (estat tròfic, règim d'estratificació i tipologia). La major part d'aquestes comunitats estava formada d'arqueus metanògens tot i que, els membres del fílum Bathyarchaeota i la classe *Thermoplasmata* eren especialment abundants en sediments de llacs càrstics estratificats. Dins els Bathyarchaeota el subgrup *Bathyarchaeota-6* fou el més abundant mentre que alguns llinatges de Thermoplasmata, com ara *Terrestrial Miscellaneous Euryarchaeota Group* i *Methanomassiliicoccales*, eren majoritaris en alguns dels sistemes caracteritzats pel seu origen antropogènic o la seva poca profunditat. La correlació observada entre l'abundància i diversitat de Bathyarchaeota i Thermoplasmata reforça la hipòtesi de l'existència d'alguna classe de lligam tròfic entre aquests dos grups.

En segon lloc, s'han estudiat els efectes de la contaminació per metalls en la distribució i abundància dels arqueus en el sediment d'un conjunt de llacs d'alta muntanya. En aquests llacs, s'han identificat els mateixos grups d'arqueus no cultivats que es trobaven en llacs càrstics, embassaments i llacunes temporals amb la diferència de la baixa contribució dels metanògens. Tot i així, l'abundància de gens específics per la metanogènesi suggereix que la producció de metà és important en els sistemes d'alta muntanya estudiats. D'altra banda, la concentració del metalls en el sediment fou el principal factor que modelava les comunitats d'arqueus, essent l'Arsènic i l'Estany els dos metalls que més explicaven les variacions en abundància i diversitat entre les comunitats. Així mateix, el Plom es va mostrar com un important efector, inversament relacionat amb la presència de gens per la metanogènesi. A més, la presència de metalls (independentment de la seva font d'origen) va tenir un efecte negatiu en relació a les abundàncies de tots els llinatges sedimentaris estudiats, amb un efecte tòxic especialment pronunciat en el cas del Cadmi.

En tercer lloc, ens va interessar especialment intentar desxifrar quins compostos orgànics eren capaços d'estimular l'activitat dels membres de Bathyarchaeota i Thermoplasmata que habitaven en els sediments anòxics. Així doncs, es van realitzar experiments controlats utilitzant microcosmos de laboratori per avaluar l'efecte de diferents fonts de carboni orgànic (diferents aminoàcids, compostos aromàtic i polisacàrids complexes) sobre la composició i activitat d'arqueus que habitaven el sediment i biofilms adherits sobre fullaraca en descomposició. Tots els compostos assajats (L- i D-Arginina, triptòfan, protocatecuat, àcids húmics i pectina) van estimular els membres de Bathyarchaeota i Thermoplasmata durant els 30 dies que va durar l'experiment. Diferents subgrups dins aquests llinatges foren responsables de les diferències observades al comparar les comunitats en funció de la fracció estudiada (DNA o RNA), del substrat (sediment/biofilm) o del temps d'incubació (7 dies/30 dies). La resposta a les fonts de carboni fou diferent però en funció del tipus de comunitat. Les del biofilm van mostrar estimulació a curt termini (7 dies d'incubació) i especialment en resposta als àcids húmics. Les del sediment, en canvi, van mostrar una resposta més clara a llarg termini (30 dies d'incubació) en resposta a l'addició de triptòfan. A més, la fracció activa d'alguns taxons va mostrar variacions significatives en la microdiversitat a nivell d'OTU quan es comparaven les fonts de carboni d'origen proteic (aminoàcids) i les d'origen vegetal o aromàtic (pectina i àcids húmics).

Els resultats d'aquest treball són clau per entendre millor les preferències metabòliques dels grups d'arqueus majoritaris en els sediments d'aigua dolça com a primer pas per resoldre la seva contribució en el reciclatge del carboni orgànic en aquests hàbitats.

RESUMEN

Las arqueas son ubicuas en sedimentos de todo el mundo. Pero su baja cultivabilidad hace que la mayoría de estudios se basen en técnicas moleculares centradas en la identificación y el recuento de genes específicos para estos microorganismos. Estudios recientes basados en la metagenómica han revelado que las arqueas disponen de un amplio potencial metabólico y, por tanto, de un repertorio funcional inimaginable hasta hace poco. Aún así, la mayoría de los estudios se han centrado en sedimentos marinos y hay menos información sobre los sedimentos de agua dulce.

En esta tesis hemos estudiado en primer lugar la abundancia y la composición de las comunidades de arqueas de sedimentos de agua dulce en sistemas caracterizados por un amplio abanico de variables ambientales (estado trófico, régimen de estratificación y tipología). La mayor parte de estas comunidades estaba formada de arqueas metanógenas aunque, los miembros del filo Bathyarchaeota y la clase *Thermoplasmata* eran especialmente abundantes en sedimentos de lagos cársticos estratificados. Dentro de los Bathyarchaeota el subgrupo *Bathyarchaeota-6* fue el más abundante mientras que algunos linajes de Thermoplasmata, como *Terrestrial Miscellaneous Euryarchaeota Group* o *Methanomassiliicoccales*, eran mayoritarios en algunos de los sistemas caracterizados por su origen antropogénico o su poca profundidad. La correlación observada entre la abundancia y diversidad de Bathyarchaeota y Thermoplasmata refuerza la hipótesis de la existencia de alguna clase de vínculo trófico entre estos dos grupos.

En segundo lugar, se han estudiado los efectos de la contaminación por metales en la distribución y abundancia de las arqueas en el sedimento de un conjunto de lagos de alta montaña. En estos lagos, se han identificado los mismos grupos de arqueas no cultivadas que se encontraban en los lagos cársticos, embalses y lagunas temporales con la diferencia de la baja concentración de los metanógenos. Aún así, la abundancia de genes específicos para la metanogénesis sugiere que la producción de metano es importante en los sistemas de alta montaña estudiados. Por otro lado, la concentración de metales en el sedimento fue el principal factor que modelaba las comunidades de arquea, siendo el Arsénico y el Estaño los dos metales que mas explicaban las variaciones en abundancia y diversidad entre comunidades. Asimismo, el Plomo se mostró como un importante efector, inversamente relacionado con la presencia de genes para la metanogénesis. Además, la presencia de metales (independientemente de su origen) tuvo un efecto negativo en relación a las abundancias de todos los linajes sedimentarios estudiados, con un efecto tóxico especialmente pronunciado en el caso del Cadmio.

En tercer lugar, nos interesó especialmente intentar descifrar que compuestos orgánicos eran capaces de estimular la actividad de los miembros de Bathyarchaeota y Thermoplasmata que habitaban en los sedimentos anóxicos. Así pues, se realizaron experimentos controlados utilizando microcosmos de laboratorio para evaluar el efecto de diferentes fuentes de carbono orgánico (diferentes aminoácidos, compuestoa aromáticos y polisacáridos complejos) sobre la composición y actividad de las arqueas que habitaban el sedimento y biofilm adheridos a hojarasca en descomposición. Todos los compuestos ensayados (L- y D-Arginina, Triptófano, Protocatecuato, acidos húmicos y Pectina) estimularon los miembros de Bathyarchaeota y Thermoplasmata durante los 30 días del experimento. Diferentes subgrupos dentro de los linajes fueron responsables de las diferencias observadas al comparar las comunidades en función de la fracción estudiada (DNA o RNA), del substrato (sedimento/biofilm) o del tiempo de incubación (7 o 30 días). La respuesta a las fuentes de carbono fue diferente pero en función del tipo de comunidad. Las del biofilm mostraron estimulación a corto plazo (7 días de incubación) y especialmente en respuesta a los ácidos húmicos. Las del sedimento, en cambio, mostraron una respuesta mas clara a largo plazo (30 días de incubación) en respuesta a la adición de Triptófano. Además, la fracción activa de algunos taxones mostró variaciones significativas en la microdiversidad a nivel de OTU cuando se comparaban las fuentes de carbono de origen proteico (aminoácidos) y las de origen vegetal o aromático (pectina y ácidos húmicos).

Los resultados de este trabajo son clave para entender mejor las preferencias metabólicas de los grupos de arquea mayoritarios en sedimentos de agua dulce como primer paso para resolver su contribución en el reciclaje de carbono orgánico en estos hábitats.

1. INTRODUCTION

1.1 The third domain of life

Until 60 years ago, members of the domain Archaea were misclassified as Bacteria, due to their prokaryotic morphology. However, unusual ecological features had been largely reported among some of these "bacteria", as they were able to grow in environments with extreme degrees of salinity, acidity or temperature (Kushner *et al.,* 1964; Brock *et al.,* 1967; Langworthy *et al.,* 1972). The capability to survive in such hostile environments was explained by singular morphologic traits regarding cell wall, membrane lipids or coenzymes (Woese *et al.,* 1987, and references therein). Advances in molecular biology and the implementation of the 16S rRNA gene as a molecular biomarker lead to a change of the microbial ecology paradigm. The ubiquity (related to protein synthesis) and *housekeeping* character (alterations lead to infeasibility)of this gene, together with its informative size (~1500 nucleotides) and experimental manageability (Fox *et al.,* 1977; Woese and Fox 1977) provided microbiologists with a reliable and previously unknown lens to observe the microbial biosphere. Hence, in 1977 a first division separating *Eubacteria* from a novel "hidden" branch of tree of life called *Archaeobacteria,* was proposed in basis of a sequence-comparing study of 16S rRNA gene (Woese *et al.,* 1977). This novel branch was later redefined as *Archaea*: the third domain of life (Woese *et al.,* 1990).

1.2 A continuously expanding phylogeny

Phylogenomic analyses suggest Archaea as the most ancient domain (Sun *et al.,* 2010; Kim *et al.,* 2011). Archaea was firstly composed by two phyla: Euryarchaeota, a diverse group encompassing methanogens, extreme halophiles, sulfate-reducers and some thermophiles, and Crenarchaeota, more physiologically homogeneous and, due to its thermophilic nature, closer to primitive lifeforms (Woese *et al.,* 1977; 1987; 1990; Barns *et al.,* 1996; Takai *et al.,* 1999; Huber *et al.,* 2002; Forterre *et al.,* 2002). At those times, Archaea was still thought to be a confined in extreme environments (Fuhrman *et al.,* 1992; DeLong *et al.,* 1998; Forterre *et al.,* 2002). Soon, some mesophilic *Crenarchaeota*-related sequences found in artic and the open-sea (DeLong *et al.,* 1992; 1994; Fuhrman *et al.,* 1992; McIerney *et al.,* 1995), grouped into the phylum *Korarchaeota* (Barns *et al.,* 1996). The discovering of the wide habitat distribution of mesophilic Archaea (Simon *et al.,* 2000; Ochsenreiter *et al.,* 2003; Keough *et al.,* 2003; Lepp *et al.,* 2004; DeLong *et al.,* 2005) encouraged further environmental surveys (Schleper *et al.,* 2005). Thus, the increasing number of available 16S rRNA gene sequences improved the phylogenetic resolution of newly discovered archaeons (Schleper *et al.,* 2005; Robertson *et al.,* 2005).The next step was the proposal of *Nanoarchaeota,* defined in basis of its small-genome size and composed by thermophilic symbionts (Table 1.1; Huber *et al.,* 2002; 2003).

The identification of a conserved genetic core within archaeal genomes (Marakova *et al.,* 2003; Brochier *et al.,* 2005; Garibaldo *et al.,* 2006), allowed the incorporation of comparative genomics (i.e. phylogenomics) as a parallel phylogenetic approach (Bejà *et al.,* 2002; Schleper *et al.,* 2005), strengthening subsequent phylogenies. The phylum *Thaumarchaeota* was defined relying on phylogenomics (Brochier-armanet *et al.,* 2008), this phylum is widespread and suggested to be the most abundant on earth (Pester *et al.,* 2011). The usage of genome-centric methods allowed the discovering of Micrarchaeota (Baker *et al.,* 2010) and *Aigarchaeota* (Nunoura *et al.,* 2011). The discovery of these phyla was followed by an exponential increase of phylogenetic resolution based in the study of whole genomes. Hence, the superphylum TACK (firstly composed by *Thaumarchaeota; Aigarchaeota; Crenarchaeota; Korarchaeota*) was defined in basis of functional and comparative genomics (Table 1.1; Guy *et al.,* 2011).

The use of the single cell genomic amplification allowed the identification of *Hadesarchaea,* (Baker *et al.,* 2016) and four novel phyla: *Diapherotrites, Parvarchaeota, Nanohaloarchaeota*, and *Aenigmarchaeota*, which together with *Nanoarchaeota* were firstly composing the second superphylum DPANN (Rinke *et al.,* 2013). Phyla can be composed from sequences confined in a given environment, like in the case of the

Geoarchaeota, found in high-temperature acidic iron mats (Kozubal *et al.,* 2013). Or may also be composed by sequences which fail to cluster within cultured groups (Eme *et al.,* 2015), like in the case of Bathyarchaeota (Meng *et al.,* 2014), which was firstly defined as *Miscellaneous Crenarchaeotic Group* (MCG) in basis of sequences widespread accros a wide variety of environments (Inagaki *et al.,* 2003). The discovery of novel phyla was fuelled by the improving of molecular approaches: *Lokiarchaeota* (Spang *et al.,* 2015), *Thorarcheaota* (Seitz *et al.,* 2016), *Pacearchaeota* and *Woesearcheaeota* (Castelle *et al.,* 2015) leaded to a notable increase of resolution regarding archaeal phylogenetic diversity. Recently, the discovery of *Lokiarchaeota* (Spang *et al.,* 2015) and *Thorarchaeota* (Seitz *et al.,* 2016), together with Odinarchaeota and *Hemidallarchaeota* led to the definition of the third superphylum ASGARD (Table 1.1; Zaremba *et al.,* 2017). Alongside that, the definition of novel phylogenetic relationships are not free of discrepancies (Brochier *et al.,* 2005; Robertson *et al.,* 2005; Williams-Embley *et al.,* 2014), and controversies regarding the stated groups are still fuelling the debate (e.g. the restructuration of the archaeal phylogenetic tree by Petitjean and co-workers; Petitjean *et al.,* 2014). The recent discovering of Verstraetearchaeota (Vanwonterghem *et al.,* 2017) together with the definition of novel classes, orders, and families (Panagiotis *et al.,* 2017; and references therein) exemplifies the unstoppable expansion of domain Archaea.

Table 1.1. Archaeal phyla defined so far. Previous names of the group and methodologies for their respective definitions are also displayed.

Superphyla	Phyla	Previous name	Methodology	Reference
-	Euryarchaeota[c]	-	Comparison of 16S rRNA gene sequences	Woese *et al.,* 1990
TACK	Crenarchaeota[c]	-		
	Korarchaeota[c]	-	Sequencing from enrichment	Barns *et al.,* 1994
DPANN	Nanoarchaeota[c]	-	Sequencing from culture	Huber *et al.,* 2002
TACK	Thaumarchaeota[c]	Crenarchaeota (Group-1)	Sequencing from enrichment	Brochier-Armanet *et al.,* 2008
	Aigarchaeota	Crenarchaeota (HWCG)	Metagenomics	Nunoura *et al.,* 2010
DPANN	Micrarchaeota	-		Baker *et al.,* 2010
-	Hadesarchaeota	Euryarchaeota (SAGMEG)	Single cell genomics	Baker *et al.,* 2016
DPANN	Diapherotrites	Euryarchaeota (MEG)		Rinke *et al.,* 2013
	Parvarchaeota	(ARMAN-4 and -5)		
	Nanohaloarchaeota	-		
	Aenigmarchaeota	Euryarchaeota (DSEG)		
TACK	Geoarchaeota	Crenarchaeota (NAG-1)	Metagenomics	Kozubal *et al.,* 2013
	Bathyarchaeota	MCG		Meng *et al.,* 2014
-	Proteoarchaeota	-	Metanalysis	Petitjean *et al.,* 2014
ASGARD	Lokiarchaeota	Thaumar-chaeota (DSAG/MBG-B)	Metagenomics	Spang *et al.,* 2015
DPANN	Pacearchaeota	Euryarchaeota (DHVEG-6)		Castelle 2015
	Woesearchaeota	Euryarchaeota (DHVEG-6)		
-	Verstraetearchaeota			Vanwonterghem *et al.,* 2016
ASGARD	Thorarchaeota	-		Seitz *et al.,* 2016
	Odinarchaeota	-		Zaremba *et al.,* 2017
	Hemidallarchaeota	-		

[c] Cultured representatives.

1.3 Ecological distribution

Members of Archaea were firstly discovered in extreme habitats (Kusher *et al.,* 1964; Brock *et al.,* 1967; Langworthy *et al.,* 1972), generally affiliated to hyperthermophilic lineages placed at the shortest and deeply-rooted branches of the archaeal phylogenetic tree. Nowadays, Archaea are still consistently found in extreme niches, generally located on seafloor: deep sea vents (Takai *et al.,* 1999), black smokers (Takai *et al.,* 2001), hydrate ridges (Marlow *et al.,* 2010), hydrothermal vents (Zierenberg *et al.,* 2000) and methane seeps (Dang *et al.,* 2010) among others. However, their ability to thrive under energy stress (Valentine *et al.,* 2007) combined with their prokaryotic life-style-characteristics: large population sizes, high genetic diversity and potential for long-range passive dispersal (Pedros-alio *et al.,* 2006; Polz *et al.,* 2006; Falkowski *et al.,* 2008; López-García *et al.,* 2008; Logares *et al.,* 2009), enabled archaeal cells to colonize a wide range of disparate environments during their phylogenetic diversification.

The first change of the archaeal ecology paradigm came with the discovery that up to 2% of the total rDNA sequences retrieved from temperate marine waters belonged to mesophilic Archaea (DeLong *et al.,* 1992). Further studies stated the wide distribution of Archaea in sea waters (Fuhrman *et al.,* 1992; López-García *et al.,* 2001; Fuhrman *et al.,* 2009; Bowskill *et al.,* 2012), concluding that 1.10^{28} cells (20% of the total picoplankton) belongs to a pelagic *Crenarchaeota* clade (Karner *et al.,* 2001). Nowadays, Archaea is reported and widely represented from coastal to wide-sea waters at any latitude (Fuhrman *et al.,* 1992; Massana *et al.,* 2000; López-García 2001; Kirchman *et al.,* 2007; Galand *et al.,* 2010; Beman *et al.,* 2011). Their members also encompass up to 6.3% of ribosomal signatures in surficial waters of high-mountain lakes (Ortiz-Álvarez *et al.,* 2016) and up to 4.5 % of DAPI counts in water column of the volcanic lake Kivu (Llirós *et al.,* 2010). Subsequent surveys further confirmed the ubiquity of Archaea in freshwater columns from inland systems (Restrepo-Ortiz *et al.,* 2014; Hugoni *et al.,* 2015). Likewise, global surveys demonstrated that Archaea inhabit, virtually, in any soil of the planet, found in disparate niches such as: tundra soils (Blaud *et al.,* 2015), Tibetan plateau dry land (Wang *et al.,* 2015) or arable fields (Bengston *et al.,* 2012). In average, 2% (and up to 10%) of soil rRNA signatures are archaeal in origin (Ochsenreiter *et al.,* 2003; Bates *et al.,* 2011). The presence of these archaeal transcripts have been noticed even in interstitial water from deep soil strata (Shimizu *et al.,* 2006; 2007).

Considering that subseafloor is one of the bigger compartments at planetary scale, archaeal lineages recurrently found in marine sediments (Biddle *et al.,* 2006,

Teske *et al.,* 2008, Kubo *et al.,* 2012; Breuker *et al.,* 2013; Koyano *et al.,* 2014) may be between the most abundant living organisms on Earth. In subseafloor, Archaea is widespread among sediment typologies (e.g. pelagic clay-rich sediment and/or volcanic ash layers; Inagaki 2003) and sedimentary horizons (Schippers *et al.,* 2012; Jorgensen *et al.,* 2013; Li *et al.,* 2015), regardless of the organic matter content (Durbin *et al.,* 2012). Same marine lineages are found in sediments from rivers (Jiang *et al.,* 2011), estuaries (Webster *et al.,* 2015) and freshwater lakes (Jiang *et al.,* 2008; Fillol *et al.,* 2015). Little information is available about Archaea in freshwater sediments, although being abundant in lakes differing in stratification regimes (Schwartz *et al.,* 2007; Bhattarai *et al.,* 2012), having monimolimnia rich in methane (Bhattarai *et al.,* 2012) or sulfate (Fillol *et al.,* 2015), with seasonal ice covering, karstic nature, subalpine placement or oxbow typology (Lim *et al.,* 2011; Casamayor *et al.,* 2012; Conrad *et al.,* 2014; Coci *et al.,* 2015; Fillol *et al.,* 2015). Additionally, archaeal signatures were retrieved from suboxic ponds (Briée *et al.,* 2006) and shallow lakes ranging from oligotrophy to eutrophy (Liu *et al.,* 2012; Lim *et al.,* 2011; Mandic-Mulec *et al.,* 2012; Chen *et al.,* 2015; Yang *et al.,* 2016) or man-made reservoirs encompassing different basin sizes and depths (Green *et al.,* 2012; Garças *et al.,* 2011; Lymperoloupou *et al.,* 2012).

1.4 The masters of sediment realm

Some archaeal groups are repeatedly found in sediments worldwide (Kubo *et al.,* 2012; Zhang *et al.,* 2015; Fillol *et al.,* 2016). Among them, two of the most representative are the phylum Bathyarchaeota (Meng *et al.,* 2014) and the class *Thermoplasmata* (Bergery manual 2002). However, their lack of cultured representatives prevents from reaching bona fide conclusions about metabolic functions. Hence, molecular-based surveys of 16S rDNA signatures, distinguished both lineages as core generalists in sediments, finding co-occurrence patterns between them, and suggesting a possible metabolic partnership (Fillol *et al.,* 2016; See Chapter 1). Unfortunately, the guild diversities and ecological functions of these worldwide distributed groups are still enigmatic.

1.5 Setting up a cosmopolitan phylum: Bathyarchaeota

The first phylotypes belonging to this group came from non-extreme environments, and were clustered under the provisional name Group 1 (divided in subgroups C1, C2 and C3; Delong *et al.,* 1998) of moderate archaea. The discovery of closely related sequences from Okhotsk seafloor, lead to a second rename of the group as *Miscellaneous Crenarchaeotic Group* (MCG; Inagaki *et al.,* 2003). From the very beginning the wide distribution of its signatures combined with complex phylogeny suggested a high inter-group diversity (Sørensen *et al.,* 2005; Webster *et al.,* 2006). Its signatures account between 40 – 80% of the clones from anoxic marine sediments (Fry *et al.,* 2008), were they are widespread (Lloyd *et al.,* 2013; Kubo *et al.,* 2012; Breuker *et al.,* 2013; Jorgensen *et al.,* 2013; Mahmoudi *et al.,* 2015) and between 5.9 and 93% of archaeal signatures from brackish sediments (e.g. estuaries; Jiang *et al.,* 2011; Meador *et al.,* 2015).

In inland sediments, Bathyarchaeota accounts for a major fraction of archaea in Tibetan plateau oligotrophic lakes (Zhang *et al.,* 2015) being well represented in mesotrophic and eutrophic (between 23 and 36 % of Archaea; Yang *et al.,* 2016; Fan *et al.,* 2016), and encompassing a wide variety of geomorphologies, stratification regimes (Jiang *et al.,* 2008; Ferrer *et al.,* 2011; Bhattarai *et al.,* 2012; Rodrígues *et al.,* 2014; Fillol *et al.,* 2015), and salinity levels (Fillol *et al.,* 2016; Dorador *et al.,* 2010; Liu *et al.,* 2016). The increasing number of sequences, led to a succession of divisions in smaller and more manageable subgroups: PM-1 to PM-8 (Parkes 2005), MCG-1 to MCG-4 (Sørensen *et al.,* 2006), A to G (Jiang *et al.,* 2011) and MCG-1 to MCG-17 (Kubo *et al.,* 2012). After that metagenomic studies raised the group at phylum category, and renamed as Bathyarchaeota (Meng *et al.,* 2014). Bathyarchaeota is suggested to be phylogenetically diverse (with some 16S rRNA identities as low as 76%; Kubo *et al.,* 2012) and phylogenetically close to Thaumarchaeota (Rinke *et al.,* 2013). The most updated classification encompasses 21 subgroups (Figure 1.1; Fillol *et al.,* 2016).

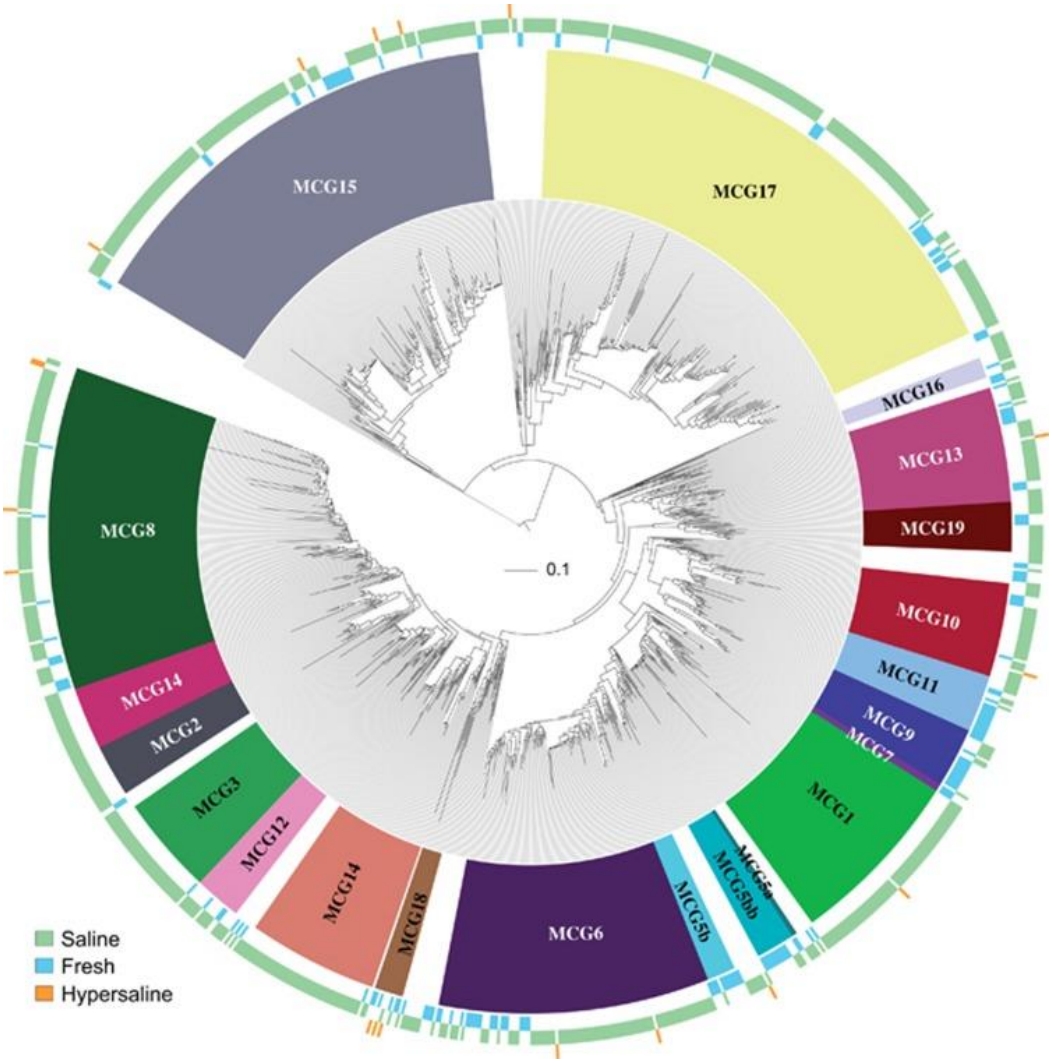

Figure 1.1 Phylogentic tree made in basis of ribosomal gene sequences that represents the 21 Bathyarchaeota subgroups defined by Fillol and co-workers so far.

1.6 The consortium of heterotrophic guilds

Environmental surveys on Bathyarchaeotal 16S rRNA and 16S rDNA signatures pointed out to the heterotrophic nature its members (Table 1.2; Sørensen *et al.,* 2005; Inagaki *et al.,* 2006). Different preferences of Bathyarchaeota subgroups among ecological niches were suggested by the low similarities of their ribosomal signatures (76% identity; Kubo *et al.,* 2012) and confirmed in further studies in brackish and freshwater niches (Lazar *et al.,* 2015; Fillol *et al.,* 2015). Some subgroups are also defined as ecological indicators for freshwater or saline sediments (Fillol *et al.,* 2016). Along with that, their high intra-group diversity converges also with different metabolic preferences.

Stable isotope probing (SIP) analyses confirmed the organic carbon uptake by *Bathyarchaeota-6*, *-8* and *-15*, all of them abundantly found in seabed, salt-marshes, coastal sediments or estuaries (Biddle *et al.,* 2006; Webster *et al.,* 2010; Seyler *et al.,* 2014; Na *et al.,* 2015). Other cultivation-based approaches depicted the increase of signatures from *Bathyarchaeota-4* and *-8* thriving in estuaries after supplementation with organic substrates, suggesting the tolerance of these subgroups to mild oxic conditions (Gagen *et al.,* 2013). The advance in molecular methods allowed the transition from 16S rRNA-centred to genome-wide approaches, improving the metabolic inferences through the genomic scope (Table 1.2). Fosmid libraries envisaged the Bathyarchaeota potentialities in lipid biosynthesis and oxidation of various aliphatic and aromatic compounds (e.g. protocatechuate by *Bathyarchaeota-8*; Ping-Yi *et al.,* 2012; Meng *et al.,* 2014). Single cell genomics (SCG) and metagenomics unveiled the potential degradation of detrital proteins and acetate incorporation of *Bathyarchaeota-15* thriving in estuaries and seafloor (Lloyd *et al.,* 2013; Lazar *et al.,* 2016), the breakdown of cellulose by *Bathyarcheota-6* (Lazar *et al.,* 2016). Other authors stated the capability of *Bathyarchaeota-16* to carry out homoacetogenesis (He *et al.,* 2016) or of some bathyarchaeons to perform methanogenesis in aquifers (Evans *et al.,* 2015).

Some combined studies, carried out by genomic analysis and carbon supplementations set strong hypothesis regarding the Bathyarchaeota lifestyle (Meng *et al.,* 2014). But by now, more cultivation efforts and microcosm analyses are required besides genomic approaches, in order to verify the potential metabolic capabilities of the members of the phylum Bathyarchaeota.

1.7 Resolving an ancient puzzle: *Thermoplasmata*

Prior to the rise of Archaea as a domain, several isolates were retrieved from environments characterized by extremely low ph and high temperature (e.g. *Thermoplasma acidophilum*; Darland *et al.,* 1970). Phylogenetic analyses placed their ribosomal sequences between the two existing archaeal branches, composed by methanogens, extreme halophiles and thermophilic sulfur-oxidizers (Woese *et al.,* 1984; Fox *et al.,* 1980). Related sequences were retrieved from sea waters and marine sediment (Massana *et al.,* 1997; Inagaki *et al.,* 2003). The discovery of more of them in polluted aquifers (Dojka *et al.,* 1998), groundwater, (Takai *et al.,* 2001), rivers (Dumestre *et al.,* 2001), freshwater lakes and ponds of diverging nature (Casamayor *et al.,* 2000; Briée *et al.,* 2006; Auguet *et al.,* 2008) suggested a wider occupancy of this novel clade among freshwater sites. The group was finally defined as the class Thermoplasmata and placed within the phylum Euryarchaeota (Reysenbach 2002).

Subsequent surveys reported Thermoplasmata sequences in sediments from many inland water bodies (On *et al.,* 2005; Mandic-Mulec *et al.,* 2012; Ma *et al.,* 2015; Fillol *et al.,* 2015; Zhang *et al.,* 2015; Fan *et al.,* 2016; Yang *et al.,* 2016). The wide representation of these signatures in anoxic sediments is congruent with posterior genomic studies that inferred the capability of some members of Thermoplasmata to mineralize organic matter in sediments (Iverson *et al.,* 2012; Lloyd *et al.,* 2013; Lin *et al.,* 2015). Besides, their recurrence in digestion processes (Xia *et al.,* 2012; Gannoun *et al.,* 2013; Mirjafari *et al.,* 2016) further reinforces the hypothesis of them as outstanding decomposers in environmental mineralization processes.

1.8. Dipping into the archaeal dark matter: the *Thermoplasmatales*

Thermoplasmatales (Reysenbach 2002), was the first Thermoplasmata order. This complex and deeply branching clade is mainly composed by uncultured facultative anaerobes (Segerer *et al.,* 1988). Some of these cells present a sulfur-based metabolism (Reysenbach 2002) and no genes responsible for methanogenesis have been detected among them (Reysenbach 2002; Paul *et al.,* 2012; Lloyd *et al.,* 2013; Borrel *et al.,* 2014). Some Thermoplasmatales families recurrently found in sediments are the *Marine Benthic Group D* (MBG-D), AMOS1A-4113-D04, CCA47, *Terrestrial Miscellaneous Euryarchaeota group* (TMEG) and ASC21.

1.8.1. The Marine Benthic Group D

The discovery that in seafloor clone libraries, about 4% or reads were unaffiliated but with group-specific regions, lead to the definition of MBG-D as a separated Euryarchaeota clade (Vetriani *et al.,* 1999). Their members are ubiquitous (Inagaki *et al.,* 2006; Lloyd *et al.,* 2013; Parkes *et al.,* 2014), and potentially active (Sørensen *et al.,* 2005; Weigold *et al.,* 2016) in seabed, encompassing 40 % and 56 % of archaeal reads in surficial and deep sedimentary strata, respectively (Koyano *et al.,* 2014; Choi *et al.,* 2016). Family *Thermoplasmatales* may contribute in a great extent to the nutrient cycling suggested in marine sediments (Kallmeyer *et al.,* 2009). Considered one of the most numerous archaeal taxa in marine and continental saline niches (Lloyd *et al.,* 2013; Weigold *et al.,* 2016), their members are reported in methane seeps (Dang *et al.,* 2010), estuaries (Jiang *et al.,* 2011), account 40.5% of archaeal reads in microbial mats (Lazar *et al.,* 2011), and 68% of plankton in the clipperton atoll (Galand *et al.,* 2011) also dominant in deeper layers of hypersaline microbial mats (Schneider *et al.,* 2013).

Dramatic changes on the diversity of MBG-D are driven by environmental variations (e.g. available electron acceptors, sediments depth; Schubert *et al.,* 2011; Cruaud *et al.,* 2015), explaining the ability of its members to disseminate among more dynamic freshwater sedimentary niches. MBG-D signatures have been also found in sediments moving from oligotrophic high-mountain lakes (See chapter 2) to sulfur-rich eutrophic lakes (Hamilton *et al.,* 2016). Their occupancy among freshwater sediments, tempted some authors to suggest MBG-D to perform (of have performed) methanogenesis during its evolutionary lifespan (according with the presence of the gene *mcrA* in their genomes; Paul *et al.,* 2012; Borrel *et al.,* 2014). However, metagenomic results combined with the divergent segregation patterns of MBG-D and other methanogens (Borrel *et al.,* 2012) contradicted this hypothesis. So far, the lack of isolated MBG-D archaeons

hampers the possibility to formulate reliable hypothesis about their metabolic requirements.

1.8.2. The AMOS1A-4113-D04 and CCA47

These groups are generally found as satellite taxa in marine subsurface or saline habitats (Sørensen and Teske 2005; Sørensen *et al.,* 2005; Breuker *et al.,* 2013; Koyano *et al.,* 2014). For instance, CCA47 was firstly defined from a cloned sequence from anoxic sediments from a salt marsh (Stoeck *et al.,* 2003). While AMOS1A-4113-D04 (recently renamed TMEG-5; Lanzén *et al.,* 2012) was defined in basis of rare seafloor sequences (Breuker *et al.,* 2013). Their members are scarce, occasionally found just in wide-range surveys conducted either in seafloor (Breuker *et al.,* 2013; Koyano *et al.,* 2014). They are also found in continental saline sediments as rare taxa, were AMOS1A-4113-D04 and CCA47 encompass 2.13 and 0.91% of archaeal reads, respectively (Liu *et al.,* 2016).

The dominance of AMOS1A-4113-D04 in Euryarchaeota populations from marine snow blowers (Meyer *et al.,* 2013) and sediments from freshwater karstic systems (from where they have been reported for first time; See Chapter 1) further arise questions about their ecologic requirements and role. The salinity preferences of the latter group are shared by CCA47 whose members are able to live even in hypersaline microbial mats (Sørensen *et al.,* 2005; Schneider *et al.,* 2013), in addition to sea waters (Wemheuer *et al.,* 2012) and mangroves (Bhattacharyya *et al.,* 2015). CCA47 is dominant in sub-saline sediments with high sulfur-related activity (79% of archaeal reads; Ferrer *et al.,* 2011) which suggests their ability to reduce sulfate compounds. Nonetheless further molecular approaches would contribute to the understanding of their yet-unknown capabilities of these elusive taxa.

1.8.3. The Terrestrial Miscellaneous Crenarchaeota Group (TMEG)

This diverging group was based on sequences from South African Gold Mines (encompassing sequences affiliated to previous mesophilic group C2; Takai *et al.,* 2001). It was defined in order to circumscribe the "soil miscellany" among uncultured taxa. Up to date TMEG phylotypes span the terrestrial-aquatic divide. Over time TMEG agglutinated phylotypes from soils (Tupinambá *et al.,* 2016), surficial estuarine sediments (Jiang *et al.,* 2011; Li *et al.,* 2012), lakes with differing levels of salinity (Jiang *et al.,* 2007; Dorador *et al.,* 2010; Bar-Or *et al.,* 2015), and hypersaline microbial mats (Schneider *et al.,* 2013). Despite not being widespread in seafloor, it has been found as rare taxa in consecutive surveys carried out across the globe (Teske *et al.,* 2006; Wang *et al.,* 2010; Vigneron *et al.,* 2014; Choi *et al.,* 2016). Thus, TMEG is known to be a sis-

ter lineage of MBG-D (Teske *et al.,* 2006) with a distribution comparable to that for Bathyarchaeota (Teske *et al.,* 2008). The resolution of its intragroup phylogeny has been attempted, however TMEG is still underrepresented in the databases and the two TMEG-related subgroups obtained had high identities with sequences from a very wide spectrum of sites (aquifers polluted by hydrocarbon and chlorinated compounds, mangrove soils, sulfur springs; Dorador *et al.,* 2010). Some phylotypes are phylogenetically related to methanogenic Thermoplasmata (i.e. *Methanomassiliicoccales*; Kuroda *et al.,* 2014), but regarding the miscellaneous nature of the group, this issue still merits further efforts.

The study of the metabolic requirements of TMEG is really just beginning, however their genomic potential for the degradation of fatty acids and the reduction of sulfite and/or organosulfonate (Lin *et al.,* 2015), makes sense with their relation with sulfur-rich environments (Fillol *et al.,* 2015). It is also known that TMEG abundance is affected by soil properties (Tupinambá *et al.,* 2016) and their members are hypothesized thrive in nutrient constrained environments (Mirjafari *et al.,* 2016), such as bioreactors (Kuroda *et al.,* 2014), were can be naturally enriched along time (Mirjafari *et al.,* 2016).

1.8.4. The ASC21

Members of this rare clade have been mainly found in polluted soils (Kasai *et al.,* 2005; Ashby *et al.,* 2007; Nishizawa *et al.,* 2008; Cao *et al.,* 2012), freshwater iron-rich flocs (Kato *et al.,* 2013), mud volcanoes (Wrede *et al.,* 2012) and saline sediments from inland or marine sites (Inagaki *et al.,* 2003; Dorador *et al.,* 2010). Its low abundance and lack of specific molecular tools has hampered the assessment their ecological preferences and genomic potential. The ASC21 has rarely been reported in freshwater sediments (Fillol *et al.,* 2015; Fan *et al.,* 2016). Interestingly, some of the studied systems in the present work have a high abundance of ASC21 (See Chapter 1), being promising sites for the conduction of further studies.

1.8.5. The Methanomassiliicoccales

This order has been defined as a sister clade of Thermoplasmatales (Borrel *et al.,* 2014). It was firstly envisaged in basis of the phylogenetic divergence of a cluster of ribosomal signatures from human stools, in relation to other methanogenic lineages (Mihajlovsky *et al.,* 2008). After the finding of related sequences in subgingival plaque (Horz *et al.,* 2012) gastrointestinal tracts (Scanlan *et al.,* 2008; Evans *et al.,* 2009; Mihajlovsky *et al.,* 2010), and two arthropod-specific phylotypes from termite gut (Paul *et al.,* 2012), the clade was preliminarily named as *Methanoplasmatales* (Paul *et al.,*

2012). Further clues came with the isolation of *Methanomassiliicoccus luminiensis* (Dridi *et al.,* 2012) and the enrichments of *Candidatus Methanomassiliicoccus Alvus* and *Candidatus* Methanogranum caenicola (Borrel *et al.,* 2012; Iino *et al.,* 2013). After that, the group was considered monophyletic and renamed as *Methanomassiliicoccales* (Iino *et al.,* 2013; Castelle *et al.,* 2015).

Methanomassiliicoccales are putative H_2-dependent methylotrophs (Borrel *et al.,* 2012; 2013; Lang *et al.,* 2015), sharing ancestry with MBG-D (Borrel *et al.,* 2013), and being phylogenetically closer to *Marine Group II* (MG-II) than to any other methanogenic order (Paul *et al.,* 2012; Borrel *et al.,* 2014). Members of *Methanomassiliicoccales* participate in anaerobic digestion processes (Chouari *et al.,* 2015; Dziewit *et al.,* 2017), and their genomic features inferred them as potentially able to thrive in soils and sediments (Borrel *et al.,* 2014). Their prevalence among gut microbiota (Söllinger *et al.,* 2015), combined with the high occupancy among the methane-producing environments (e.g. wetlands; Borrel *et al.,* 2013; Söllinger *et al.,* 2015), suggests an outstanding role of *Methanomassiliicoccales* in methanogenic guild. Phylogenomic analyses unveiled its intragroup low diversity, with just two subgroups defined so far: the gastrointestinal and the environmental clades (Söllinger *et al.,* 2016). Methanomassiliicoccales-related sequences from seafloor (Nunoura *et al.,* 2016), soils (Kemnitz *et al.,* 2005) and peatlands (Galand *et al.,* 2003) suggests them as a rare lineage in freshwater niches. Interestingly, it encompasses large fractions of Thermoplasmata in some lacustrine sediments (See chapter 1).

Table 1.2. Metabolic traits of uncultured archaeal taxa, inferred by different molecular and genomic approaches.

Studied group	Sampled site	Approach	Compounds	Inferred metabolism	References
Bathyarchaeota Woesearchaeota Marine Benthic Group D TMEG	Peru basin subsurface	RT-qPCR Cloning	-	Heterotrophy	Sorensen 2005
Bathyarchaeota Woesearchaeota	Peru basin subsurface	FISH analysis Chromatografic analysis of lipids Cloning	-	Heterotrophy	Biddle 2006
Bathyarchaeota-15 *Marine Group I* *Marine Benthic Group D*	Severn estuary sediment	13C-supplemented microcosms DGGE Cloning	Glucose, Acetate, CO_2	Glucose (MGI) and Acetate (Bathyarchaeota and MBG-D) utilization	Webster 2010
Bathyarchaeota Marine Group I	South China sea sediment	Fosmid library RFLP	-	Oxidation of aromatic and alifatic aldehydrates	Ping-Yi 2012
Methanomassilicoccales	Human feces	Enrichment cultures	Methanol	Methylotrophy	Borrel 2012
Bathyarchaeota	White Oak river estuarine sediment	Enrichment cultures qPCR Cloning	Acetate, Glucose aminoacid mix, methanol, protochuateic acid, pyruvate, glycero, complex organic compounds	Heterotrophy under "in vitro" conditions	Gagen 2013
Bathyarchaeota Marine Benthic Group D	Aarhus bay sediment	Single-cell genomics	-	Degradation of detrital proteins	Lloyd 2013
Bathyarchaeota-8	Pearl rivers estuary sediment	Metagenomics Enrichment cultures	-	Protochatechuate degradation	Meng 2014
Bathyarchaeota-6 *Bathyarchaeota-8* *Marine Group I*	Hooks creed salt marsh sediments	13C-supplemented microcosms TRFLLP Cloning	Acetate, Glycine, Urea, Lipids, Protein mix, bicarbonate, artifical meduim	Heterotrophy	Seyler 2014

Table 1.2. Continued

Studied group	Sampled site	Approach	Compounds	Inferred metabolism	References
Methanomassilicoccales	Termite guts	Enrichment High-troughput sequencing	-	Methylotrophy Gluconeogenesis	Lang 2015
Bathyarchaeota	Coal bed from Surat basin	Metagenomics	-	Methanogenesis	Evans 2015
Bathyarchaeota-15	Aarhus bay sediment	13C-supplemented microcosms High-troughput sequencing q PCR	Acetate	Acetogenesis	Na 2015
TMEG	Peat soil	Metagenomics	-	Degradation of fatty acids Organosulfonate reduction	Lin 2015
Thorarchaeota	White Oak river estuarine sediment	Metagenomics	-	Protein degradation Reduction of elemental sulfur	Seitz 2016
Bathyarchaeota	Guayamas Basin sediment	q PCR Metagenomics	-	Homoacetogenesis Degradation of aromatic compounds	He 2016
Bathyarchaeota	White Oak river estuarine sediment	Metagenomics	-	Hydorlisis of plant-derived carbohidrates Degradation of detrital proteins Acetogenesis Nitrite reduction Ethanol fermentation	Lazar 2016
Marine Benthic Group D Theionarchaea	White Oak river estuarine sediment	Metagenomics	-	Acetogenesis Sugar and aminoacid degradation	Lazar 2017

1.9 Interactions with abiotic factors

1.9.1. Salinity

Considering that microbial habitats are chemical in nature (Robertson *et al.,* 2005), environmental parameters (e.g. light, salinity, pH, availability of electron donors and acceptors, etc.) are key drivers regarding the segregation among archaeal taxa (Auguet *et al.,* 2010; 2013; Berdjeb *et al.,* 2013; Xie *et al.,* 2014). Salinity has been described as the most important driver regarding microbial dissemination (Oren *et al.,* 2001; Lozupone *et al.,* 2007; Logares *et al.,* 2009). The high or low levels of salt mainly affect the prokaryotic cells by osmotic stress. This occurs when the concentration of molecules in solution outside the cell is different than those ones inside of it. When such concentration changes occur water flows either into or out the cell, affecting the intracellular environment. Regarding this, the rising levels of salt represent an increasing stress, able to diminish the richness of prokaryotic communities (Simachew *et al.,* 2016). Halophilic cells have developed mechanisms to overcome the osmotic stress. Some of them are the high content of GC nucleotide (Dym *et al.,* 1995, Soppa *et al.,* 2006), the elevated frequencies of negative-charged residues (Kennedy *et al.,* 2001; Fukuchi *et al.,* 2003) or the lower propensity for protein helix formations compared with the higher preference for coil structure (Paul *et al.,* 2008).

Archaeal cells are able to live along the whole salinity spectrum. Besides, they replace the Bacteria along the salinity gradients (Simachew *et al.,* 2016), and some of the most saline places in the world are mainly inhabited by Archaea. Even so, salinity gradients produce structural changes in archaeal communities (Webster *et al.,* 2015; Liu *et al.,* 2016). Seafloor Archaea overcame the saline-freshwater boundary through their evolutionary race (Auguet *et al.,* 2010; Fillol *et al.,* 2016) as marine lineages can be found in freshwater sediments (Jiang *et al.,* 2008; Lymperoloupou *et al.,* 2012; Zhang *et al.,* 2015; Fillol *et al.,* 2015; Liu *et al.,* 2016). Interestingly, their presence in continental hypersaline lakes (Dorador *et al.,* 2010; Borsodi *et al.,* 2013; Abdallah *et al.,* 2016) may indicate a second freshwater-saline transition. This second transition has been clearly confirmed for the members of the phylum Bathyarchaeota (Fillol *et al.,* 2016), with subgroups *Bathyarchaeota-5b* and *-11* defined as indicator species for freshwater sediments, and *Bathyarchaeota-1* and *-8* for saline ones (Fillol *et al.,* 2016). Despite Thermoplasmata is widespread, some groups namely CCA47 and AMOS1A-4113-D04 are recurrently associated to high salinity environments (Ferrer *et al.,* 2011; Liu *et al.,* 2016). In the present work some of them have been found in large numbers in some of the studied sediments (See Chapter 1).

1.9.2. Oxygen

After salinity, oxygen is a key driver for Archaea (Liu *et al.,* 2015). Oxygenic photosynthesis represented a major transition in the history of life, confining (up to date) many prokaryotes into anoxic environments (Raymond *et al.,* 2006). Primitive Archaeal cells throve solely in extreme niches (McIerney *et al.,* 1995; Edwards *et al.,* 2000; López-García *et al.,* 2001; Zierenberg *et al.,* 2001; Dorador *et al.,* 2010) using simplified metabolic networks (Caetano-Anollés *et al.,* 2009). Thus, the diversification of archaeal metabolisms cannot be understood without considering the rise in atmospheric oxygen levels. This diversification leaded to a variety of aerobic (e.g. ammonia oxidation) or anaerobic (e.g. methanogenesis, sulfate reduction, denitrification) metabolisms (Offre *et al.,* 2013).

Oxygen has been described as one of the main mediators in the biogeochemical processes (Falkowsky *et al.,* 2008). Many prokaryotes use it as final electron acceptor as it is associated with high-energetic reactions and confer an ecological advantage to aerobes. Therefore, oxygen is depleted during the mineralization of organic matter and anoxia can arise wherever organic matter from surrounding oxic places accumulates. Marine sediments (anoxic below the first milimeters) and bottoms of freshwater lakes exemplify these processes (Fenchel *et al.,* 1991). Opposite to oxic environments where organic matter can be mineralized by a single organism, anaerobic prokaryotes must work syntrophically in order to completely mineralize carbon compounds (Fenchel *et al.,* 1991). A comparison of ecologic niche typologies based on the structure of their respective uncultured Archaea, distinguish anoxia as a key parameter, able to cluster marine samples with diverging physicochemical factors and different compartments (i.e. water column, sediment) of anoxic lakes (Auguet *et al.,* 2010). So far, most information regarding oxygen-depleted sediments refers to bacterial guilds (e.g. sulfate reducers, fermenters), the functions of their archaeal counterparts are still undetermined.

Bathyarchaeota is highly represented in anoxic seafloor (Kubo *et al.,* 2012; Breuker *et al.,* 2013 and references therein) and lake sediments (Borrel *et al.,* 2012; Zhang *et al.,* 2015; Yang *et al.,* 2016) while Thermoplasmata is especially abundant in euxinic sediments of karstic lakes (Fillol *et al.,* 2015; See Chapter 1). The microdiversity of both groups is affected by the presence / absence of oxygen. For instance, Bathyarchaeota-6 prefers anoxic environments (Lazar *et al.,* 2015), while Bathyarchaeota-4 tolerates microaerophilic conditions (Gagen *et al.,* 2013). Almost all Thermoplasmata lineages, were firstly defined from anoxic or low-oxygen sites and they have been continuously associated to these environments (Ferrer *et al.,* 2011; Koyano *et al.,* 2014).

1.9.3. Organic matter

Together with the recurrent anoxia, large amounts of deposited organic matter are characteristic in sedimentary compartments (especially freshwater ones; Dean *et al.,* 1998; Fry *et al.,* 2008). Those conditions make sediments suitable places for microbial heterotrophy to take place. Microorganisms play a pivotal role in the nutrient turnover at global scale (Falkovsky *et al.,* 2008), and Archaea actively contributes to nutrient cycling in both marine (Meng *et al.,* 2015) and freshwater (Hugoni *et al.,* 2015) sediments.

Heterotrophy has been stated as a dominant lifestyle in seafloor (Biddle *et al.,* 2006; Lloyd *et al.,* 2013), being also being a prominent metabolism in sediments from freshwater lakes (Torres *et al.,* 2011; Morales-Pineda *et al.,* 2016). Organic matter may be deposited in several forms, encompassing a spectrum of reactivity, moving from molecules with very fast turnover to recalcitrant compounds (Hansell *et al.,* 2013). In seafloor the vast majority of chemoheterotrophic cells thrive under extreme energy limitation (Jorgensen *et al.,* 2007) using sulfur compounds as electron acceptors (Jorgensen *et al.,* 2007; Hansell *et al.,* 2013). In those environments heterotrophy is limited by the recalcitrance of organic matter (Fry *et al.,* 2008). In their freshwater sedimentary counterparts the carbon turnover is much higher (Dean *et al.,* 1998; Tranvik *et al.,* 2009). In the latter sediments, the typology of the organic carbon inputs depends on the ratio of terrestrial and autochthonous organic matter (i.e. planktonic or plant-derived; Sampei *et al.,* 2001).

The ubiquity of Bathyarchaeota and *Thermoplasmata* members among sediments (Fillol *et al.,* 2016) makes sense with their capability to grow using allochthonous carbon compounds (Lloyd *et al.,* 2013; Meng *et al.,* 2014; Lin *et al.,* 2015; Lazar *et al.,* 2016). In seabed, the abundance of both groups has been related with total organic content (Oni *et al.,* 2015). In freshwater lakes, the abundance and microdiversity of Bathyarchaeota has been related with C/N ratios (Zhang *et al.,* 2015; Fan *et al.,* 2016). The lack of electron acceptors leads to the cleavage (i.e. fermentation) of recalcitrant molecules to more bioavailable compounds (Offre *et al.,* 2013), thus, some Bathyarchaeota and Thermoplasmata lineages may trigger this process (e.g. *Bathyarchaeota-6*), providing labile compounds used by other groups (e.g. *Bahyarchaeota-15,* MBG-D; Lloyd *et al.,* 2013; Fillol *et al.,* 2015; Lazar *et al.,* 2016; 2017).

The abundance of organic matter may have a direct effect over the abundance of sedimentary Archaea, while their source and bioavailability may favour the prevalence of concrete lineages.

1.9.4. Metal pollution

The capability of salinity, oxygen and organic matter inputs to shape abundances and microdiversity of archaeal lineages are largely known. Metals are other type of natural compounds, naturally present in trace amounts in sediments (Bacardit *et al.,* 2012; Martinez-Cortizas *et al.,* 2016), and required as cofactors for prokaryotes (Jacquot *et al.,* 2014; Sorichetti *et al.,* 2016). Some of them are specially needed for archaeal metabolisms (e.g. Cu; Andreini *et al.,* 2008).

In the last decades the expelling of metals as a side-product of industrialization has leaded to an increase of metal pollution (Camarero *et al.,* 2009; Bacardit *et al.,* 2012). Sedimentary environments may be impacted when exogen metal particles from atmospheric depositions or local activities (Thevenon *et al.,* 2011; Kuwae *et al.,* 2013) accumulate in sediments (Catalan *et al.,* 2006; Xia *et al.,* 2011; Zaaboub *et al.,* 2015), surpassing basal levels and becoming toxic. Although archaeal cells are able to survive in such metal-contaminated sediments (Bruneel *et al.,* 2008; Gough *et al.,* 2011; Besaury *et al.,* 2014) large amounts metals have effects over them (Almeida *et al.,* 2008; Halter *et al.,* 2011; Ma 2016), decreassing their biomass (Azarbad *et al.,* 2013; Niemeyer *et al.,* 2014; Besaury *et al.,* 2014). Differing toxicity levels have been stated among microbial clades (Rastogui *et al.,* 2009; Ma *et al.,* 2016; Ni *et al.,* 2016) changing community structures (Yin *et al.,* 2015) and leading to a community specialization (MacDonald *et al.,* 2011). Regarding Archaea, such changes may affect nutrient cycling (Niemeyer *et al.,* 2012; Ni *et al.,* 2016). In spite of all of that, the main part of studies addressing the effects of metals over archaeal communities have focused over one or several metals (McDonald *et al.,* 2011; Besaury *et al.,* 2012; Ma *et al.,* 2016). Hence, more exploratory studies are required in order to assess the individual or combined effects of metals among the archaeal uncultured clades thriving in sediments.

Due to their isolate location, high-mountain lakes are little affected by exogenous sources of pollution, besides, they are placed on convergence zones, where the metal depositions are specially accute (Loewen *et al.,* 2005; Wegmann *et al.,* 2006). Thus, sediments of these pristine systems are model niches for exploratory studies in order to assess the concrete effects of the natural diversity of metals over uncultured sedimentary archaea (See Chapter 2).

1.10 Towards genome-centric approaches

Initial 16S rRNA gene surveys highlighted the importance of archaea in sedimentary environments, posing pressing questions about their metabolic capabilities and ecological roles. As 16S rRNA-centred surveys cannot solve these questions, genomic approaches gained importance in the microbial ecologist toolbox. Nowadays, whole-genome techniques are indispensable to complement the 16S rRNA gene analyses (Poretsky *et al.,* 2014; Logares *et al.,* 2015). Exemplifying this, single cell genomics (SCG) and metagenomics allowed the discovering of potential degradation of detritic proteins by some Bathyarchaeota and MBG-D in the cold sediments of Aarhus Bay and White Oak river (WOR) estuary (Lloyd *et al.,* 2013; Lazar *et al.,* 2017), while Lin and co workers used metagenomics to unveil the potential of TMEG to degrade long-chain fatty acids in anoxic peat layers (Lin *et al.,* 2015). Many of these studies have focused on several subgroups of the ubiquitous Bathyarchaeota. The potential of *Bathyarchaeota-8* to degrade recalcitrant compounds in seafloor has been predicted by fosmid approaches (Meng *et al.,* 2014). Subsequent metagenomic surveys ascertained the different Carbon preferences among Bathyarchaeota subgroups (Lazar *et al.,* 2015). Same technique has been useful to determine the ability of *Bathyarchaeota-6* to hydrolyze plant-derived carbohydrates (Lazar *et al.,* 2016) and of *Bathyarchaeota-16* to produce homoacetogenesis, a metabolism thought to be restricted to Bacteria (He *et al.,* 2016). Methagenomic results arised even surprising quest, namely with the suggestion of Bathyarchaeons thriving in a deep aquifer to carry out methanogenesis (Evans *et al.,* 2015).

The 16 rRNA gene still is a valuable biomarker for the quantification of archaeal clades and elucidation of their underlying phylogenetic relationships. However, inherent biases on primer coverage (Klindworth *et al.,* 2013; Poretsky *et al.,* 2014) may result in an artefactual selection of some taxa (Cruaud *et al.,* 2014) or may influence quantitative abundance estimators as well (Tremblay *et al.,* 2015; Parada *et al.,* 2016). The conjunctive use of several gene biomarkers enabled the strengthening of the defined phylogenetic relationships. This phylogenomic approach has been crucial in the definition and refinement of novel clades (Brochier-Armanet *et al.,* 2008; Rinke *et al.,* 2013; Borrel *et al.,* 2013; Castelle *et al.,* 2015; Seitz *et al.,* 2016).

1.11. Moving from in silico to in vitro

The methodology optimization and recent advances in the genomic field (Venter *et al.,* 2004) attracted an increasing attention by many researchers (Tyson *et al.,* 2005; Pedrós-Alió *et al.,* 2006). Even so, cultivation is the last goal in the characterization of microbial species. Microbial isolates are mandatory for the description of novel species. Besides, pure cultures allow reliable "in vitro" analysis of their physiology and metabolic capabilities, providing basis for the annotation of metagenomic datasets (Stefani *et al.,* 2015). Finally, it must be kept in mind that is impossible to discover new genes involved in novel pathways just from the available sequence data (Steward *et al.,* 2012). However, according to the "great plate count anomaly", just a small portion of microbes are cultured and microbial dark matter still represents a hole in the knowledge.

The impossibility to cultivate microbial species is due to the inability to replicate the microbial interactions (Schink *et al.,* 2002) or the environmental variables (e.g. pH, nutrients, osmotic conditions, temperature) existing in natural environments (Steward *et al.,* 2012). In addition, laboratory-scale manipulations may lead to artefactual selection, for instance of fast-growing species due to excessive inputs of nutrients (Ferrari *et al.,* 2005). The co-cultivation of multiple species and the transfer of a part of the environment to laboratory (mesocosms) may help to cover the environmental necessities of uncultured lineages (Steward *et al.,* 2012). The latter approach has been successfully used to determine metabolic requirements of several archaal taxa (Table 1.2; Webster *et al.,* 2010; Gagen *et al.,* 2013; Seyler *et al.,* 2014; Meng *et al.,* 2014).

Nowadays, cultivation experiments are still valuable for the study of environmental niches with a large fraction of rare or unknown taxa (e.g. gut microbiome; Lagier *et al.,* 2015; Sommer *et al.,* 2015). That makes mesocom assays a useful previous step, aiming to provide information to further cultivation efforts in order to unveil the metabolic features of the still-unknown microbes.

2. OBJECTIVES AND OUTLINE OF THE THESIS

This PhD thesis is focused in the study of uncultured archaeal lineages in freshwater sediments putting an especial attention to two core groups: the phylum Bathyarchaeota and the class Thermoplasmata. We aimed to assess their ecological and metabolic role in freshwater environments, and to determine the main environmental variables that drive their distribution and abundance. To address these issues we established three main objectives:

Objective 1. To assess the abundance and composition of sedimentary archaeal communities in freshwater sediments with different typologies and trophic status.

Objective 2. To determine the effects that the emergent metal pollution from different sources have on these communities taking as a reference undisturbed freshwater environments: the high-mountain lakes.

Objective 3. To identify the metabolic preferences that biofilm- and sediment-dwelling archaea of a karstic lake have among different sources of organic carbon sources.

To assess the distribution patterns of the target lineages (**Objective 1**) we carried out a molecular screening of 21 freshwater systems of the Iberian Peninsula encompassing a wide range of trophic status and different typologies (**Chapter 1**). We examined the representation and the dominant subgroups of each one of the target lineages among the archaeal communities of each system. Moreover, we stated the dominance of classical methanogenic lineages in our system set and identified specific uncultured lineages particularly abundant in systems with concrete characteristics (e.g. high representation of Bathyarchaeota and Thermoplasmata in karstic stratified systems). Finally, we reinforced the hypothesis of a metabolic linkage between both groups by comparing them in terms of abundance and diversity.

The effects that metal pollution may have on archaeal communities were assessed by carrying a second molecular survey in sediments of 18 pristine high-mountain lakes (**Chapter 2**) with different levels of anthropogenic pollution (**Objective 2**). We realized that Bathyarchaeota and Thermoplasmata are more abundant in high-mountain lake sediments than in sediments collected from their low land counterparts (**Chapter 1**). In addition, these systems presented low abundances of methanogenic groups and low abundances of genes associated to methanogesis. We also resolved that metals were the main factor affecting the composition and abundance of archaeal communities. Furthermore, we identified which metals had a toxic effect on the studied lineages,

which ones were susceptible to inhibit methanogesis, and which environmental variables were positively or negatively correlated with the abundance of Bathyarchaeota, Thermoplasmata and some of their most prominent subgroups.

The metabolic preferences of Bathyarchaeota and Thermoplasmata (**Objective 3**) have been addressed in a laboratory scale microcosm experiment (**Chapter 3**). Sediment and leave-attached biofilm from their anoxic bottom were supplemented with different aminoacidic and plant-derived compounds and incubated under anaerobic conditions. We confirmed the responsiveness of both studied group under the treatments, stating the community specialization taking place during the incubation time. Large disparities (in terms of composition and diversity) were also observed between the bulk and active communities and in a minor extend, between substrates (biofilm and sediment) and incubation time. Lastly, we identified the sub lineages responsible of the observed community changes and we realized that some of them experienced changes in their microdiversity (in terms of OTU abundances) when supplemented either with aminoacidic or plant-derived compounds.

3. RESULTS AND DISCUSSION

CHAPTER 1

Abundance and Co-Distribution of Widespread Marine Archaeal Lineages in Surface Sediments of Freshwater Water Bodies across the Iberian Peninsula

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Abstract

Archaea inhabiting marine and freshwater sediments have a relevant role in organic carbon mineralization, affecting carbon fluxes at a global scale. Despite current evidences suggesting that freshwater sediments largely contribute to this process, few large-scale surveys have been addressed to uncover archaeal diversity and abundance in freshwater sedimentary habitats. In this work, we quantified and high-throughput sequenced the archaeal 16S rRNA gene from surficial sediments collected in 21 inland waterbodies across the Iberian Peninsula differing in typology and trophic status. Whereas methanogenic groups were dominant in most of the studied systems, especially in organic-rich sediments, archaea affiliated to widespread marine lineages (the Bathyarchaeota and the Thermoplasmata) were also ubiquitous and particularly abundant in euxinic sediments. In these systems, Bathyarchaeota communities were dominated by subgroups Bathyarchaeota-6 (87.95 ± 12.71%) and Bathyarchaeota-15 (8.17 ± 9.2%) whereas communities of Thermoplasmata were mainly composed of members of the order Thermoplasmatales. Our results also indicate that Archaea accounted for a minor fraction of sedimentary prokaryotes despite remarkable exceptions in reservoirs and some stratified lakes. Copy numbers of archaeal and bathyarchaeotal 16S rRNA genes were significantly different when compared according to system type (i.e., lakes, ponds, and reservoirs), but no differences were obtained when compared according to their trophic status (from oligotrophy to eutrophy). Interestingly, we obtained significant correlations between the abundance of reads (Spearman $r = 0.5$, $p = 0.021$) and OTU richness (Spearman $r = 0.677$, $p < 0.001$) of Bathyarchaeota and Thermoplasmata across systems, reinforcing the hypothesis of a potential syntrophic interaction between members of both lineages.

Keywords

Bathyarchaeota; Euxinic sediments; Thermoplasmata; Uncultured archaea

CHAPTER 2

Metal Contaminations Impact Archaeal Community Composition, Abundance and function in Remote Alpine Lakes.

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Metal contaminations impact archaeal community composition, biomass and function in remote alpine lakes

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Summary

Using the 16S rRNA and *mcrA* genes, we investigated the composition, abundance and activity of sediment archaeal communities within 18 high-mountain lakes under contrasted metal levels from different origins (bedrock erosion, past-mining activities and atmospheric depositions).

Bathyarchaeota, Euryarchaeota and *Woesearchaeota* were the major phyla found at the meta-community scale, representing 48%, 18.3% and 15.2% of the archaeal community, respectively. Metals were equally important as physicochemical variables in explaining the assemblage of archaeal communities and their abundance. Methanogenesis appeared as a process of central importance in the carbon cycle within sediments of alpine lakes as indicated by the absolute abundance of methanogen 16S rRNA and *mcrA* gene transcripts (10^5 to 10^9 copies g^{-1}). We showed that methanogen abundance and activity were significantly reduced with increasing concentrations of Pb and Cd, two indicators of airborne metal contaminations. Considering the ecological importance of methanogenesis in sediment habitats, these metal contaminations may have system wide implications even in remote area such as alpine lakes.

Overall, this work was pioneer in integrating the effect of long-range atmospheric depositions on archaeal communities and indicated that metal contamination might significantly compromise the contribution of Archaea to the carbon cycling of the mountain lake sediments.

Introduction

Alpine lakes are natural sensors of regional and global environmental change (Williamson *et al.*, 2009; Battarbee *et al.*, 2012). Given their remoteness from areas with high anthropogenic activities and their pristine nature, the effects of large scale changes, such as climatic and background diffuse contamination, are not hidden by local perturbations. Thus, they act like natural sentinels, being able to record climatic changes and variations in the levels of contaminants (Camarero *et al.* 2009; Kuwae *et al.* 2013; Martínez Cortizas *et al.* 2016). In a large-scale study covering 275 alpine lakes across Europe, Camarero et al. (2009) showed that the sediment concentrations of trace metals such as Pb, Zn, Cd, Hg, Cu and Se were comparable to concentrations found in moderately to intensely contaminated systems. Metals within lake sediments have a natural origin as they are constituents of the Earth's crust and they may be released from rocks by weathering processes (Bacardit *et al.*, 2012; Li *et al.*, 2013; Bing *et al.*, 2016). However, anthropogenic inputs can alter these natural cycles, increasing the concentration of metals, and contributing to their accumulation. These inputs can be local due to ore-extracting activities or long range in the case of high temperature anthropogenic processes such as smelting and fossil fuel combustion. With the exceptions of lead, which has been emitted in huge amounts over Europe since Antiquity (Camarero *et al.*, 1998), the rate of emissions of metals has been low during the pre-industrial period, due to their low volatility. However, the high temperature of modern anthropogenic processes such as smelting and fossil fuel combustion led to increased emissions, and resulted in the increase of metal concentration in the atmosphere. Once there, metals adsorb to particles and can be dispersed by atmospheric currents, being deposited in remote ecosystems placed far from their source of origin (Brimblecombe 1993; Thevenon *et al.* 2011; Bacardit *et al.* 2012). Organic matter is an efficient scavenger of metals (Davis 1984) and metals tend to adsorb more efficiently and accumulate in lake sediments. From a study of 74 lakes in the Pyrenees it has been estimated that about half of the lakes have lead concentrations in sediments above the level at which the first biological harmful effects are detected. The same has been observed for mercury, zinc and cadmium, although in a lower percentage of lakes. In some cases, these same four elements even exceed the level for which serious biological effects may occur (Camarero *et al.*, 2009). Despite these alarming levels of metal contaminations, no study has been undertaken to investigate their effect on alpine lake microbiota.

Mountain regions represent approximately one fifth of the Earth's surface with one tenth of the world's population, and provide a large amount of goods and services (Monz, 1999). In alpine lake sediments, these services are supported by diverse micro

bial communities insuring the recycling of carbon, nitrogen, phosphorus and sulfur back to the water column (Ingvorsen *et al.*, 1981; Billen, 1982; Holmer *et al.,* 2001). Among these microbes, sediments harbour a large diversity and abundance of uncultured Archaea (Auguet *et al.*, 2010; Borrel *et al.*, 2012; Fillol *et al.*, 2015; Compte-Port *et al.*, 2017). Some of these taxa, namely: *Bathyarchaeota, Thermoplasmata* (and their lineages *Marine Benthic Group D* and *Methanomassilicoccales*) have the genomic potentialities for the degradation of organic compounds and the production of methane, a greenhouse gas (GHG) with a global warming potential 28-fold higher that of carbon dioxide (Lloyd *et al.*, 2013; Borrel *et al.*, 2014; Meng *et al.*, 2014; Evans *et al.*, 2015; Lin *et al.*, 2015; Lazar *et al.*, 2016). Between 6 and 16% of total natural methane emissions on a global scale would originate from lacustrine environments (Rahalkar *et al.*, 2009). In addition to their role in GHG emissions, methanogens participate to the mineralization of organic matter within anoxic sediments making them a key functional guild in freshwater habitats (Pedersen and Sayler, 1981). The methyl coenzyme M reductase (*mcrA*) that is responsible for the last step in all methanogenic pathways is typically used as functional gene marker for analysis of methanogenic communities along with the 16S rRNA gene (Luton *et al.*, 2002). Besides their pivotal role in methanogenesis, Archaea are also involved in other global biogeochemical processes such as methane oxidation (Ahila *et al.*, 2014), sulphate reduction (Hocking *et al.*, 2014) and ammonia oxidation (Liu *et al.*, 2014; Zhou *et al.*, 2016). Changes in prokaryotic community composition in relation to local metal contaminations have been observed previously (O'Sullivan *et al.*, 2013; Costa *et al.*, 2015; Yin *et al.*, 2015), and several exploratory studies pointed out the effects of metals on the abundance (Besaury *et al.*, 2014), and diversity (Haller *et al.*, 2011) of Archaea. Metals were also shown to adversely impact methanogenesis in laboratory experiments (Muñoz *et al.*, 1996) and environmental surveys (Geets *et al.* 2006; Gough and Stahl 2011). However, none of these studies have investigated the effect of long range atmospheric deposition of metals on microorganisms and particularly on Archaea. Because of their important ecological role, any effect on their diversity, abundance or function may have implications on the nutrient cycling for the whole lake habitat.

Considering the documented toxicity of metals on Archaea and the observed levels of metals in the sediment of alpine Pyrenean Lakes, we hypothesized a potential effect of local and atmospheric metal contaminations on archaeal communities and particularly on methanogens. The aim of the present study was to test this hypothesis using the 16S rRNA and *mcrA* genes to investigate the composition, abundance and activity of sediment archaeal communities within 18 high-mountain lakes contaminated by varying levels of metals of different origin (bedrock erosion, past-mining activities and

atmospheric depositions). By assessing the physicochemical variables and geomorphological traits of each lake, we also estimated the contribution of metals compared to well-known drivers of archaeal diversity and function.

Results

Physico-chemical characterization of the sediments

On the basis of metal concentrations measured in alpine lake superficial sediments of a precedent study (Camarero, 1993), we classified the 18 sampled lakes in three categories corresponding to their level of metal contamination. In agreement with this *a priori* classification, we observed a significant increase ($p < 0.05$, Kruskal-Wallis test) in the total amount of metals from lakes with low metal contents to lakes with local contaminations (Figure S1). The three most abundant metals found in lake sediments were Al (24239.2 ± 8825.1 mg Kg^{-1}, average for all lakes), Fe (33877.8 ± 24361.6 mg Kg^{-1}) and Mg (4624.15 ± 1833.23 mg Kg^{-1}), while many other metals (Se, Mo, Ag, Cd, Sn, Sb, W, Hg, Tl) were found in trace amounts (generally >1 mg Kg^{-1}; Table S1). Considering sediment quality guidelines for heavy metals (i.e. see values for Pb, Cd, Cu, Zn, Hg, As and Se in table S1), toxic effects might be expected in 11 out of the 18 lakes as one or more of the metal concentrations exceeded the threshold value for severe biological effect (OMOE, 1992; Delvalls *et al.,* 1998; Camarero *et al.* 2009). In order to compare each lake based on their heavy metal composition, sediment samples were sorted in an ordination plot (nMDS, Bray-Curtis distance). Lakes with low metal contents (L lakes) formed a clear cluster and tended to be very similar in their heavy metal composition (Figure 1). Heavy metal composition in lakes affected by atmospheric depositions (A lakes) and lakes with local contaminations (i.e., H lakes) were much more variable but significantly different from L lakes as evidenced by pairwise comparisons ($p < 0.05$). A perMANOVA analysis confirmed that the level of metal contamination explained 33.8 % of the variance ($p < 0.01$). No effect of the layer of sampling was detected on metal composition ($p > 0.05$).

Acidity tended to be higher in lakes affected by local sources of metals (pH = 5.86 ± 1.21) compared to L and A lakes (pH = 6.83 ± 0.56 and 6.3 ± 0.36, respectively; Table S2). On average, higher concentrations of Chl *a* were found in L lakes (3.67 ± 3.02 μg L^{-1}) compared to A and H lakes (1.21 ± 0.72 and 1.37 ± 1.35 μg L^{-1}, respectively). Significant higher levels of SO_4^{2-} were found in H lakes compared with L ones (Mann-Whitney test; W= 32; $p = 0.022$) with remarkably high levels found in lake Baiau (56.1 and 29.6 μM for the upper and lower sediment layers, respectively). Nitrogen was mainly found in its reduced form (2.71 ± 1.68 μM NH_3; 0.64 ± 1.59 μM NO_3^{-2}) across

the whole dataset (Table S2). No ecologically meaningful differences were observed in relation to geo-morphological parameters (Table S3).

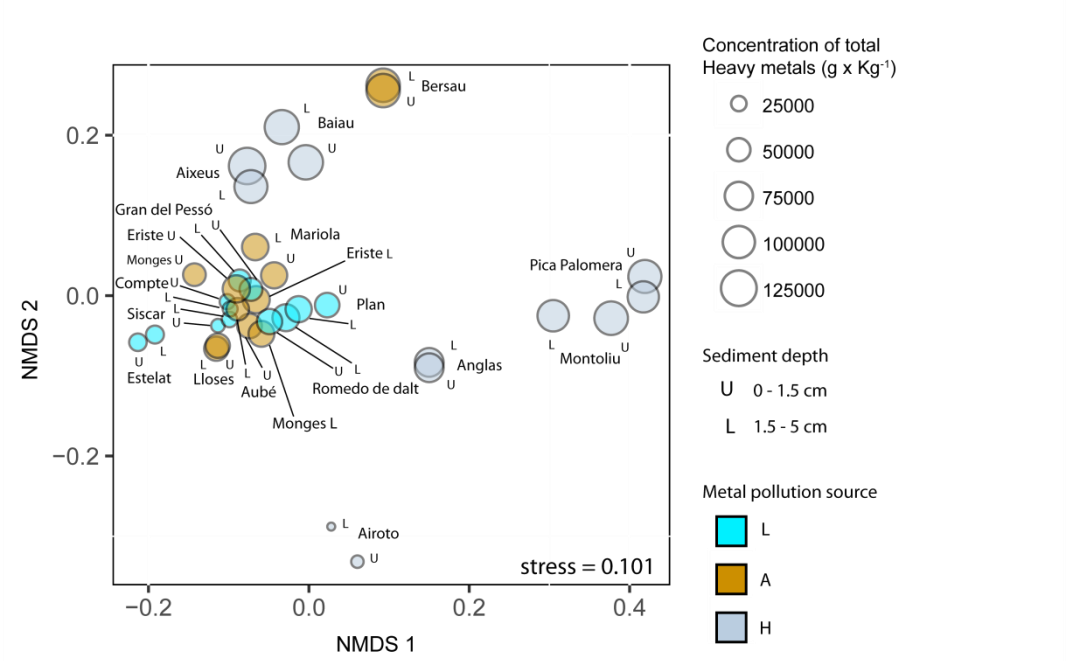

Figure 1. Non-metric Multidimensional Scaling (nMDS; Bray-Curtis dissimilarity) of the sediment samples, based on the concentrations (mg Kg^{-1} sediment) of heavy metals. Each site is represented by a circle. Circle sizes represent the concentration of heavy metals (i.e. Pb, Zn, Cd, Cu, As, Hg, Se) and colours represent the level of metal contamination: L (low contaminations), A (atmospheric depositions), H (local contamination sources). Superficial (S) and underlying (U) sediment samples are represented for each lake.

Effect of metals on archaeal diversity and abundance

No significant differences were observed in taxonomic compositional diversity (TCD, 127.7 ± 53.5 OTU's), and taxonomic structural diversity (TSD, Shannon Index; 3.02 ± 0.77) between the different levels of metal contamination (Figure 2 and Table S4). Using stepwise multiple linear regressions, we found that the main descriptors for TCD were latitude (positively related and explaining 24 % of variance) and PO_4^{3-} (positively related, explaining a 21.1 %), while the best descriptors of the variance in TSD were latitude (positively related, explaining 25.9 %) and SO_4^{2-} (negatively related, explaining 15.8 %; Figure 2).

In order to unveil a potential structuration effect of metals on archaeal communities, these communities were plotted into an ordination (nMDS) according to their

structural similarity (i.e., taking into accounting OTU abundances, Figure 3). Lakes from the same level of contamination tended to group together indicating that they shared more similar archaeal communities. Dissimilarities in the archaeal community structure were significantly driven by the level of metal contamination (p = 0.011, per-MANOVA), which explained 10.5% of the variance. The effect of metals was not driven only by local contaminations as evidenced by pairwise comparisons. Indeed, archaeal community composition from L and A lakes were different ($p < 0.05$), indicating a significant influence of atmospheric deposition. In contrast, the sampling layer had no significant effect on community composition. Nonetheless, part of the variance explained may be also due to other environmental variables correlated with the level of metal contamination. Hence, the relative importance of the sets of explanatory variables (i.e., metals, physico-chemical parameters and geomorphology) on variations in archaeal community composition was tested by RDA using Sørensen dissimilarities and Hellinger distances in order to give more weight to rare and abundant OTUs, respectively (Figure S2). The three sets of explanatory variables accounted for a similar amount of variance (approx. 39 %) in archaeal community composition for the two matrices. Among the sets of explanatory variables, metals were the main contributors to variations in archaeal community composition when abundance of OTUs were taken into account. Their contribution was also significant on the Sørensen matrix (4.7 %) but to a lower extent than the contributions of physico-chemical variables (i.e., SO_4^{2-}, NO_3^{2-}, PO_4^{3-}, NH_4, Chla, pH and temperature, 14.9 %) and geomorphology (i.e., altitude, area, depth, longitude and latitude, 8.1%). Metals also appeared to have a significant influence on archaeal community composition through their interactions with geomorphology. This is particularly true for rare OTUs for whom 7.7 % of the variance is explained by this interaction (Figure S2).

In order to determine the strongest determinant of community composition among metals, physico-chemical parameters and geomorphology were included as covariables in a pRDA (using Hellinger distances), effectively eliminating the variance associated with them (Figure 4). The remaining variance was assumed to be associated only to metals. Only the first axis of the pRDA was significant (p = 0.012) and explained 6.81% of the variance in community composition. The automated selection procedure followed by an ANOVA-like permutation test identified (in order of importance) Sn, As, Cu, Cd, Tl, Sr and Pb as the best contributors to the first axis of the pRDA. Three OTUs were the main contributors to this axis and represented more than 30 % of total archaeal sequences. These OTUs were classified as *Batyarchaeota-6* (OTU 1), *Bathyarchaeota-5b* (OTU 2) and *Thaumarchaeota* from the SAGMCG1 lineage (OTU 4, Figure 4).

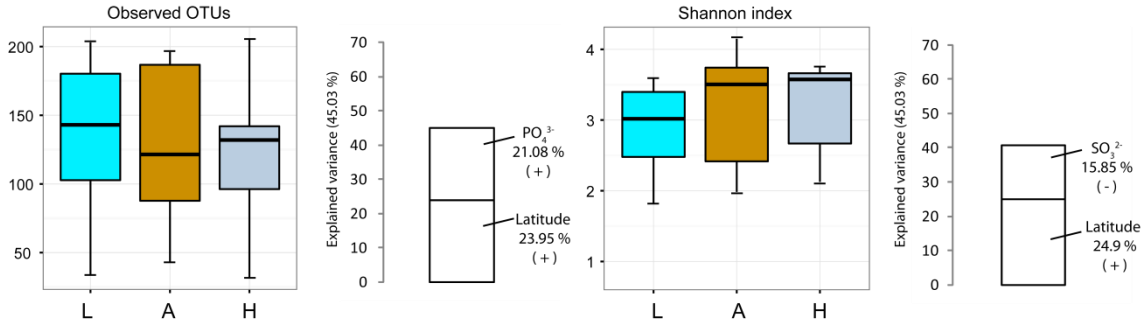

Figure 2. Boxplots for the taxonomic compositional and structural facets of alpha diversity (observed OTUs and Shannon index, respectively). Values are grouped in basis of the level of metal contamination (L, A, H). Relationship between alpha diversity indices and metal contamination levels have been tested using a Kruskal Wallis test followed by a Nemenyi post-hoc tests. The main environmental variables significantly related to the variances of both indexes in multiple linear regressions are displayed, together with the amount (%) of variance explained by each of them.

Lastly, the effect of metals on total archaeal abundances was assessed by qPCR (Table S5). A significant higher number of archaeal 16S rRNA gene copies were found in lakes with low metal content compared to lakes exposed to local or atmospheric contaminations (Figure 5). However, PO_4^{3-} and SO_4^{2-} were the main descriptors (accounting for 61.2 % of the variance) related to 16S rRNA gene copy numbers of total Archaea as evidenced in stepwise multiple linear regressions (Figure 5).

Effect of metal contaminations on methanogen abundance and activity

A comprehensive picture of the composition of archaeal communities was achieved by sequencing the 16S rRNA gene (Figure 6). The most abundant phyla were *Bathyarchaeota* (47.96 ± 21.94 %), *Euryarchaeota* (18.25 ± 11.75 %) and *Woesearchaeota* (15.2 ± 9.49 %). *Euryarchaeota* was mainly composed by members of class *Thermoplasmatales* with "historic" methanogenic lineages (i.e. *Methanomicrobia* and *Methanobacteria*) representing a minor fraction (9.64 ± 16.25 % of *Euryarchaeota* reads). The order MBG-D was dominant within the class *Thermoplasmatales* (51.58 ± 23.27 % of reads).

Archaeal communities being dominated by potentially methanogenic lineages (i.e. *Bathyarchaeota, Thermoplasmata,* MBG-D and *Methanomassiliicoccales*), we assessed the effect of metals on this functional guild by quantifying their absolute abu-

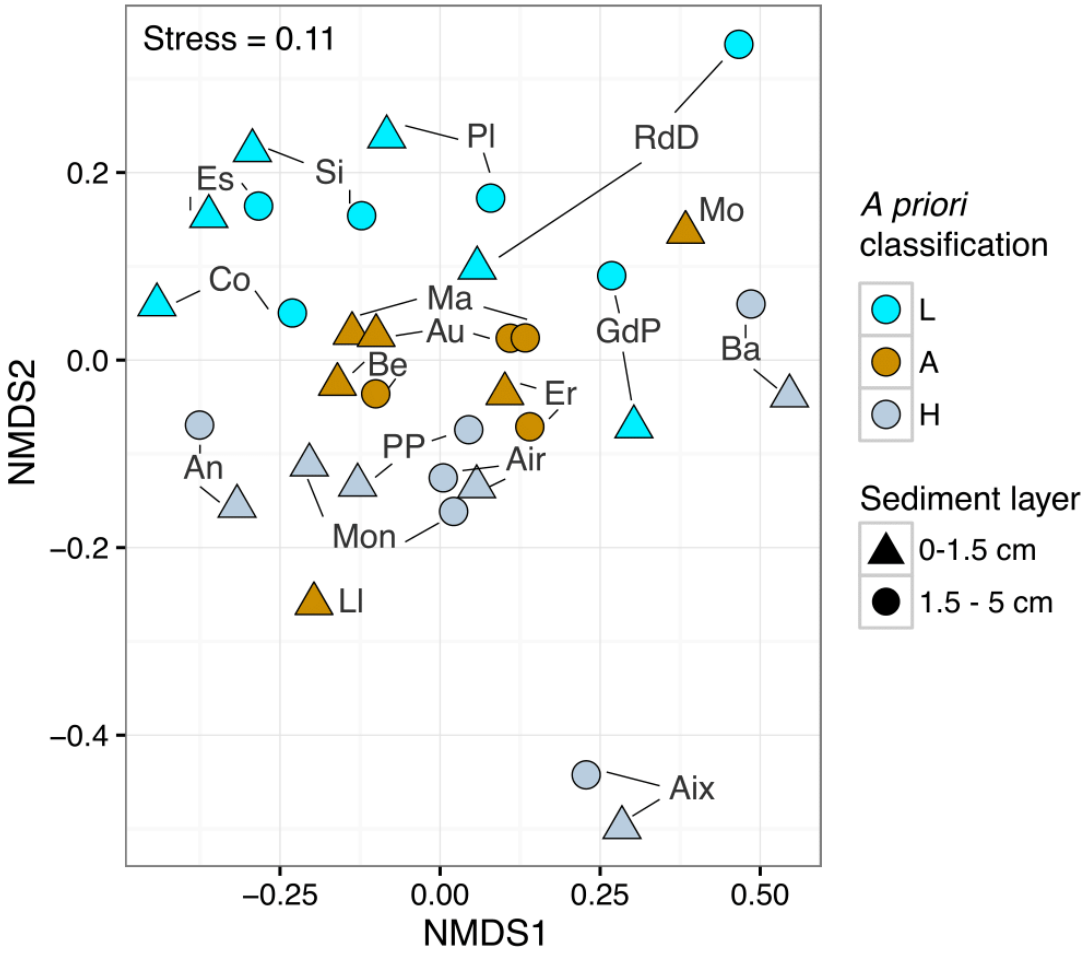

Figure 3. Non-metric MultiDimentional Scaling ordination (nMDS; Hellinger distances) of sampled sediments, based on the abundances of the different archaeal OTUs. The level of metal contamination for the different samples is color-coded. Abbreviations for lake names are as follow: Aix for Aixeus, An for Anglas, Mon for Montoliú, PP for Pica Palomera, Air for Airoto, Ba for Baiau Superior, Er for Eriste, Be for Bersau, Au for Aubé, Ma for Mariola, Mo for Monges, Ll for Llosas, Co for Compte, Es for Estelat, Si for Siscar, Pl for Plan, RdD for Romedo de Dalt, GdP for Gran del Pessó.

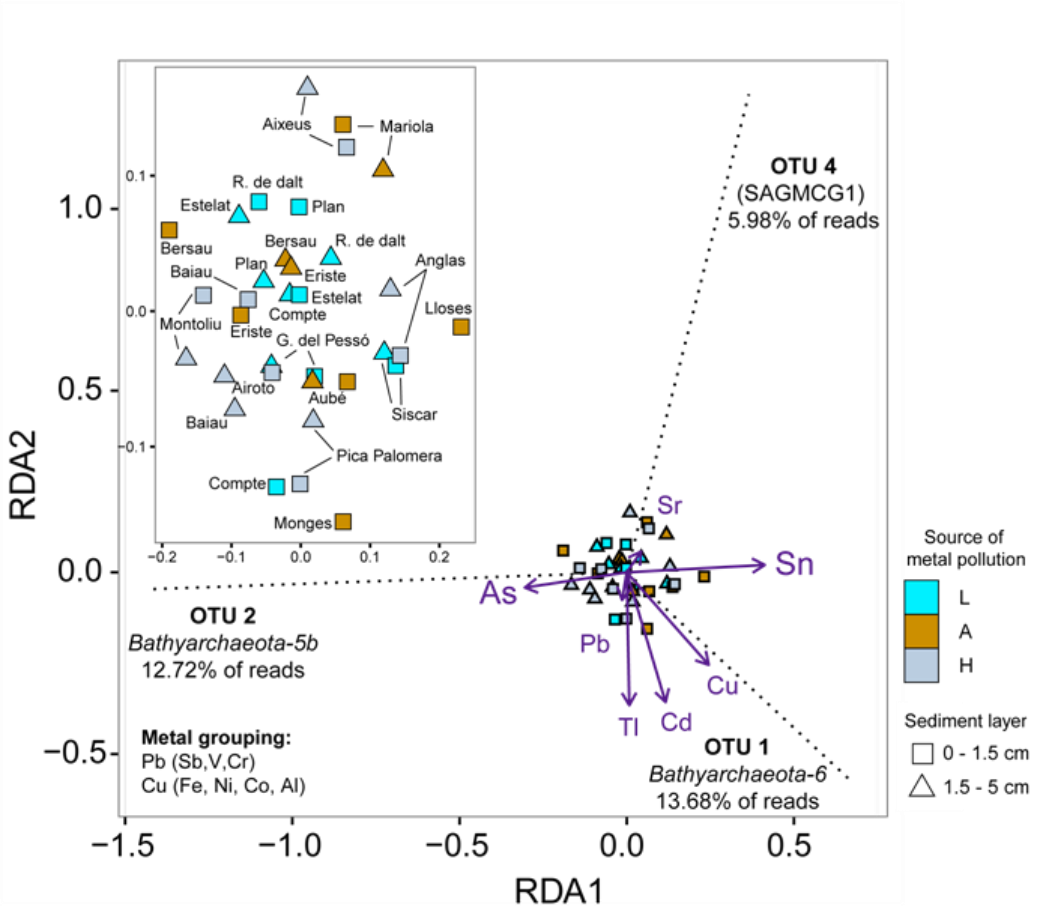

Figure 4. Ordination plot of the partial Redundancy Analysis (pRDA) ran on the OTU abundance matrix (Hellinger distances). Physico-chemical parameters and geo-morphology were included as co-variables. Arrows represent those metals that better explain the ordination according to the automated procedure of the ordistep function (vegan package in R). Dotted lines represent the main OTUs contributors to the first axis. The significance of the axes was tested with an Anova-like permutational test. Colours represent metal contamination source and shape represent the sediment layer.

ndance in qPCR assays. In addition, and as a proxy of the methanogenic activity, we also quantified methanogens 16S rDNA gene transcripts and *mcrA* gene transcripts. *Bathyarchaeota* copy numbers were at least one order of magnitude lower than those of total Archaea and presented disparate abundances across the sample set (Table S5). In agreement with NGS data, MBG-D ($1.05\times10^8 \pm 2.82\times10^8$ Copies × g sediment^{-1}) represented a larger proportion of *Thermoplasmata* ($1.4\times10^8 \pm 2.26\times10^8$ copies x g sediment^{-1}) than *Methanomassiliicoccales* ($2.15\times10^7 \pm 4.58\times10^7$ Copies × g sediment^{-1}). Spearman correlations showed a high co-occurrence of 16Sr RNA gene copies for *Bathyarchaeota* with *Thermoplasmata* (r = 0.74) and *Methanomassiliicoccales* (r = 0.88). For all methanogenic lineages, the number of 16S rRNA gene copies was significantly reduced ($p < 0.05$) in lakes exposed to local or atmospheric contaminations compared to lakes with low metal content (Figure 5). The same trend was observed for methanogenesis activity proxies with significantly more methanogens 16S rDNA and *mcrA* gene transcripts in low metal content lakes compared to locally contaminated lakes (Figure 5).

Parsimonious models obtained using stepwise multiple linear regressions, explained between 30% and 54% of the variances in absolute abundances (Figure 5). PO_4^{3-} was an important descriptor (accounting between 22.3 and 43.7 % of the variance), positively related to 16S rRNA gene copy numbers of *Bathyarchaeota, Thermoplasmata* and *Methanomassilicoccales*. SO_4^{2-} has always a negative effect on the abundance of 16S rRNA gene or methanogenesis biomarkers, capturing between 5.0 and 26.3 % of the variance. Metals also explained a significant proportion of the variance in archaeal lineage abundance and particularly for methanogen activity proxies, for which Pb was an important descriptor negatively explaining 11% of their variance (Figure 5).

Discussion

Methanogens: a dominant guild in sedimentary archaeal communities

Uncultured *Archaea* are widely distributed in the water column (Auguet *et al.,* 2008, 2013; Ortiz-Alvarez *et al.,* 2016) of alpine lakes where they play an important role in the nitrogen biogeochemical cycle (Pouliot *et al.*, 2009; Hu *et al.*, 2010; Auguet *et al.*, 2011, 2012). In contrast, the ecology of Archaea in the sediment compartment of these habitats have received much less attention (Jiang *et al.*, 2009; Liu *et al.*, 2014, 2016; Zhang *et al.*, 2015). The high rates of organic matter mineralization taking place in lake sediments (Tranvik *et al.* 2009; and references therein) leads to the conclusion that archaeal lineages could play a pivotal role in nutrient cycling. In the present work,

Bathyarchaeota and *Euryarchaeota* were the dominant sedimentary phyla representing, respectively, 48% and 18.3% of the archaeal meta-community. *Bathyarchaeota* is a core generalist phylum in both marine and freshwater sediments (Fillol *et al.*, 2016) and its members have been found abundant in a previous high mountain lake survey (Zhang *et al.*, 2015). Although the full understanding of the metabolic potential of this phylum is far from being resolved, evidences for heterotrophy based on assimilation of organic matter such as aromatic compounds or detrital proteins has been obtained (Biddle *et al.*, 2006; Lloyd *et al.*, 2013; Meng *et al.*, 2014). More recently, divergent homologs of the genes necessary for methane metabolism have also been found in two *Bathyarchaeota* genomes suggesting that members of this phylum may contribute to methane cycling in sediments (Evans *et al.*, 2015). Similarly, *mcrA* gene copies have been found in genomes from the order MBG-D (Paul *et al.*, 2012), which represented a major fraction of the phylum *Euryarchaeota* in our samples. In contrast, well-known methanogens affiliated to the phylum *Euryarchaeota* (i.e., *Methanobacteriales, Methanocellales, Methanomicrobiales* and *Methanosarcinales*), represented a small proportion of the archaeal meta-community (2.2% of all the sequences) if compared with low land meso- and eutrophic aquatic systems (65.3%, Compte-Port *et al.* 2017). However, typical methanogens (dominated by the *Methanomicrobiales*) were detected in the 18 alpine lakes under study and the absolute abundance of methanogen 16S rRNA and *mcrA* gene transcripts (10^5 to 10^9 copies g^{-1}) were in the range of abundances previously found in freshwater sediments (Freitag *et al.* 2009; Bae *et al.*, 2014; García-Maldonado *et al.*, 2014; Morris *et al.*, 2014), indicating a recurrent methanogenic activity in these sediments. Hence, similarly to meso- and eutrophic lakes, methanogenesis appears as a central process in the carbon cycle within sediments of ultra oligotrophic alpine lakes.

Metal contaminations as drivers of archaeal community composition

High mountain lakes are seen as valuable sentinel ecosystems, providing signals that reflect anthropogenic long-range chemical pollution (Williamson *et al.*, 2009). Particularly, concentrations of trace elements in European alpine lakes were found to be comparable to those reported in aquatic sediments receiving higher contamination loads (Camarero *et al.*, 2009). The high enrichment factors found indicated an atmospheric origin for these contaminations with Pb showing the highest contamination levels (Camarero *et al.*, 2009). When compared to sediment quality guidelines (SQGs), the concentrations of trace heavy metals measured in this study were above threshold levels for which biological effects may become severe in 11 out of the 18 lakes. Although SQGs are usually obtained using macro-organisms, we hypothesized a potential effect

of metals also on the composition of archaeal communities. While metals had no effect on archaeal alpha diversity, we found that they could have as much effect as typical physico-chemical or geo-morphological parameters on the structuring of archaeal communities (Figure 3 and 4). Furthermore, this effect was more pronounced on the dominant members of archaeal communities and particularly on two typical freshwater subgroups of the phylum *Bathyarchaeota* (Figure 4). Indeed, metals play a pivotal role in the microbiological functioning of aquatic niches, as their bio-available forms can be used as cofactor when present in trace amount (Jacquot *et al.*, 2014; Sorichetti *et al.*, 2016), allowing nutrient cycling and organic matter transformation. However, the inability of microbial communities to degrade them may lead to an accumulation until reaching toxic levels. Hence, the effect of high metal concentrations found in locally contaminated lakes is not original *per se*, since previous studies have linked local metal contamination (i.e., as a result of industrial activities) with variations in sediment or soil prokaryotic communities (Haller *et al.*, 2011; O'Sullivan *et al.*, 2013; Besaury *et al.*, 2014; Costa *et al.*, 2015; Yin *et al.*, 2015; Ma *et al.*, 2016; Zhang *et al.*, 2016).

The true originality of this work lies in the introduction of the effect of long-range contaminations due to atmospheric depositions and is illustrated by Pb and Cd being one of the strongest determinants of the archaeal community composition and abundance among all metals. This result is particularly compelling since freshwater sediments have suffered an increase of metal contamination worldwide, as a consequence of anthropogenic activities (Bing *et al.*, 2011; Thevenon *et al.*, 2011; Kuwae *et al.*, 2013). Even ecosystems such as alpine lakes, which are localized far from sources of metal pollution can be affected by atmospheric depositions reaching these remote areas (Camarero *et al.*, 2009; Bing *et al.*, 2016). Concretely, and due to cold condensation, alpine regions can act as convergence zones (Wegmann *et al.*, 2006) were atmospheric pollutants may be trapped. This phenomenon has been observed in the Pyrenean area (Camarero *et al.*, 2009; Bacardit *et al.,* 2010; Bacardit *et al.*, 2012) but this is the first time that the biological consequences of these contaminations are evidenced.

Influence of metals on the abundance and activity of sedimentary archaeal lineages

PO_4^{3-} and/or SO_4^{2-} were the main descriptors for all the enumerated archaeal groups, explaining between 5 and 54.3 % of the archaeal abundances (Figure 5). These results corroborate the importance of phosphorous, a key element regarding freshwater productivity (Sterner, 2008; McMahon *et al.*, 2013), with archaeal life thriving in lake sediments playing an outstanding role in its cycle (Hupfer *et al.*, 2004; McMahon *et al.,* 2013). Several studies have highlighted the importance of phosphorous on archaeal abundances. In Tibetan lakes, the abundance of sedimentary Archaea has been corre

lated with the amounts of phosphorous (Yang *et al.*, 2016). In addition, the novel phylum *Woesearchaeota* (a well-represented phylum in the studied meta-community) has been suggested to be highly responsive to phosphorous inputs, taking an active role to biomass transformation (Fan *et al.*, 2016).

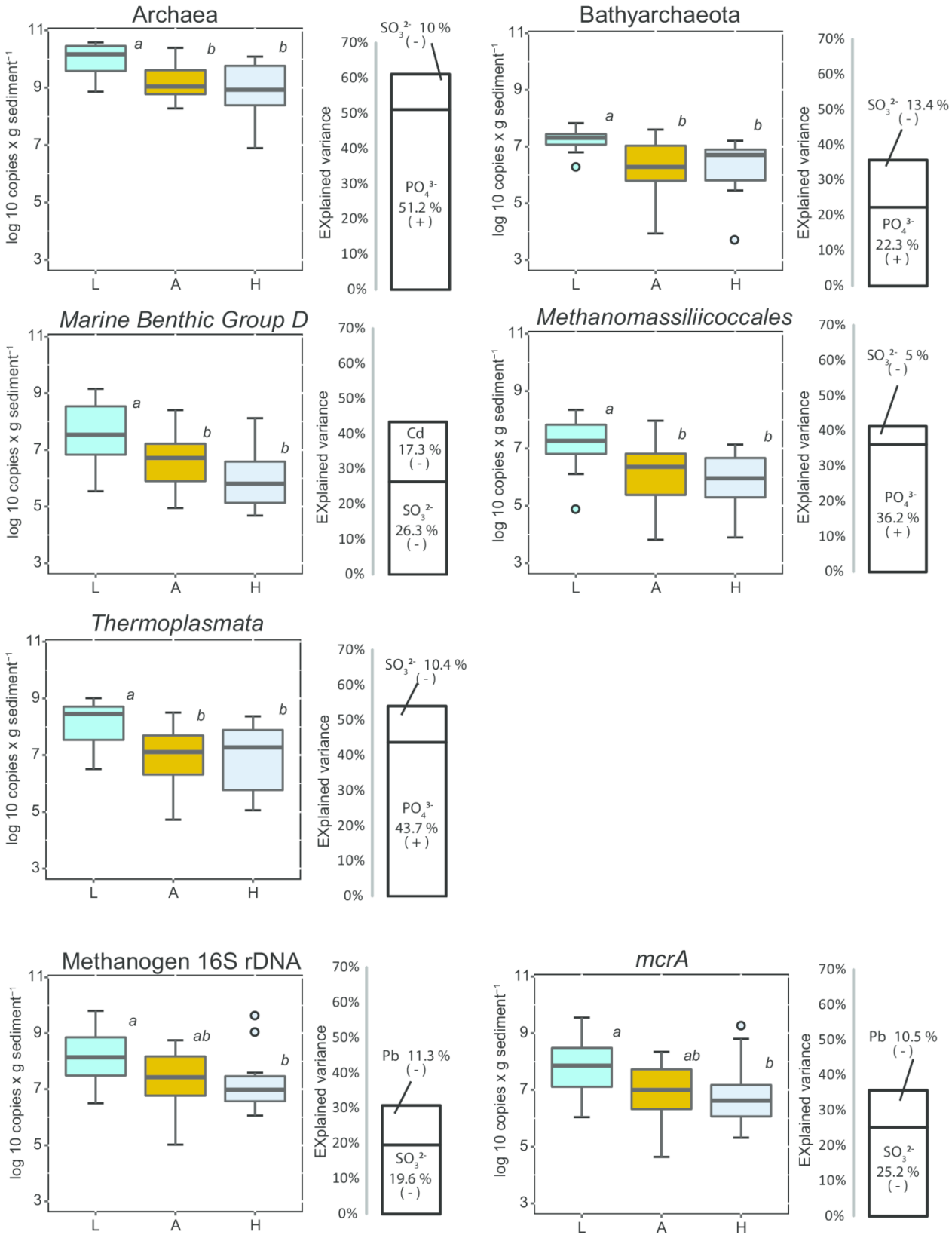

Figure 5.(Previous page) Boxplots representing the abundance of 16S rRNA and *mcrA* gene copies in relation to the level of metal contamination (L, A, H). Significant differences have been tested using a Kruskal-Wallis test followed by a Nemenyi post-hoc test. Main environmental variables significantly explaining the variances of 16S rRNA and *mcrA* gene copies in stepwise multiple regressions are displayed. The amount of variance (%) explained by each variable and the direction of the relation (either positive, + or negative -) are shown.

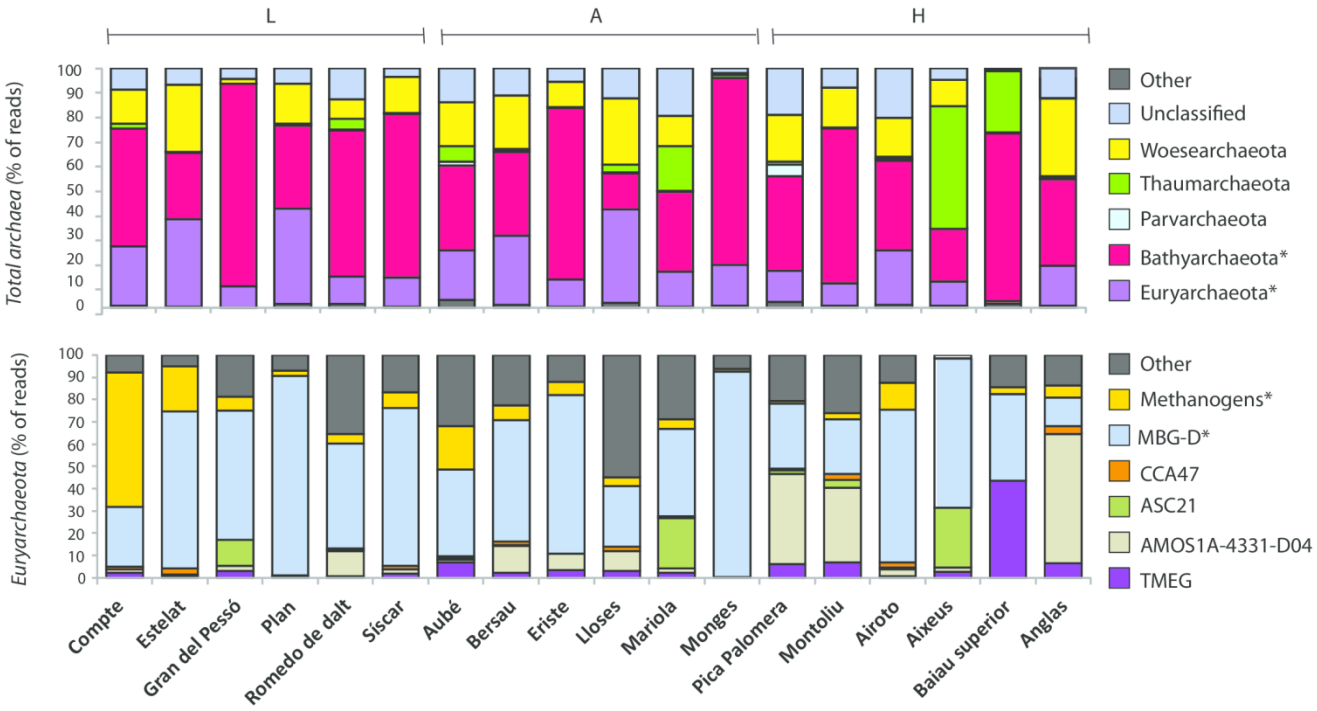

Figure 6. Composition of the whole archaeal community and diversity of the phylum *Euryarchaeota* found in each lake. Sites are arranged according to their metal contamination source, and relative abundances are represented as averages between both sediment depths (0-1.5 and 1.5-5 cm). Minor groups or unclassified OTUs have been merged and displayed as "Other". Methanogens (i.e. *Methanomicrobia* and *Methanobacteria*) and potentially methanogen lineages are indicated with an asterisk.

The negative relation between sulfate concentrations and the abundance of methanogenesis biomarkers or methanogens (*Bathyarchaeota, Thermoplasmata, Methanomassilicoccales*) confirmed a well-established pattern of vertical segregation in the distribution of methanogen within natural sediments. The presence of SO_4^{2-} in sediments allows the oxidation of organic compounds or molecular hydrogen, coupled to the reduction of sulfate to hydrogen sulfide (H_2S). As sulfate-reduction is thermodynamically favourable compared to methanogenesis (Lovley *et al.*, 1982), the sulphate-reducers are able to outcompete methanogens by the depletion of methanogenesis precursors (i.e. H_2, acetate) in sedimentary habitats (Mountfort *et al.*, 1980; Lovley *et al.,* 1983). Finally, the negative relation between sulfate concentrations and the abundance of total Archaea corroborate the fact that methanogens were a dominant guild in sediment of alpine lakes.

Although metals were not the primary drivers of the different archaeal group abundances, they explained a significant proportion of their variance (17.3 % in the case of MBG-D; Figure 5). Metal inputs (e.g. as a result of industrial activities) can have strong effects over the abundance of microbial clades (Rastogi *et al.*, 2009; Ma *et al.*, 2016; Ni *et al.*, 2016), which potentially can alter nutrient cycling (Niemeyer *et al.* 2014; Ni *et al.,* 2016). Our results were in line with previous findings (Gough *et al.,* Stahl *et al.*, 2011; Niemeyer *et al.*, 2012) and revealed a recurrent decrease of 16S rRNA copy numbers for all the enumerated microbial groups in those lakes with metal contamination (Figure 5), independently of their source (i.e. atmospheric depositions or local). Two trace metals were found to intervene significantly in the explanation of the enumerated microbial group abundances and methanogenesis biomarkers: cadmium and lead (Figure 5). In agreement with its toxic potential (Oremland *et al.*, 2003), cadmium exerted a negative effect on the abundances of MBG-D. Similarly, lead exerted a negative effect on methanogenesis biomarkers and to our knowledge, this is the first report of this trend in a natural environment. Using batch experiments, Muñoz et al. (1996) observed the deleterious effect of lead additions on methanogenesis. In addition to these laboratory experiments, two studies reported the low abundance of methanogen populations in anoxic metal-contaminated environments (Geets *et al.*, 2006; Gough *et al.*, 2011). Lead and Cadmium are mainly deposited as airborne pollution (Bacardit *et al.*, 2010). Hence, our results underlined again the critical ecological consequences of long range metal contaminations, which have reduced the abundance and activity of methanogens in impacted sediments. A huge amount of methane is produced by the process of microbial methanogenesis in lacustrine sediments (Liu *et al.*, 2017; Yang *et al.*, 2017). During this process, methanogens play a key role in the mineralization of organic matter by removing the excess hydrogen and fermentation products

generated by other forms of anaerobic respiration. Reduction in methanogenesis rates would result in hydrogen and carbon accumulation within the sediments and lower methane emissions. Owing to the ecological importance of this function in the sediment habitat, these metal contaminations may hence have system wide implications even in remote area such as alpine lakes.

Conclusions

Our findings clearly showed that the metal contaminations observed in pristine and remote alpine habitats had biological and ecological consequences. Among them, we showed a significant reduction of methanogen abundance and activity with increasing concentrations of Pb and Cd, two indicators of airborne metal contaminations. Taking into account that these metal contaminations have been observed not only in lake sediments but also in the whole catchment area and particularly in soils (Bacardit *et al.* 2012), and considering that methanogenesis is a key process in the organic matter mineralization taking place in sediments and soils, the accumulation of metals brought by atmospheric depositions in high-mountain ecosystems might have system-wide effects by interfering in the cycling of carbon.

Experimental procedures

Study sites and sampling

Sediments were collected in September 2013 from a set of 18 mountain lakes, located above 1700 meters above sea level along a West to East transect of 200 kilometers across the French and Spanish Pyrenees (Table S3). Alpine lakes were classified *a priori* on the basis of metal concentrations measured in their superficial sediment during a precedent study (Camarero, 1993). According to these concentrations, lakes were classified in three groups: lakes with low metal concentration (L), lakes with a high concentration of metals coming from the local lithology (H), and lakes with a high concentration derived from atmospheric pollution (A). H lakes lied on metamorphic and detritic rocks, which can contribute with most of the metals measured in this study, or on plutonic rocks bearing minerals that contribute with As, Cu and probably Se. Their high metal concentration is of natural origin, but in some cases (Pica Palomèra, Montoliu and Anglas) the natural contribution has been enhanced by past ore- extracting (i.e., zinc) activities within their catchments. L lakes lied on catchments on plutonic rocks releasing low metal amounts, but where the atmospheric inputs (specially Pb, Zn and Cd) were significant.

Sediment cores were collected from a plastic boat using a gravity sediment plastic corer. In order to overcome the problem of in-lake sediment heterogeneity, we sampled in triplicate at the deepest parts of each lake (i.e., where depositional conditions are similar). We acknowledge that triplicates did not represent the whole in-lake sediment variability, however, in such small mountain lakes, the variability is presumably larger among lakes than within lakes (Camarero *et al.*, 2009). Immediately after collec

tion, the sediment cores were divided in two different layers (i.e., 0-1.5 cm and 1.5-5.0 cm) using plastic tools to avoid metal contamination.

Physico-chemical analyses

For metal and metalloids analysis, approximately 20 g of the superficial (0-1.5 cm) layer and the next underlying (1.5-5.0 cm) layer were put into clean plastic bags and transported to the laboratory in portable cool boxes at 4 °C to reduce the effects of microbiological activity. Once in the lab, the sediment samples were frozen and then lyophilized in a Cryodos apparatus from Telstar. Finally, samples were sieved to assure a maximum particle size of 65 □m and kept in the refrigerator at 4 °C until analysis. The concentration of metal and metalloids in the samples was measured by inductively coupled plasma mass spectrometry (ICP-MS) after ultrasound assisted acid extraction (de Vallejuelo *et al.*, 2009). Briefly, about 0.5 g of dried sediment was transferred to an extraction vessel with 20 mL of HNO_3/HCl (45/55) (Tracepur, Meck) acid mix. Ultrasound energy was applied for 6 min. by means of a HD 2070 Sonopuls Ultrasonic Homogenizer from Bandelin, equipped with a 6 mm glass probe. The extract was filtered through a 0.45 □m filter and diluted with MilliQ water. Before analysis by ICP-MS (NexION 300, Perkin Elmer, Ontario, Canada), internal standards (Sc, Y, In, Bi, and Ge) were added to the diluted samples. Calibrant solutions and internal standards were purchased from Alfa Aesar. Blanks were processed in a similar way. All the aliquots were stored at 4 °C and analyzed within 24 h in a Class 100 clean room. The argon (99.999%) used in the ICP-MS measurements was supplied by Praxair.
For the analysis of PO_4^{3-}, Cl^-, SO_4^{2-}, NH_4^+, and NO_3^-, porewater was extracted anaerobically from 10 g of sediment. Quantification of major nutrients was performed by ion chromatography. Quantification of PO_4^{3-} and NH_4^+ was performed by spectrophotometry (690 nm) using respectively molybdenum blue after acidic hydrolyses and blue indophenols after reaction with hypochlorite and salicylate in the presence of sodium nitroprusside. The concentration of Cl^-, SO_4^{2-} and NO_3^- was measured by capillary electrophoresis according to Method 6500 of the Environmental Protection Agency (Environmental Protection Agency, 2007). Lake water samples were collected 1 m above the bottom of each lake with a Ruthner-type sampler. At the lab, two 0.5 L subsamples were filtered through GF/F glass fiber filters to collect phytoplankton for Chlorophyll-*a* determination. Photosynthetic pigments were extracted from the filters using acetone (90%, v/v) at 4 °C overnight, and the extracts were analyzed by the spectrophotometric method using Jeffrey and Humphrey's (1975) equations. Another water subsample was used to measure pH, using a CRISON 5221 electrode (suitable for low ionic strength solutions) and an Orion 720 meter. pH was measured immediately upon arri-

val to the lab, and care was taken to avoid equilibration of the sample with the ambient air during transport and measuring. Temperature profiles of the water column were measured using a PT10-type thermistor.

Values for metal and metalloids concentrations, physico-chemical variables and geo-morphological parameters are summarized in Tables S1, S2 and S3).

DNA extraction, amplification and sequencing

Sediment samples were transported within 3 h in sterile 2 mL cryotubes (ice dry cooled and in the dark) to the laboratory where they were flash-frozen in liquid nitrogen and conserved at -80 °C until further processing. DNA was extracted from 200 mg of sediment, using a commercial DNA extraction kit PowerSoil®DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA), following the manufacturer instructions. DNA was quantified by fluorescence using the Qubit dsDNA BR Assay kit (Invitrogen, Carlsbad, USA) and the Qubit 3.0 Fluorometer. Concentrations averaged 154 ng μL^{-1} (± 56, n = 36). DNA quality was assessed by spectrophotometry (Nanodrop 1000, Wilmington,USA).Values of A_{260nm} / A_{280nm} and A_{260nm} / A_{230nm} averaged 1.83 (± 0.25) and 1.54 (± 0.45), respectively. DNAs were diluted to 1.5 ng μL^{-1} for subsequent molecular analyses.

The universal primer A519F (S-D-Arch-0519-a-S-15, 5'-CAG CMG CCG CGG TAA-3') and archaeal specific primer 1017R (S-D-Arch-1041-a-A-18, 5'-GGC CAT GCA CCW CCT CTC-3') were used to amplify a ~540 bp fragment corresponding to the V4-V5-V6 region of the 16S rRNA gene (Klindworth *et al.*, 2013). On average, 87 ± 13% of the archaeal sequences from the Silva SSU r132 database and belonging to the main archaeal phyla were retrieved by this set of primers (Figure S3). The reaction mixture included 1 µL of each primer at 10 µM, 25 µL of Amplitaq Gold 360 master mix (Thermo Fisher Scientific), 10 ng of DNA template and sterilized MilliQ water to give a 50 µL final volume. PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Calif., USA) under the conditions summarized here: initial denaturation at 95°C for 10 min, followed by 30 cycles at 95°C for 30 s, 54°C for 45 s and 72°C C for 60 s, with a final extension of 72°C for 7 min. Thirty four of 36 samples were successfully amplified. The amplicons were mixed in equal amounts of DNA, and subsequently the 16S rRNA gene was sequenced on an Illumina platform using the 2 X 300 bp MiSeq chemistry. We obtained a total of 698,067 reads from the 34 samples. The complete data set was deposited in the NCBI Sequence Read Archive (SRA) database under project Accession PRJNA416782.

Sequence processing, taxonomic affiliation and phylogenetic analyses

Sequences were processed following the Mothur Illumina Standard Operating Procedure (Kozich *et al.*, 2013). Briefly, reads were demultiplexed and joined into contigs using the make.contig command and a base quality score > 25. Sequences were filtered (homopolymers < 8 bp), trimmed to discard very short sequences (<480 bp) and dereplicated to conserve only unique sequences. Up to 297 636 unique sequences with an average length of 507 bp were retained. Sequences were then aligned against the SILVA 123 database (July 2015). Chimera were detected and eliminated using UCHIME (Edgar *et al.* 2011). Taxonomic classification of each sequence was performed using the Ribosomal Database Project II Classifier (Wang *et al.*, 2007) against the SILVA 123 database. Sequences presenting more than 97% identity were clustered. Sequence clustering resulted in a contingency table of 3423 archaeal OTUs. All statistical analyses were performed on a random subsample of 1919 sequences, corresponding to the smaller number of sequences per sample in the datasets, after trimming and quality processing.

A representative sequence (i.e., the sequence with the minimum distance to the other sequences in the OTU) for each OTU was selected and a phylogenetic tree was reconstructed using FastTree 2 (Price *et al.*, 2009). The tree was rooted using a set of six bacterial 16S RNA gene sequences obtained from the SILVA 123 database (Quast *et al.*, 2013). A chronogram was then adjusted on the phylogenetic tree using the 'chronos' function (*discrete* model, 20 evolution rates) provided in the R package *ape* (Paradis *et al.*, 2004). This function provides a dated ultrametric tree using a maximum-likelihood algorithm and calibration points.

Diversity computation

The OTU contingency table, the environmental table, the taxonomy file and the phylogenetic tree were merged into a single R object using "phyloseq" package (McMurdie and Holmes, 2013).

Alpha diversity was described by 2 complementary indices, describing taxonomic compositional diversity (i.e. taxonomic richness based on presence/absence of OTUs), and taxonomic structural diversity (i.e. taking account of relative abundances of OTUs). Taxonomic compositional diversity (TCD) was assessed as the number of different OTUs in each sample (i.e., richness) while taxonomic structural diversity (TSD) was assessed using Shannon alpha diversity (Shannon, 1948). Phylogenetic richness (Faith's PD), based on the sum of branch lengths of the phylogenetic tree grouping

OTUs present in the sample, was calculated using the *Picante* R-package (Kembel *et al.*, 2010) . Phylogenetic structural diversity was assessed using Allen alpha diversity (Allen *et al.*, 2009) and calculated with the 'ChaoPD' function of *entropart* package (Marcon *et al.,* 2015). Taking into account the phylogenetic facet of diversity for alpha diversity did not change significantly the results. Hence, only results obtained for the taxonomical facet of diversity are presented in this study.

Dissimilarities between archaeal communities (i.e., the beta diversity) were assessed considering each component of diversity (compositional and structural). Compositional taxonomic (based on presence/absence OTU table) and structural taxonomic (accounting for OTU abundances) beta diversities were assessed by computation of two matrices based on Sørensen dissimilarities and Hellinger distances, respectively.

Quantification of major sediment archaeal lineages

The absolute abundance of the whole Archaea and 4 major archaeal lineages commonly found in aquatic sediments (i.e., *Bathyarchaeota, Thermoplasmata*, *Marine Benthic Group D* and *Methanomassiliicoccales)* were assessed by quantitative PCR (qPCR) using the primer sets and the PCR program summarized in Table S6. In addition, and as a proxy of the methanogenic activity, we also quantified methanogens 16S rRNA gene transcripts and *mcrA* gene transcripts. The two sets of primers used for this purpose had a good coverage for euryarchaeotal methanogens but may be less efficient for methanogens that have evolved in other phyla (Figure S3). RNA was extracted from 0.25 g of sample using the RNeasy PowerSoil total RNA kit (Qiagen; formerly the RNA PowerSoil total RNA isolation kit [MO BIO Laboratories, Carlsbad, CA]). RNA samples were tested for the presence of contaminating genomic DNA by PCR and then reverse-transcribed with random primers using the SuperScript III Reverse Transcriptase kit (Invitrogen).

DNA standards for Archaea and *Methanomassiliicoccales* were made using genomic extracts of *Sulfolobus solfataricus* (DSM 1616) and *Methanomassilicoccus lumyensis* (DSM 25720) respectively. For lineages *Bathyarchaeota, Thermoplasmata* and *Marine Benthic Group D* and methanogens, plasmids containing 16S rRNA and *mcrA* genes were used as standards. Preparation was performed as follows: PCR products from natural environments were amplified with general archaeal and *mcrA* primers, purified using QIAQuick Purification kit (QIAGEN, Manchester, UK), and cloned using the TOPO TA cloning kit for Sequencing (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Clones were picked and checked for the correct size inserts. Valid clones (i.e. those showing the correct size insert) were amplifed 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 (Applied Biosystems, Carlsbad, CA, USA) and classified by Mothur software (http://www.mothur.org, (Schloss *et al.* 2009) using the SILVA (version 123; Quast *et al.* 2013) and the Yang and coworkers *mcrA* (Yang *et al.*, 2014) databases for 16S rRNA and *mcrA* clones, respectively.

Absolute 16S rRNA and *mcrA* gene copies μL^{-1} were quantified in triplicate from DNA extracts and standard DNA dilution curves by quantitative PCR (qPCR, LightCycler 480, Roche). In each qPCR run, amplification of control water samples was done by triplicate in order to confirm the lack of exogenous DNA contaminations. DNA distribution and reactive dispensing was done in 384 well plates, using an automatic pipetting system (Echo 525, Labcyte. Inc, CA, USA), that allowed the miniaturization of the assays in a final volume of 1.5 µL (0.5 µL of DNA at 0.5 ng μL^{-1} and 1 µL of reactive mix). Reactive mix solutions were prepared mixing each primer pair (10 mM, Table S2) with ready-to-use hot start reaction mix LightCycler® 480 probes master (Roche) and molecular grade water to a final primer concentration of 0.8 mM. Inhibited samples (i.e., samples giving no PCR amplification) were purified using Wizard® DNA clean-up system (Promega), and no further inhibitions were found. After quantifications, absolute abundances (nº copies/g sediment) for all quantified genes and transcripts were calculated. Specificity of reactions was confirmed by melting curve analyses. All qPCR analyses carried out followed MIQE rules for qPCR analyses (Bustin *et al.*, 2009) and all essential information have been taken in consideration.

Statistical analyses

All analyses were conducted with the RStudio software (R version 3.2.5; R core Team 2015). Prior to sequence analyses, collinearities in the environmental variables were tested. Variables with collinearity up to 0.7 according to Spearman correlations ($p<0.05$) were grouped together, and proxies of each group were used as explanatory variables (Table S7). All explanatory variables were standardized in order to avoid scale effect in subsequent multivariate analysis and stepwise multiple regressions. We used the coefficient of variation (CV = (SD/mean) X 100) as a standardized measure of dispersion for each environmental parameter (Tables S1; S2; S3).

The similarity of samples according to metal profiles was assessed by calculating Bray Curtis dissimilarities based on heavy metal concentrations and plotted as Non-metric Multidimensional scaling (nMDS) using the function metaMDS in the vegan package (Oksanen *et al.*, 2017).

Kruskal Wallis tests were used to investigate the relationship between alpha diversity indices or gene copy abundances with the level of metal contamination. Local versus atmospheric contamination effects were separated using Nemenyi post-hoc tests. Metals and other non-collinear environmental variable contributions to variance in alpha diversity indices or gene copy abundances were investigated using stepwise multiple regressions (stepAIC function of the mass package, Venables *et al.,* 2002). The contribution of each explanatory variable to the most parsimonious models was calculated using the function calc.relimp (relaimpo package; Grömping 2006).

For beta diversity analysis, biologic dissimilarity / distance matrices were represented in an nMDS in order to get a first ordination of the samples. To assess the sources of variation in the distance matrices, we used permutational multivariate analysis of variance (PERMANOVA) based on 1,000 permutations (Mcardle *et al.*, 2014). Where applicable, pairwise differences between metal contamination levels were assessed with the function pairwise.perm.manova from the package RVAideMemoire (Hervé M., 2016). Subsequently a redundancy analysis (RDA) was used to explore the dissimilarity / distance matrices and partition their variance between the following group of non-collinear explanatory variables: metals, geomorphologic (depth, altitude, area, longitude and latitude) and physico-chemical variables (SO_4^{2-}, NO_3^-, PO_4^{3-}, Chl *a*, Cl, pH, NH_4 and temperature). Explanatory variables were obtained after a stepwise model selection using the ordistep function of the vegan package (Oksanen *et al.*, 2017). Then, the pure effects of each set of explanatory variables and each individual parameter were tested with an Anova-like permutation test for canonical analyses (anova.cca function in vegan package). Partial RDA (pRDA) was used to remove variability effects due to geomorphologic and physico-chemical variables, and the remaining variability was assumed to be due to metals (Borcard *et al.*, 1992; Peres-neto *et al.*, 2006).

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Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER 3

Response of freshwater sedimentary archaea to organic carbon amendments

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Response of freshwater sedimentary archaea to organic carbon amendments

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ABSTRACT

Recent studies using both genome-centric approaches and cultivation-based techniques have shed light on the identity, metabolic capabilities and potential role of Bathyarchaeota and Thermoplasmata in marine sediments. However, less information is available regarding their substrate preferences in freshwater habitats. Here we used laboratory-controlled microcosms inoculated with either the leave-attached biofilms or the bare sediment from a karstic lake to assess the changes in the composition and responsiveness of archaeal communities to simple aminoacids (D- and L-arginine and tryptophan), plant-derived polysaccharides (pectin) and complex aromatics (protocatechuate and humic acids). Changes in the composition of the overall archaeal communities (at phylum level) and their microdiversity (at OTU level) were assessed from both DNA and cDNA libraries after 7 days (short-term) and 30 days (long-term) of incubation using both high-throughput sequencing of the archaeal 16S rRNA gene and qPCR using specific primers. Sediment and biofilm archaeal communities exhibited a reduction in alpha diversity through time that pointed to a specialization of the community in response to carbon amendments. Besides, Bathyarchaeota, Thermoplasmata and Woesearchaeota showed non-random changes in their microdiversity in relation to carbon sources (aminoacids *vs.* polysaccharides/aromatics). In biofilms, all carbon sources but pectin caused a positive stimulation of the Bathyarchaeota and Thermoplasmata at short term (7 days) whereas only humic acids (and tryptophan in the case of Bathyarchaeota) sustained this response at long-term (30 days). In sediments, such response was only observed in microcosms amended with tryptophan after 30 days of incubation thus suggesting that archaeal communities inhabiting both niches differ in their substrate preferences. Our results agree well with the wide metabolic versatility of members of the Bathyarchaeota and the Thermoplasmata derived from recent genome-centric approaches and argue for their active role on the mineralization of organic carbon that accumulates in freshwater and marine sediments.

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6. GENERAL DISCUSSION

6.1. Methodological considerations on the study of Archaea in sedimentary habitats

6.1.1. Studying distribution. Diversity and quantitative approaches.

High-throughput sequencing (HTS) technologies largely surpassed clone libraries in sequencing depth (Caporaso *et al.,* 2012; Kozich *et al.,* 2013), time demands and cost-effectiveness (Cruaud et al., 2014), but they add a level of analytical complexity as a trade-off (Klindworth et al., 2013). Drawbacks of HTS methods such as deficient curation of raw sequence datasets to get rid of bad reads (Youssef et al., 2015), differential sampling effort across samples (Schloss et al., 2016) and pyrosequencing-associated errors (e.g. homopolymers (Kunin et al., 2010) or base-calling errors (Huse et al., 2007; Quinlan et al., 2008)) might cause strong biases in diversity estimates (Cardenas et al., 2008) and inflate OTU richness (Quince et al., 2009; Kunin et al., 2010). These methodological limitations can be partially overcome by the use of specific primer pairs (DeLong *et al.,* 1992; Klindworth et al., 2013) and adequate bioinformatic pipelines (Schloss et al., 2009; Caporaso et al., 2012). In the current study, we applied domain-specific primers for both chemistries used, that is, 454 pyrosequencing (340F-958R primer pair targeting hypervariable regions V3-V5 of the 16S rRNA archaeal gene; Ovreas *et al.,* 1997; DeLong *et al.,* 1992) and Illumina MiSeq (519F-1017R for regions V4-V6; Loman et al., et al., 2012; Teeling et al., 2012). Notwithstanding this, several pitfalls such as incomplete databases (Youssef et al., 2012; Gies et al., 2014), variable taxa names in reference taxonomies (DeSantis et al., 2006; Meng et al., 2014; Petitjean et al., 2014) or misclassification of short-reads when aligned against full-length sequences (Schloss et al., 2016) are still major concerns. As stated by Youssef and co-workers (2012), the curation of ribosomal databases does not keep up with the rapid pace of description of novel archaeal taxa. The present work does not expect to be an accurate description of the phylogeny of sedimentary Archaea, but provide useful data for subsequent analyses.

The study of relevant ecosystem processes rely on the proper quantification of microbial groups involved (Allison et al., 2013). In this regard, the semi-quantitative character of HTS techniques is of little help (Amend et al., 2010; Props et al., 2017) and complementary, quantitative approaches are needed. Quantitative PCR (qPCR) has a paramount importance in the study of microbial communities (Smith et al., 2009), and its combination with HTS technologies is of great help as far as both techniques use the same DNA extract to eliminate methodological biases (Props et al., 2017). qPCR quantifies phylogenetic or functional gene markers (Petersen et al., 2012) and has successfully been applied in environmental surveys to quantify the archaeal contribution to nutrient

cycling (Bollmann et al., 2014; Billard et al., 2015; Pelikan et al., 2016; Dziewit et al., 2017). For instance, quantification of functional genes (e.g. *amoA*, *dsrA*, *nosZ*) provides valuable indexes of activity (Petersen et al., 2012; especially in cDNA fractions) that may complement taxonomy-centred approaches and help in obtaining clues about which processes are relevant in a given environment (Xu et al., 2014). In this regard, quantitative and compositional analyses are complementary and the usage of both warrants a more accurate description of the distribution and abundance of uncultured archaea in freshwater sediments.

The application of complementary approaches is not free from difficulties especially when contrasting results become apparent (Birtel et al., 2015; Lloyd et al., 2015). In our case, the discrepancies observed between the relative abundances for Bathyarchaeota and Thermoplasmata using either HTS or qPCR (Chapters 1 and et al., 2) are of special concern. These variations might be explained both by the different number of copies of ribosomal gene operons in different archaeal genomes (Dlott et al., 2015) and by the inherent biases of PCR amplification shared by both techniques. For instance, primer-related biases prevent an accurate assessment of relative abundances (Pinto et al., 2012) and, regarding qPCR, other amplification-associated biases such as differential efficiencies (Brankatschk et al., 2012), different GC content or target sequences (Abtahi et al., 2011), annealing temperatures (Sipos et al., 2007) or primer-template mismatches (Bru et al., 2008) may aggravate error rates. Finally, the use of different qPCR primer pairs for Archaea, Bathyarchaeota and Thermoplasmata represents a cumulative source of error. Accordingly, it must be kept in mind that a direct comparison of the archaeal abundance obtained by different techniques is risky, if not erroneous.

6.1.2. Studying activity. Future approaches for function analysis.

In nature, microbial-mediated processes are usually studied through the assessment of the functional capabilities of ecologically meaningful groups (Burke et al., 2011; Xu et al., 2014). Traditionally it has been assumed that the abundance of 16S rRNA molecules in a given sample is proportional to number of ribosomes in a cell and then it might be indicative of protein synthesis and cell activity (Blazewicz et al., 2013). Accordingly, quantification of rRNA in both pure cultures and mixed communities has been regularly used to identify the active fraction of microbes and their responsiveness to changing environmental conditions (Barnard et al., 2013; Mikkonen et al., 2014; De Vrieze et al., 2016).

Despite its wide use in microbial ecology, limitations such as the high lability of RNA molecules, the presence of rRNA in dormant cells or the different rates of RNA synthesis among taxa have been identified as factors that seriously compromise the interpretation of the data (Blazewicz et al., 2013; Dlott et al., 2015; Steven et al., 2017).

Due to technical limitations, in this thesis we maintained the archaeal RNA as biomarker to identify carbon sources were able to stimulate the activity of prevalent groups (Bathyarchaeota and Thermoplasmata) in sediment and biofilm samples, thus assuming all the inconveniences. Even so, more sophisticated methods would overcome the RNA-related drawbacks, such as the measurement of metabolic by-products using radiotracers, gas- or ion-chromatography (Lamarche Gagnon et al., 2014; Xu et al., 2015; Mills et al., 2016; McKay et al., 2016), the addition of bromodesoxiuridine (BrdU; Urbach *et al.,* 1999; Artursson et al., 2003) or isotopically-labelled substrates (Stable Isotope Probing, SIP; Boschker *et al.,* 1998). Anyway, we would like to highly recommend these complementary approaches in further studies, if possible.

Particularly, SIP has been used to infer metabolic traits for Bathyarchaeota (Biddle et al., 2006; Webster et al., 2010; Seyler et al., 2014). In our group, SIP was used in previous exploratory analyses, aiming to detect ^{13}C incorporation into Bathyarchaeota membrane lipids. Unfortunately, ^{13}C incorporation was not detected because the extracts did not reach the detection limit. Hence, it would be advisable to use longer incubation times and/or higher concentrations of labelled substrates in subsequent attempts, as SIP-centred analyses are still a good option. Similarly, the application of Nanoscale Secondary Ion Mass Spectrometry (NANO-SIMS), which combines labelled probes and SIP, may help on the assessment of metabolic routes carried out by uncultured Bathyarchaeota and Thermoplasmata in sediments similar to studies focused on sedimentary bacterial communities (Li et al., 2008; Herrmann et al., 2007; Musat et

al., 2012; Carpenter et al., 2013). The identification of Cisó leaves as natural enrichments for Bathyarchaeota is very promising if considering further research studies.

Recent advances in metatranscriptomics may also be useful to unveil expression of archaeal genes from target groups in response to different nutrient amendments. In this regard, the current bias towards dominant taxa in sequence databases restrains the study of rare archaeal groups such as the Bathyarchaeota and the Thermoplasmata. Other drawbacks are related to the extraction and processing of RNA from complex samples, bad gene annotations and incomplete reference gene databases (Moran et al., 2009; Gosalbes et al., 2011; Carvalhais et al., 2012; Bikel et al., 2015). Assuming these limitations, a pre-enrichment of target lineages (e.g. in microcosms; Carvalhais et al., 2012) would be desirable to properly apply a metatranscriptomic approach to provide clues on how Bathyarchaeota and Thermoplasmata respond to environmental changes (Moran et al., 2013). As metatranscriptomic expression profiles can be normalized by units of mass or volume in environmental studies (Gilford et al., 2011) it would be interesting to use this approach to further evaluate the responsiveness of members of both lineages under "in-vitro" conditions. Conversely, NANO-SIMS studies would be more suitable to be performed in environmental samples as they have the ability to discern specific taxa.

6.1.3. Genome-based approaches to by-pass cultivation-based limitations

Despite some well-known examples, microorganisms are difficult to cultivate under controlled conditions in the laboratory even when all their metabolic requirements are known and fulfilled (Kaeberlein et al., 2002; Steward et al., 2012). Moreover, precise metabolic requirements for many environmental microbes are not even known. The inability to stimulate the growth of members of the phylum Bathyarchaeota and the class Thermoplasmata in our microcosm-scale experiment (Chapter 3) is a good example of such incapacity. Up to date, cultivation techniques recovered less than 1% of microbial diversity (Ferrari et al., 2005; Epstein et al., 2013) although novel cultivation strategies such as diffusion chambers, membrane systems or "in-situ" microbial traps (Kaeberlein et al., 2002; Ferrari et al., 2005; Gavrish et al., 2008; Epstein et al., 2013) are a step-forward in the field. These advances have not successfully been applied yet for the study of uncultured Archaea and only few papers aimed to enrich and isolate any member of the Bathyarchaeota and the Thermoplasmata have recently been published (Gagen et al., 2013; Seyler et al., 2014; Chapter 3).

In the last years, genome-centring approaches such as single cell genomics and short-read genome assembly have provided many clues on the potential metabolic traits of members of the Bathyarchaeota and the Thermoplasmata (Lloyd et al., 2013; Meng et al., 2014; He et al., 2015; Evans et al., 2015; Lin et al., 2015; Lazar et al., 2016; 2017). Despite these data, the lack of complete reference genomes limits the correct annotation of putative gene products obtained in genome-centric studies, including metagenomic and metatranscriptomic explorations (Olson et al., 2017). This problem is even more severe for members of the Thermoplasmata, which are underrepresented in reference databases and information about their potential metabolism and physiological requirements is lacking. As genomic approaches are non-targeted, the proper selection of sampling sites naturally enriched in the target group is crucial (Lazar et al., 2015). In this regard, results from Chapters 1 and et al., 2 regarding the composition of archaeal communities in freshwater sediments across the Iberian Peninsula and the Pyrenees, respectively, identified several lakes as suitable abundance hotspots of rare Thermoplasmata lineages such as the AMOS1A-4113-D04, the ASC21 (lakes Vilar and Cisó), the MBG-D (lakes Plan and Estelat) and Bathyarchaeota subgroups such as the *Bathyarchaeota-5b* in lakes Montoliu and Baiau Superior. These hotspots would be interesting starting points for future studies on rare archaea.

Despite the great potential of genome-centric approaches to provide clues about the metabolic capabilities of uncultured archaeal groups (Nichols et al., 2007; Morales et

al., 2010; Steward et al., 2012; Stefani et al., 2015), we agree with recent opinions claiming that cultivation should not be abandoned (Lagier et al., 2015). The complete understanding of the function and role of microorganisms in the ecosystem may only be obtained by the combination of gene-centric approaches and the study of isolates under well-controlled laboratory conditions (Konopka et al., 2009; Steward et al., 2012; Epstein et al., 2013).

6.2. Contribution of uncultured Archaea to the organic carbon recycling in freshwater sediments

6.2.1. Sediments as Carbon sinks. The role of uncultured Archaea in Carbon cycling

Inland waters are large sinks of organic matter (Tranvik et al., 2009; Battin et al., 2009). Conversely to the seafloor, where the large amount of sulfate fuels anaerobic sulfate respiration (Mountfort *et al.,* 1980), the low sulfate concentration in freshwater sediments (karstic lakes being the exception), the anoxic conditions and the accumulation of organic matter pave the way to methanogens (Chaudhary et al., 2013; Billard et al., 2015). In fact, archaea thriving in freshwater sediments have traditionally been related to methanogenic groups (Green et al., 2012; Lymperoloupou et al., 2012; Dong-lin et al., 2012; Conrad et al., 2014). Results from Chapters 1 and 2, demonstrate, however, that archaeal groups prevalent in freshwater sediments are not affiliated to well-known methanogenic lineages but to uncultured lineages characterized by their wide metabolic versatility regarding the use of organic carbon sources. It must be kept in mind that our results refer only to surficial sediments and no information is available on deep sediment layer. Considering freshwater waterbodies as complex systems where the quality and quantity of settled organic matter is expected to vary along sediment depth profiles, the lack of such data is a clear limitation of our work. In fact, previous studies revealed that the composition of archaeal communities greatly vary along sediment layers (Borrel et al., 2012; Lazar et al., 2015) and thus, similar variations are expected to occur in the studied systems. In order to elucidate this, more research is needed to elucidate these variations and to identify prevalent lineages at each sediment strata in relation to changes in physico-chemical conditions and quality of organic matter.

Likewise, freshwater systems receive varying amounts of organic matter of different sources (Dean 1998). Thus, the identification of links between these carbon sources and their potential use by different archaeal groups is mandatory to assess their activity on the organic carbon recycling. It is also worthy to consider that Carbon cycling is not just mediated by sedimentary microbes but also occurring in the water column (Pace et al., 2004; Bogard et al., 2014; Morana et al., 2015; Alfreider et al., 2017) where uncultured archaeal lineages are also abundant (Llirós et al., 2008; Hugoni et al., 2015; Fillol et al., 2015; Ortiz-Álvarez et al., 2016). Planktonic Archaea should be thus considered in future studies aimed to resolve the role of uncultured archaea on the global Carbon cycle.

6.2.2. Biofilms, unsuspected reservoirs of active Archaea

Like their bacterial counterparts, archaeal cells are able to grow in biofilms to cope with environmental stressors such as desiccation, pH shifts or UV light (Fröls et al., 2013; Poshlshroder et al., 2015). Archaea thriving in biofilms affiliate to various phylogenetical orders such as: *Halobacteriales*, *Methanobacteriales* and *Thermoplasmatales* (Fröls et al., 2013). Pioneering studies reporting the presence of Archaea in biofilms were related to cold salt marshes and marine sulfide chimneys (Schrenk et al., 2003; Koch et al., 2006), radioactive thermal springs (Weidler et al., 2008) and biofilms from an acidic cave and methane-rich marine sediments (Macalady et al., 2007; Briggs et al., 2011). These studies began to call attention to the fact that Archaea might probably be a common component of biofilm assemblages where they would have a key but still unexplored role in nutrient cycling (Koerdt et al., 2010; Di Meglio et al., 2014; Chimileski et al., 2014; Courdeau et al., 2012; Fröls et al., 2013; Hansel et al., 2016).

In the present work we have unveil a hitherto unknown habitat for freshwater Bathyarchaeota, which were prevalent in biofilms growing on the surface of plant debris in the anoxic, sulfide-rich sediment of a Lake Cisó (Chapter 3). In this habitat, Bathyarchaeota may be playing a key role in the degradation of the abundant recalcitrant organic compounds derived from accumulated leaf litter (e.g. aromatics). The studied biofilms are 'natural enrichments' of one specific subgroup of the phylum Bathyarchaeota (*Bathyarchaeota-6*) and thus might be regarded as priceless samples to address future experimental approaches aimed to resolve several questions that remain unanswered such as those related to substrate preferences, growth requirements and cultivability. Similarly, the abundance of biofilm-associated MBG-D and AMOS1A-4113-D04 suggests that these groups make up a not negligible amount of archaeal biomass in the studied biofilms. In fact, the role that MBG-D plays in sediments could also be assumed for populations thriving in biofilms. Intriguingly, the exclusivity of AMOS1A-4113-D04 in bulk-community with almost no presence in the active has no conclusive explanation yet. Another interesting group to mention is *Bathyarchaota-15,* being almost negligible in bulk-community while largely prevalent in the active fraction of biofilm community. In spite of being scarce, *Bathyarchaeota-15* might anyway be a "backbone" of the heterotrophic guild in leave-attached biofilms of Lake Cisó. Thus, the composition of the archaeal assemblages may comprise largely represented but dormant groups together with other ones likely to play relevant roles in carbon mineralization, despite their low abundance.

Besides, it is likely that same (or other) archaeal lineages than those studied in this work, like the epipelagic Woesearchaeota (Ortiz-Álvarez et al., 2016), may have a prominent presence in biofilm niches from other habitats (e.g. epiphytic, epilithic or epipsammic biofilms in aquatic habitats). This consideration opens a wide number of research directions to assess the role of uncultured archaea in the whole variety of biofilm-associated freshwater environments.

6.3. Great unknowns: the Bathyarchaeota and the Thermoplasmata

6.3.1. Ubiquity, phylogenetic diversity and metabolic versatility

Results presented in this thesis highlight the co-occurrence of Bathyarchaeota and Thermoplasmata in sediment habitats agreeing with previous studies that demonstrate that both lineages represent core groups in sediments worldwide (Fillol 2016). Assuming that the occupancy of a species is related to its local abundance (Brown *et al.,* 1984), the ecological success (considering occupancy and abundance) of Bathyarchaeota and Thermoplasmata could be explained in terms of their metabolic versatility (Lin et al., 2015; Lazar et al., 2016; Spang et al., 2017). Also, the latter is known to mirror the diversification through evolution (Panagiotis et al., 2017). The phylum Bathyarchaeota is known to be extremely diverse (Kubo et al., 2012; Fillol et al., 2016) and its phylogenetic complexity required the subdivision of the phylum into smaller subgroups (Teske et al., 2008; Fillol et al., 2016). As previously suggested by other authors (Seyler et al., 2014; Lazar et al., 2016; Fillol et al., 2016), diversification into such number of different subgroups must probably come along with different adaptations to disparate environmental conditions and substrate preferences. Intriguingly, almost all the Bathyarchaeota reads recovered in the dataset of 39 lakes studied here, affiliated to *Bathyarchaeota-6*, *-15* and, to a minor extent, to *Bathy-5b*. Many questions remain open regarding why these groups, and no others, prevail in lake sediments, or which are the factors that explain their dominance in euxinic freshwater sediments. It should be emphasized that the phylogeny of Bathyarchaeota has just been resolved at class- or order-level, being still far from being completely understood. It is likely that the possible system-related phylogenetic variations remain masked by the fact that the so called "Bathyarchaota subgroups" are still resolved at a low phylogenetic resolution. A more accurate description of the phylogenetic relationships among all Bathyarchaeota subgroups would allow a more complete understanding of the effects of the environmental variables over the composition of bathyarchaeotal communities. The amount of data derived from genome-centric studies are currently shedding some light over this and other questions (Evans et al., 2015). Presumably, the rapid pace of discovery will clarify these issues in the next years.

Similar to the Bathyarchaeota, members of the Thermoplasmata were also prevalent in the sampled sediments agreeing with previous studies carried out in freshwater systems (Zhang et al., 2014; Bar Or et al., 2015; Fillol et al., 2016). This is of special interest since most Thermoplasmata identified so far have also been identified in extreme environments (Reysenbach et al., 2006; Rangon et al., 2013; Lucheta et al., 2013; Cerqueira et al., 2015). Most members of class *Thermoplasmata* are chemohet-

erotrophic, being able to thrive under a wide variety of environmental conditions (Spang et al., 2017). Besides, their intragroup phylogenetic diversity is also high and similar to that for the phylum Bathyarchaeota. This fact and its widespread distribution across disparate niches hinders the complete understanding of their ecologic preferences. In fact, members of the Thermoplasmata were detected in almost all sediments studied in this work (Chapters 1 and 2).

The ubiquity of MBG-D in freshwater sediments (Chapters 1 and 2) combined with its responsiveness to a variety of organic compounds (Chapter 3) makes MBG-D an interesting group to focus in future research projects. Although primers targeting MBG-D have been designed (Vetriani *et al.,* 1999), the design of new primers and probes is very necessary, and warrants interesting discoveries. Further quantitative studies centred to MBG-D would be useful in order to elucidate the ecological functions of their members in natural sedimentary systems. Although MBG-D has been related to the degradation of proteins in saline and freshwater sedimentary niches (Lloyd et al., 2013; Lazar et al., 2016), its responsiveness to other compounds such as intermediates of lignin degradation such as protocatechuate or benzoate, humic acids or other aromatics makes it tempting to speculate that members of MBG-D may have various facultative metabolisms and other ecological roles. Hitherto, the information of this group is still very preliminary.

The identification of bioreactor- and gut-associated lineages such as TMEG and *Methanomassilicoccales* in the studied sediments is also intriguing considering their high relative abundance (especially in reservoirs and ponds). Söllinger and co-workers (2015) addressed the study of the spatial distribution for *Methanomassilicoccales* across wetlands and the gastrointestinal tract of several mammal and reptile species, also making the first attempt to resolve the phylogeny of this group. The work here presented will represent a further step in this direction. TMEG has been related to the degradation of several organic compounds (mainly fatty acids; Lin et al., 2015) but their ribosomal signatures have been reported in a wide range of different type of soils and sediments (Koyano et al., 2014; Bar Or et al., 2015; Xiang et al., 2017). The "miscellaneous" character of TMEG suggests that it has no clear phylogenetic boundaries, comprising unclassified sequences of distantly-related organisms. Hence, TMEG signatures will continue to be found in disparate niches and the repertory of its potential metabolisms is expected to keep growing.

6.3.2. Bathyarchaeota and Thermoplasmata: True lovers or random partners

The high correlations found in relative abundances and OTU richness between the Bathyarchaeota and Thermoplasmata point to some kind of metabolic linkage between them reinforcing the idea of a potential syntrophy between members of both lineages (Fillol et al., 2016).

It is well known that the division of work also occurs among microorganisms (Costa et al., 2006). In fact, this type of cooperation is crucial for global carbon cycling since it allows the degradation of complex compounds by different community members (Sieber et al., 2012). The results presented here agree with previous studies that showed that members of the Bathyarchaeota and the Thermoplasmata are able to degrade aromatic compounds and detrital proteins thus allowing the recycling of organic carbon in both marine and freshwater sediments (Lloyd et al., 2013; Meng et al., 2014; Lin et al., 2015; Lazar et al., 2016). The complexity of the metabolic networks aimed to degrade complex organic compounds is especially relevant under anoxic conditions (Jessen et al., 2017 and references therein). Under these conditions, complete degradation of recalcitrant organic matter is only accomplished through a complex network of microbial interactions (Schink et al., 2002; Morris et al., 2013; Nobu et al., 2015).

Even so, it is important to remember that coincidence does not imply causality and not all the co-occurrences observed in microbial world have to be interpreted as effective interactions. Although relevant, the observed co-distribution between members of the Bathyarchaeota and the Thermoplasmata may result from: *i)* stochastic processes (Hawkins et al., 2003); *ii)* co-varying richness (i.e. number of OTUs), which may reflect similar environmental responses (Wolters et al., 2006); and *iii)* misclassification caused by technical biases or uncomplete taxonomic databases (Faust et al., 2012).

Another limitation when defining a metabolic linkage is the phylogenetic level to which the studied groups are defined. At that point is also important to remember that both of the lineages studied here have deeply branching phylogenies. A true syntrophic interaction between Bathyarchaeota and Thermoplasmata might probably imply just some subgroups (e.g. *Bathyarchaeota-6*, *-15*, TMEG or MBG-D) not only because their high phylogenetic diversity (Kubo et al., 2012; Castelle et al., 2015; Fillol et al., 2016; Panagiotis et al., 2017; Spang et al., 2017) but also because not all subgroups within each lineage share the same metabolic lifestyles (Lloyd et al., 2013; Meng et al., 2014; Na et al., 2015; Lin et al., 2015; Evans et al., 2015; Lazar et al., 2016; Panagiotis et al., 2017). Also, the question if this potential syntrophy is obligated or just opportunistic

still remain to be solved (Sieber et al., 2012). Finally, it should be taken into account that synthrophy is just one kind of interaction among microbes and that other types (i.e. mutualism, commensalism) could not be ruled out (Faust et al., 2012; Epstein et al., 2013; Morris et al., 2013).

7. CONCLUSIONS

The main conclusions of this work are:

I. The prevalent archaeal groups in surficial sediments of the 39 studied systems affiliate to marine lineages without cultured representatives.

II. In the studied sediments, archaea accounted for a minor fraction of total prokaryotes. Within the archaeal fraction, Bathyarchaeota (subgroups *Bathyarchaeota-6, -15* and, to a minor extent, *-5b*) and Thermoplasmata (mainly family MBG-D) were prevalent.

III. The correlation found in the abundance and richness of Bathyarchaeota and Thermoplasmata suggests a trophic linkage between both groups. If true, this link would be probably related to the syntrophic degradation of recalcitrant organic matter.

IV. In the studied sediments, the abundance of Archaea, Bathyarchaeota and Thermoplasmata could be related to system properties such as the residence time, the depth or the quality of organic matter. The metal pollution (regardless of its source) has a severe negative effect on the abundance of all archaeal lineages.

V. In sediments from high-mountain lakes, the concentration of metals has a greater effect than physicochemistry and geomorphology in shaping the composition of archaeal communities. Arsenic and Tin have the more pronounced effects, especially on the abundance of Bathyarchaeota and SAGMCG1. Cadmium had an important toxic effect over MBG-D. Regarding nutrients, PO_4^{3-} and SO_3^{2-} clearly influences the abundance of total Archaea, Bathyarchaeota, Thermoplasmata and Methanomassiliicoccales.

VI. Sediments from lakes Vilar and Cisó and, to a lesser extent, Baiau Superior and Montoliu are natural enrichments for rare taxa such as ASC21, AMOS1A-4113-D04 or *Bathyarchaeota-5b*. These sediments would be valuable samples to undertake studies aimed to recover genomes from these rare lineages using genome-centric approaches.

VII. Any of the prevalent lineages in karstic lake sediments showed a differential response to different organic carbon amendments. Notwithstanding this, some groups such as the Bathyarchaeota-6, the MBG-D and the Woesearchaeota remained active along time and exhibited changes in their OTU abundances across treatments (aminoacid vs. plant-derived polysaccharides).

VIII. Under laboratory conditions, the decrease in richness of the archaeal communities suggest that they specialize along incubation time. Moreover, the sulfate limiting conditions occurring in the experimental microcosms favoured methanogenic archaea over other archaeal lineages such as the Bathyarchaeota and the Thermoplasmata.

IX. The biofilm developed on surface of detrital leaves harbours a diverse archaeal community particularly enriched in Thermoplasmata and Bathyarchaeota (i.e. Bathyarchaeota-6).

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ANNEX

SUPPLEMENTARY INFORMATION **CHAPTER 1**

Figure S1. Location of the sampled systems across the Iberian Peninsula.

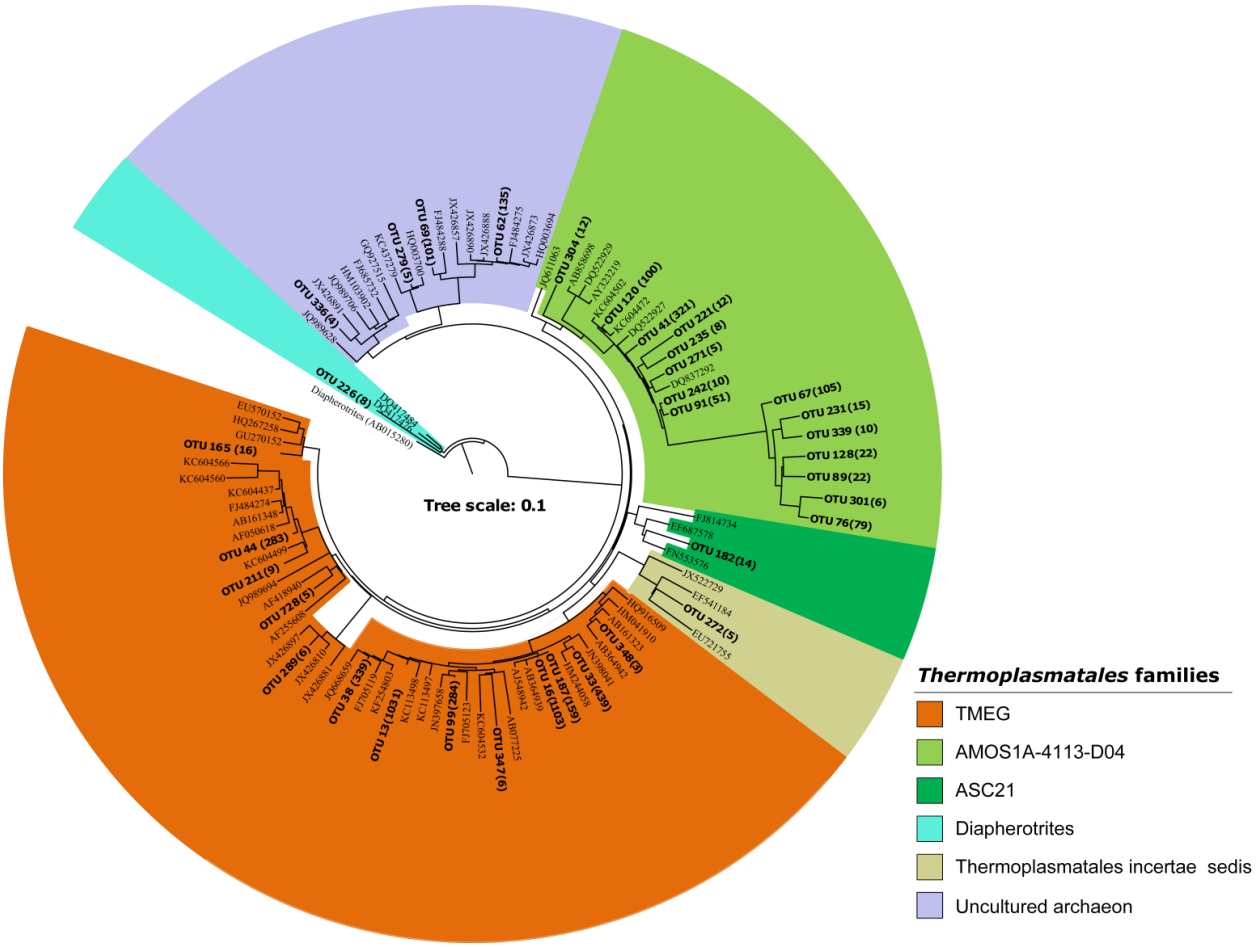

Figure S2. Maximum likelihood tree showing the affiliation of the 35 *Thermoplasmata* OTU representatives classified in QIIME as AMOS1A-4113-D04, TMEG and CCA47, values in parentheses represent the number of sequences within each OTU. The tree was constructed using the ARB tree (Silva NR SSU Reference database, release 123) as phylogenetic backbone. A random *Diapherotrites* sequence from ARB tree was used as root.

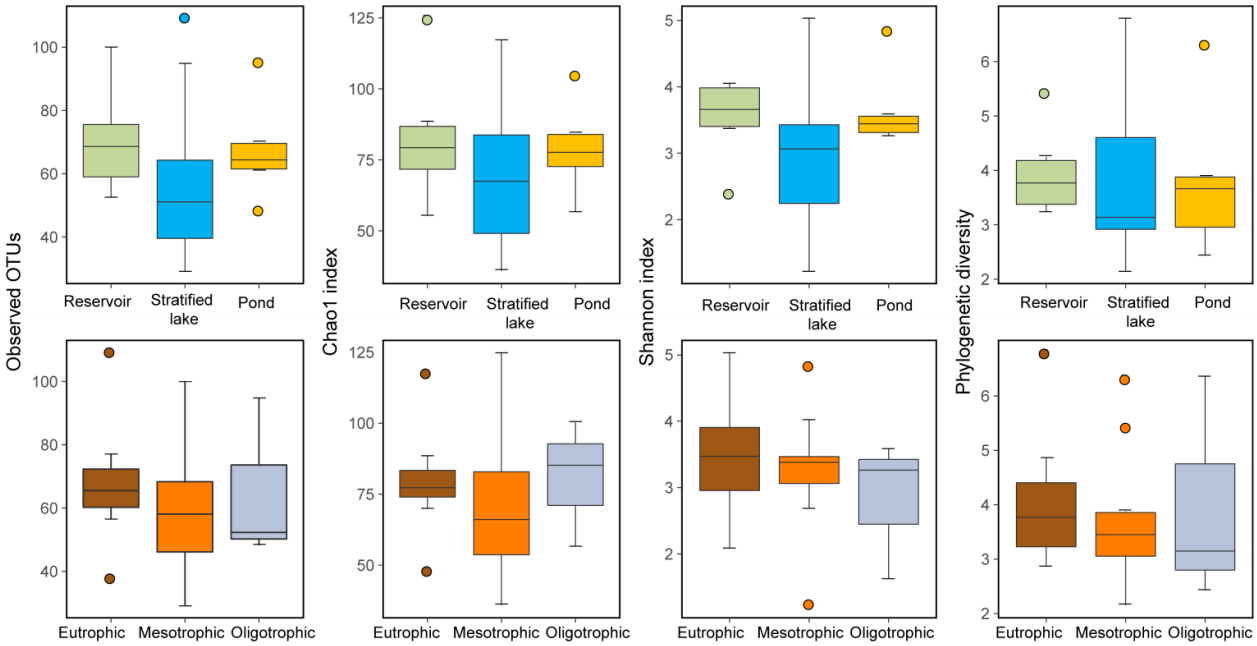

Figure S3. Boxplots representing the data distribution for observed OTUs and alpha diversity indexes (*i.e.* Chao1, Shannon and Phylogenetic diversity) of the archaeal communities retrieved in the 21 sampled sediments. The different values are shown separately on the basis of system type (upper row) and trophic status (lower row). All alpha diversity indicators were calculated after rarefying the number of reads per sample to 3,500 to normalize sequencing effort across samples. Phylogenetic diversity was calculated in QIIME according to Faith (Faith DP, 1992, Biol Conserv 61:1–10).

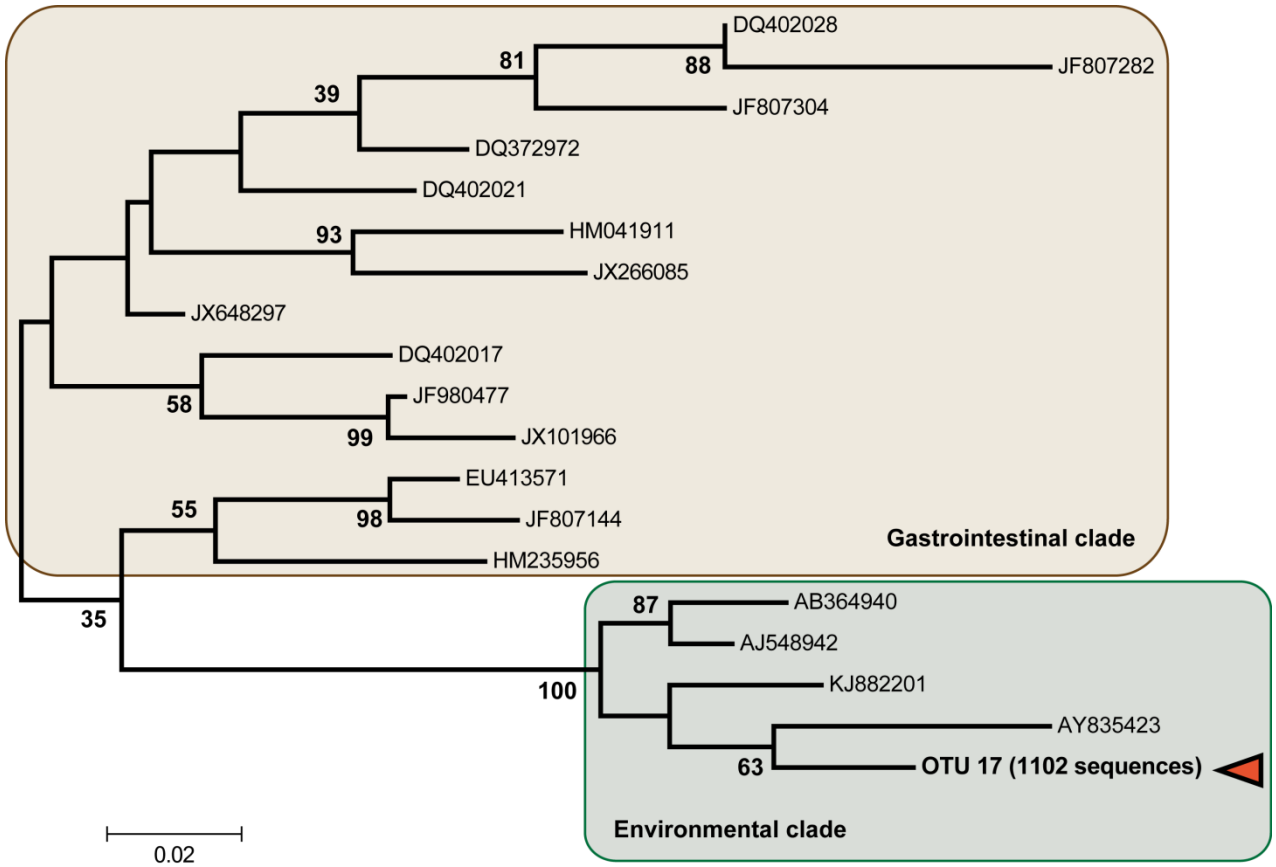

Figure S4. Maximum likelihood tree (1000 iterations; Jukes Cantor correction) showing the affiliation of OTU-17 affiliated to *Methanomassilicoccus* clustering within the environmental clade. Sequences were aligned using MEGA6 using sequences from [1] as references. Bootstrap values <35 not shown.

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Supplementary table S1. Information and location of the studied systems

System	Type	Code	Latitude	Longitude	Maximum depth (m)	Trophic status[a]	Thermo-or Chemocline depth range (m)[b]	Anoxic hypolimnion	Ref.
Sau	Reservoir	SAU	41.97761	2.519044	34.7	E	7–20 (t)	+	[1]
Santa Fe del Montseny	Reservoir	SFE	41.76854	2.469820	7.15	E	5 (t)	+	[2]
Resclosa de Serinyà	Reservoir	SRY	42.18709	2.764692	3.00	E	5 (t)		[2]
Monte de la Calzada	Reservoir	MCZ	40.04196	-5.308477	4.00	E	n.a.		[3]
Boadella-Darnius	Reservoir	BOA	42.34319	2.831382	35.0	M	7–23 (t)	+	[2]
Molí de Sant Roc	Reservoir	MSR	42.17444	2.472953	3.50	M	1 (t)		[2]
Bárcena (Antuzanos)	Lake	BAR	43.00057	-3.506999	12.0	E	n.a.	n.a.	[3]
Vilar	Karstic Lake	VIL	42.11858	2.747590	9.15	E	4.5–5 (b)	+	[4]
Cisó	Karstic Lake	CSO	42.12736	2.752375	7.30	E	1.5–2 (b)	+	[5]
Arreo	Karstic Lake	ARR	42.77798	-2.990984	24.8	M	4–6 (t)	+	[6]
Negra de Urbión	Lake	NEG	41.99936	-2.847425	8.00	M	n.a.	n.a.	[7]
Butzuberri	Lake	BUB	42.80409	-1.879080	18.0	M	n.a.	+	[8]
Zóñar	Karstic Lake	ZON	37.48391	-4.688715	13.5	M	4–6 (t)	+	[9]
Sanabria	Lake	SNB	42.12267	-6.716680	48.8	M	8–10 (t)	–	[3]
Enol	Lake	ENL	43.27245	-4.990925	22.0	O	9–10 (t)	+	[10]
Montcortès	Karstic Lake	MNC	42.33034	0.995035	30.0	O	5–10 (t)	+	[11]
Homo Morto	Pond	HOM	43.20365	-7.670416	0.45	E	–	–	[8]
Sopetón	Pond	SOP	36.95848	-6.449510	0.02	M	–	–	[8]
Dulce	Pond	DUL	37.05304	-4.835213	0.06	M	–	–	[8]
Medina	Pond	MED	36.61646	-6.050712	3.00	M	–	–	[12]
Grande de Albuera	Pond	ALB	38.68504	-6.745948	1.00	O	–	–	[13]

[a] Trophic status: (E) Eutrophic; (M) Mesotrophic; (O) Oligotrophic. [b]: (t): thermocline; (c): chemocline; (b) both; n.a. not available.

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Supplementary table S2. Quantitative PCR used primers and conditions. In all cases denaturalization stages of 95°C for 20 seconds and annealing steps of 60 seconds were performed. Efficiencies, R^2, and slopes of the standard curves are displayed as intervals.

Target lineage	Primer	Sequence (5' - 3')	Reference	Annealing temperature	Efficiencies (%)
Archaea	806F 915R	CACAGCGTTTACACCTAG GTGCTCCCCCGCCAATTCCT	Takai *et al*., 2000 Stahl *et al*., 1991	64 °C	83.25 - 85.2
Bathyarchaeota	242dF 678R	TDACCGGTDCGGGCCGTG AGAACGCGCCCGACGGTG	Fillol *et al*., 2014	68 °C	92.35 -101
Thermoplasmata	Thrm-f Thrm-r	GGTAAGACGGGTGGC GTATCTAATCCCGTTTGC	Compte-Port *et al*., 2017	60 °C	88.3 -89
Marine Benthic Group D	345F 490R	ATATCTGAGACACGATATCRGG CACCACTTGAGCTGCAGGTA	Vetriani *et al*,. 2014		93.15 - 96
Methanomassiliicoccales	AS1 AS2	CAGCAGTCGCGAAAACTTC AACAACTTCTCTCCGGCAC	Mihjalovsky *et al*., 2010	58 °C	89.35 - 92.5
Methanogen 16S rRNA	Met 630F Met 803R	GGATTAGATACCCSGGTAGT GTTGARTCCAATTAAACCGCA	Hook *et al*., 2009	60 °C	91 - 99
mcrA	ME3MF ME3r'	ATGTCNGGTGGHGTMGGSTTYAC TCATBGCRTAGTTDGGRTAGT	Nunoura *et al*., 2008 Hales *et al*., 1996	54 °C	84 - 97

References to Supplementary Table S2

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Supplementary table S3. Elemental measurement along our 21 sediments sample set. Carbon, Nitrogen, Phosphorus and Sulfur are displayed as dry weight percentage. Elemental ratios for C:N and C:P are also shown.

	% in sediment dry weight					
System	**TC**	**TN**	**TP**	**TS**	**C : N**	**C : P**
SAU	7.545	0.520	0.074	0.399	14.52	101.6
SFE	0.778	0.846	0.072	0.508	0.920	11.76
SRY	7.455	0.407	0.085	0.221	18.34	87.53
MCZ	4.345	0.431	0.368	0.569	10.08	11.79
BOA	4.855	0.301	0.045	0.168	16.16	109.0
MSR	5.925	0.302	0.055	0.075	19.62	108.6
BAR	12.75	0.580	0.717	0.755	21.98	17.77
VIL	5.765	0.275	0.056	0.139	20.96	103.5
CSO	7.675	0.279	0.047	0.208	27.51	165.0
ARR	16.15	1.055	0.701	0.937	15.31	23.02
NEG	16.55	2.065	1.337	2.940	8.015	12.38
BUB	11.20	0.789	0.723	0.705	14.20	15.50
ZON	11.85	0.966	0.985	0.917	12.27	12.03
SNB	18.70	2.065	1.289	3.033	9.056	14.51
ENL	8.680	0.830	0.572	0.466	10.46	15.17
MNC	19.05	1.925	1.000	1.612	9.896	19.04
HOM	11.88	1.054	0.600	1.038	11.26	19.78
SOP	8.595	0.595	0.450	0.499	14.45	19.12
DUL	2.210	0.288	0.140	0.322	7.674	15.76
MED	10.40	0.784	0.736	0.812	13.27	14.13
ALB	4.065	0.368	0.389	0.588	11.06	10.45

Supplementary table S4. Absolute abundances for the 16S rRNA gene (copy number × g sediment^{-1}) for the different studied groups (i.e. Bacteria, Archaea, Bathyarchaeota and Thermoplasmata). Quantification was done by quantitative PCR (qPCR).

System	16S rRNA gene copies × g sediment^{-1}			
	Bacteria	Archaea	Bathyarchaeota	Thermoplasmata
SAU	4.27×10^{10}	8.81×10^{9}	1.30×10^{7}	2.48×10^{7}
SFE	4.47×10^{8}	1.07×10^{8}	4.85×10^{6}	2.13×10^{6}
SRY	5.76×10^{9}	2.61×10^{9}	1.57×10^{7}	2.09×10^{7}
MCZ	3.87×10^{10}	1.89×10^{9}	1.20×10^{8}	4.45×10^{4}
BOA	3.71×10^{9}	5.85×10^{8}	3.43×10^{7}	6.43×10^{7}
MSR	9.50×10^{9}	2.35×10^{9}	2.19×10^{7}	3.35×10^{7}
BAR	2.34×10^{11}	5.02×10^{9}	5.16×10^{8}	8.19×10^{6}
VIL	3.08×10^{10}	8.35×10^{9}	1.83×10^{8}	1.07×10^{8}
CSO	1.71×10^{10}	4.03×10^{9}	1.54×10^{8}	9.90×10^{7}
ARR	1.66×10^{11}	6.85×10^{9}	8.50×10^{7}	9.05×10^{6}
NEG	5.59×10^{10}	5.44×10^{9}	1.15×10^{8}	4.24×10^{6}
BUB	6.65×10^{10}	1.05×10^{9}	5.02×10^{7}	7.58×10^{5}
ZON	2.14×10^{10}	1.74×10^{9}	2.53×10^{7}	2.59×10^{5}
SNB	1.24×10^{11}	9.90×10^{9}	3.29×10^{8}	1.36×10^{7}
ENL	1.58×10^{11}	9.72×10^{9}	1.39×10^{9}	1.54×10^{7}
MNC	7.84×10^{10}	7.95×10^{8}	1.77×10^{8}	5.75×10^{6}
HOM	1.64×10^{11}	3.72×10^{7}	2.23×10^{8}	4.25×10^{6}
SOP	4.54×10^{11}	3.80×10^{10}	1.76×10^{9}	1.45×10^{8}
DUL	5.87×10^{10}	9.35×10^{9}	3.65×10^{8}	1.82×10^{7}
MED	1.44×10^{11}	1.55×10^{10}	2.12×10^{9}	7.08×10^{7}
ALB	1.89×10^{11}	1.70×10^{10}	2.20×10^{9}	4.20×10^{7}

Supplementary table S5. In silico coverage (SILVA RefNR SSU 128) of the newly designed primers over the class *Thermoplasmata* class and over the most represented families within the order *Thermoplasmatales*.

Nº of mismatches	*Thermoplasmatales* families (in silico % coverage)					Total *Thermoplasmata*
	TMEG	ASC21	MBG-D	AMOS1A-4113-D04	CCA47	
0	86.2	80	12	1.6	0.8	24.4
1	97.1	86.3	66.1	39.3	18	59
2	97.1	90	94.5	85.2	74	75.8

Supplementary table S6. Distribution of pyrosequencing quality reads across sampled sediments. All alpha diversity indicators were calculated after rarifying the number of reads per sample to 3,500 to normalize sequencing effort across samples and avoid biases.

Total reads: 18,4570
Total OTUs: 314

System	Number of reads	Observed OTUs	Chao1	Shannon index	Phylogenetic Diversity[1]
SAU	6795	67	81.67	2.4	4.2
SFE	12057	57	69.94	3.4	3.6
SRY	3569	71	76.93	4.1	3.3
MCZ	10348	77	88.54	3.9	4
BOA	18467	100	124.7	3.5	5.4
MSR	3859	53	55.53	4	3.2
BAR	7225	38	47.78	2.1	2.9
VIL	3708	109	117.1	5	6.8
CSO	10748	65	75.36	3.1	4.9
ARR	11905	64	79.53	3.5	3.8
NEG	11097	45	53.03	2.7	3
BUB	6720	50	59.56	3	3.1
ZON	10635	33	35.91	3.3	2.2
SNB	9545	29	44.66	1.2	2.2
ENL	12098	52	85.17	1.6	3.1
MNC	6279	95	100.3	3.6	6.4
HOM	7816	62	77.67	3.6	3
SOP	7236	64	72.65	3.3	3.7
DUL	10487	70	83.98	3.4	3.9
MED	3635	95	104.6	4.8	6.3
ALB	10341	48	56.86	3.3	2.4

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SUPPLEMENTARY INFORMATION **CHAPTER 2**

Supplementary Figures

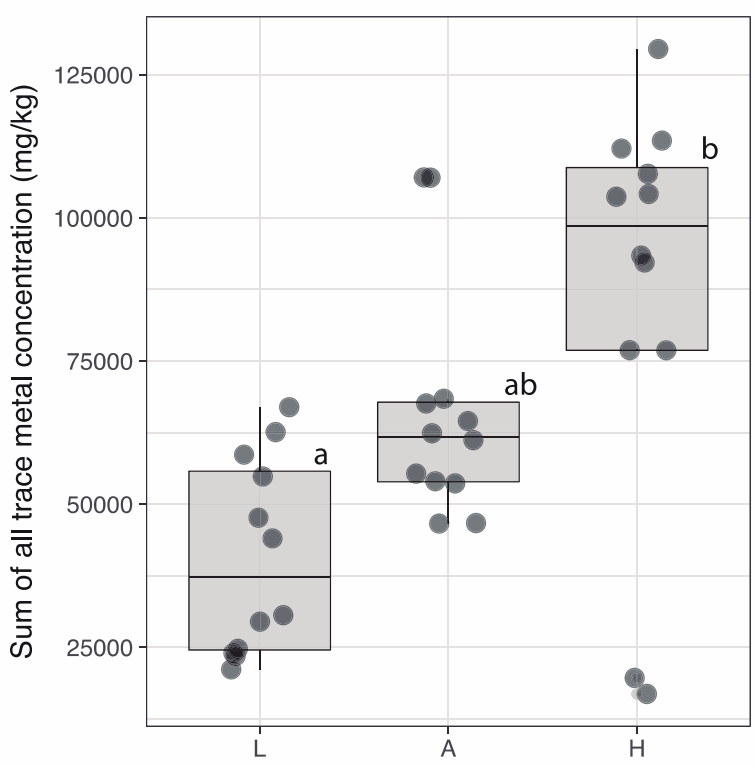

Figure S1. Boxplot representing the sum of all trace metal concentrations in the three contamination level categories (i.e., low levels, L; atmospheric depositions, A; local sources of pollution, H). Circles represent the metal concentrations in individual lake layers. Significant differences have been tested using a Kruskal-Wallis test followed by a Nemenyi post-hoc test.

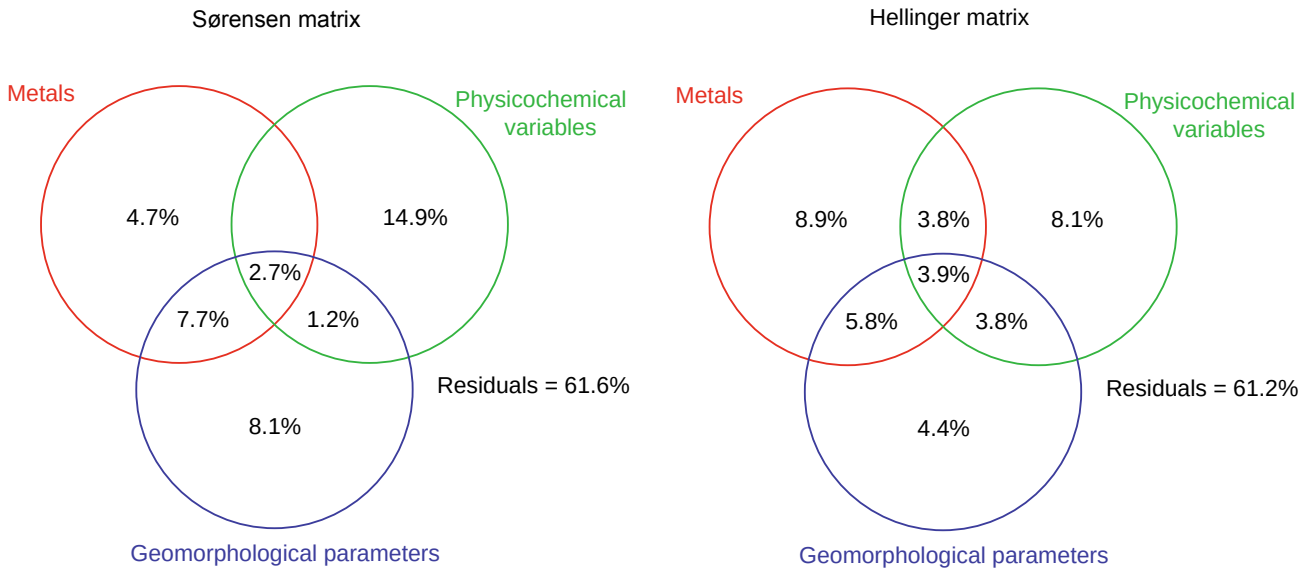

Figure S2: Venn diagrams showing variance partition (%) of beta diversity matrices (Sørensen dissimilarities and Hellinger distances) among the environmental data sets (metals, physico-chemical parameters and geo-morphologic traits).

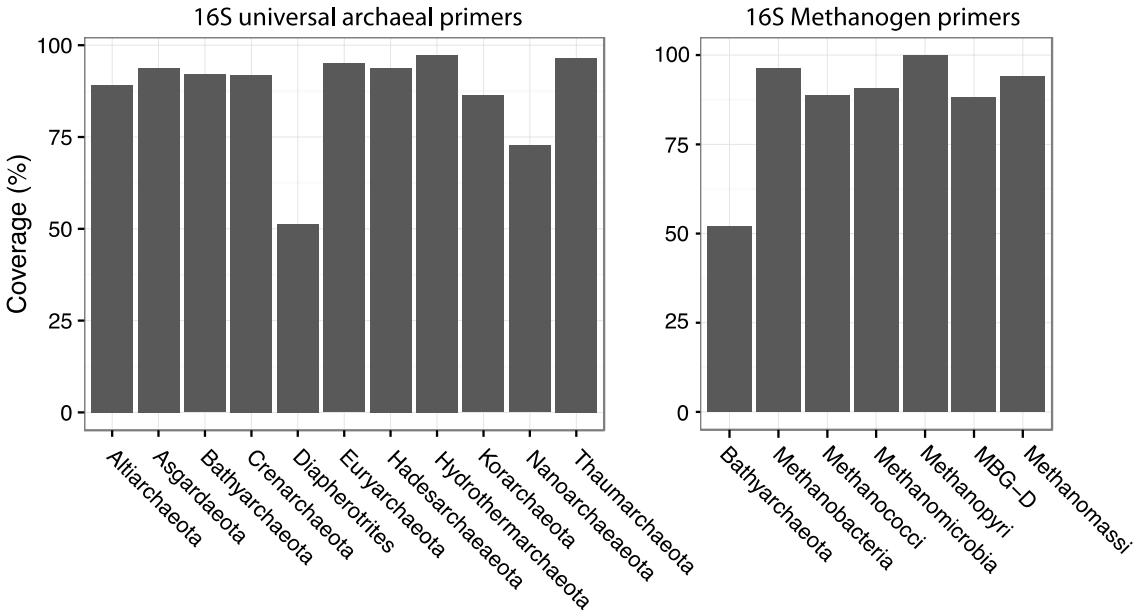

Figure S3. Proportion of archaeal sequences (i.e., coverage) matching the primer sets A519F – 1017R and Met630F – Met803R, used respectively for bacording of the whole archaeal community and quantification of methanogen activity. The results were obtained using Testprime (www.arb-silva.de/browser/ssu-132/silva-ref-nr/testprime/) on the Silva SSU r132 database and considering one mismatch. Methanomassi stands for the *Methanomassiliccocales* lineage.

Supplementary table S1. Metal abundances of the studied systems. Samples are sorted in basis of sediment depth and metal pollution source (low levels, L; atmospheric depositions, A; local sources of pollution, H).

System			mg x Kg^{-1}							
			Mg	**Al**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**
Compte	0 - 1.5 cm	L	2005.13	10544.32	365.42	11.65	12.19	106.66	7940.79	2.39
Estelat			3602.35	13074.56	1071.27	32.10	12.38	143.68	11276.49	3.67
Gran del Pessó			5129.24	20318.49	581.87	39.98	19.98	197.81	17419.12	7.57
Plan			4218.63	20847.27	413.07	37.45	23.58	157.45	28572.14	6.69
Romedo de Dalt			4524.39	31628.77	511.52	41.35	28.61	143.90	29566.24	8.28
Síscar			2316.08	12037.08	333.31	14.04	12.90	79.90	9052.28	2.86
Compte	1.5 - 5 cm		2412.24	12478.21	426.22	13.88	12.93	97.01	9080.60	2.59
Estelat			3655.76	13725.78	1091.17	35.01	13.06	142.85	11685.71	4.07
Gran del Pessó			5146.84	22546.90	638.82	46.45	21.96	197.40	18628.43	9.32
Plan			4753.68	26741.99	583.57	41.17	27.87	151.08	29681.81	8.16
Romedo de Dalt			4228.18	31380.95	509.47	36.02	26.01	129.41	21877.33	7.41
Síscar			2296.53	12508.19	362.20	14.07	12.44	73.75	8072.20	2.86
Aubé	0 - 1.5 cm	A	5814.23	30138.84	830.34	35.07	25.54	151.63	17792.22	5.54
Bersau			4332.93	36029.25	260.46	24.71	20.65	125.53	65503.78	15.99
Eriste			6302.89	25334.33	643.65	43.22	33.57	258.50	34474.48	9.38
Lloses			6337.23	22510.55	1056.73	35.28	24.17	331.08	22893.13	7.74
Mariola			6748.54	28742.83	273.83	38.83	33.00	231.71	25714.08	12.03
Monges			6228.08	34073.30	645.37	44.58	33.19	161.10	19643.04	8.12
Aubé	1.5 - 5 cm		5446.59	23687.19	631.50	27.82	19.59	155.60	16191.72	5.39
Bersau			4332.93	36029.25	260.46	24.71	20.65	125.53	65503.78	15.99
Eriste			6582.70	26764.52	703.61	43.31	34.78	266.79	33520.24	9.37
Lloses			6530.77	21819.80	1058.19	34.37	24.11	342.70	23697.79	8.07
Mariola			7070.23	30186.07	283.97	35.61	33.97	221.66	26113.79	12.44
Monges			4586.95	27168.72	462.84	32.60	24.45	113.69	14013.74	5.49
Airoto	0 - 1.5 cm	H	1724.44	8256.50	117.74	13.04	12.13	111.62	7657.64	3.30
Aixeus			6232.21	29569.02	404.70	21.17	20.36	195.75	92711.21	7.38
Anglas			7237.28	23143.13	726.03	41.68	36.92	462.58	39755.94	15.31
Baiau Superior			4324.00	41717.08	350.94	29.05	22.74	118.07	66362.75	18.71
Montoliu			3094.63	18701.70	86.26	56.43	24.78	141.74	80170.56	7.12
Pica Palomera			2447.08	22014.57	65.38	72.69	25.83	71.29	75805.38	2.42
Airoto	1.5 - 5 cm		1514.90	8203.27	119.71	10.41	10.86	76.79	5815.18	2.12
Aixeus			8295.15	31353.17	559.79	24.79	24.35	271.09	62803.41	10.46
Anglas			7237.28	23143.13	726.03	41.68	36.92	462.58	39755.94	15.31
Baiau Superior			4669.22	40057.52	344.12	28.21	23.22	131.31	66214.44	35.86
Montoliu			2771.93	32041.92	119.10	77.12	32.21	108.79	53882.49	7.31
Pica Palomera			2318.21	24093.98	63.53	70.01	26.72	68.07	60750.79	2.89
Coefficient of variation			39.6	36.4	59.4	45.6	32.8	56.2	71.9	74.7
Biological severe effect threshold *			-	-	-	-	-	-	-	-

Supplementary table S1. Continued.

System			mg x Kg^{-1} Ni	Cu	Zn	As	Se	Sr	Mo	Ag	Cd
Compte	0 - 1.5 cm	L	5.90	6.19	77.31	7.06	0.99	7.02	0.24	0.06	1.01
Estelat			5.95	11.69	63.54	8.70	0.46	23.34	0.08	0.23	0.23
Gran del Pessó			9.50	14.40	90.01	85.61	0.87	12.22	1.94	0.08	1.06
Plan			16.51	29.76	198.86	60.84	0.73	15.82	2.45	25.51	1.21
Romedo de Dalt			14.60	26.76	127.35	41.25	1.91	17.06	1.74	0.32	1.21
Síscar			8.85	8.21	48.12	5.90	0.59	7.52	0.31	0.12	0.53
Compte	1.5 - 5 cm		5.63	7.17	85.31	7.25	1.18	7.90	0.29	0.24	1.12
Estelat			6.22	12.19	65.55	8.09	0.56	17.70	0.25	0.09	0.32
Gran del Pessó			10.15	13.10	86.86	88.20	0.97	12.50	2.25	0.09	0.86
Plan			12.37	18.40	250.84	54.13	1.52	20.07	3.42	0.57	1.73
Romedo de Dalt			12.96	19.36	106.43	19.28	1.61	16.53	1.27	0.20	0.99
Síscar			7.18	8.03	55.24	4.37	0.69	5.83	0.39	0.05	0.62
Aubé	0 - 1.5 cm	A	10.96	16.17	271.90	14.38	1.65	14.64	0.89	0.27	1.66
Bersau			31.60	190.25	157.20	241.73	24.20	18.45	3.97	0.76	0.14
Eriste			15.01	19.74	105.43	181.18	0.99	9.42	2.42	0.18	0.64
Lloses			10.99	15.08	165.18	59.92	0.59	11.02	3.76	0.13	1.78
Mariola			27.87	46.14	188.44	8.78	1.68	11.47	0.58	2.49	1.01
Monges			14.48	24.64	138.31	6.22	2.03	16.43	0.87	0.51	1.03
Aubé	1.5 - 5 cm		8.55	13.16	251.10	11.02	1.08	10.38	0.66	0.17	0.97
Bersau			31.60	190.25	157.20	241.73	24.20	18.45	3.97	0.76	0.14
Eriste			15.32	18.91	110.86	179.30	1.02	9.94	2.41	0.20	0.55
Lloses			10.43	15.60	173.51	54.73	0.62	10.77	3.38	0.17	1.53
Mariola			30.12	51.33	193.13	7.87	1.31	10.91	0.55	0.24	0.78
Monges			12.07	14.12	111.46	4.50	0.81	9.57	0.29	5.90	0.37
Airoto	0 - 1.5 cm	H	5.45	42.52	148.91	1468.09	1.51	5.13	0.50	0.48	1.50
Aixeus			26.98	73.26	109.87	31.45	0.68	9.11	0.82	0.35	0.46
Anglas			34.39	21.91	4693.25	382.14	5.18	30.54	0.95	0.15	23.47
Baiau Superior			44.00	156.62	182.54	71.41	1.21	5.13	0.86	1.13	0.38
Montoliu			22.69	88.88	4319.93	79.06	2.31	14.74	17.67	0.48	22.28
Pica Palomera			10.19	252.55	2821.44	52.68	4.80	39.23	26.31	1.50	10.42
Airoto	1.5 - 5 cm		4.25	8.89	93.61	909.69	0.96	4.02	0.23	0.29	1.28
Aixeus			31.10	75.73	98.02	20.14	0.93	22.03	1.39	0.40	0.29
Anglas			34.39	21.91	4693.25	382.14	5.18	30.54	0.95	0.15	23.47
Baiau Superior			68.87	144.45	217.35	46.89	0.66	3.86	0.58	0.13	0.89
Montoliu			18.95	64.62	3535.35	82.41	1.89	42.39	16.22	0.39	16.79
Pica Palomera			9.17	211.66	4066.97	49.31	4.42	49.04	24.61	1.50	13.09
Coefficient of variation			75.9	123.3	190.4	205.5	191.0	67.9	183.0	333.3	183.5
Biological severe effect threshold			-	110	820	33	2.5	-	-	-	10

Supplementary table S1. Continued.

System			mg x Kg-1 Sn	Sb	Ba	W	Hg	Tl	Pb
Compte	0 - 1.5 cm	L	2.28	0.12	41.42	0.30	0.05	0.23	25.09
Estelat			3.53	0.20	125.25	0.45	0.05	0.13	26.31
Gran del Pessó			1.74	0.42	60.16	0.23	0.05	0.26	55.47
Plan			3.44	1.13	68.88	0.51	0.38	0.28	111.04
Romedo de Dalt			8.16	1.96	103.89	0.86	0.29	0.37	188.89
Síscar			2.79	0.18	38.73	0.05	0.05	0.13	38.92
Compte	1.5 - 5 cm		2.58	0.19	41.17	0.22	0.05	0.17	31.20
Estelat			3.82	0.18	128.43	0.30	0.05	0.17	33.06
Gran del Pessó			2.11	0.80	71.38	0.34	0.11	0.29	82.88
Plan			4.25	2.25	80.94	0.40	0.35	0.49	155.51
Romedo de Dalt			6.31	2.24	109.83	0.89	0.20	0.35	143.57
Síscar			2.59	0.26	37.77	0.05	0.05	0.16	38.93
Aubé	0 - 1.5 cm	A	9.21	1.22	81.31	0.27	0.19	0.38	135.30
Bersau			0.53	0.37	36.95	0.63	0.32	0.20	27.84
Eriste			2.86	0.85	80.89	2.60	0.12	0.40	100.27
Lloses			6.30	0.73	98.02	1.99	0.05	0.45	105.83
Mariola			5.92	1.58	99.95	0.34	0.12	0.27	198.88
Monges			3.02	1.55	98.09	1.15	0.57	1.24	63.97
Aubé	1.5 - 5 cm		4.50	0.77	57.05	0.27	0.05	0.27	88.44
Bersau			0.53	0.37	36.95	0.63	0.32	0.20	27.84
Eriste			2.95	0.80	85.08	2.15	0.05	0.40	93.55
Lloses			6.06	0.88	92.17	1.92	0.05	0.44	107.45
Mariola			3.28	1.07	97.35	0.10	0.05	0.25	148.19
Monges			2.37	0.40	92.40	0.17	0.05	0.34	51.66
Airoto	0 - 1.5 cm	H	4.35	0.87	17.60	4.96	0.28	0.22	74.69
Aixeus			1.29	0.55	46.55	0.04	0.05	0.05	40.44
Anglas			3.13	1.21	60.98	0.36	1.52	0.15	209.36
Baiau Superior			2.14	1.21	61.77	0.25	0.12	0.15	61.72
Montoliu			1.70	9.86	30.89	0.12	0.27	0.24	764.10
Pica Palomera			1.85	8.07	31.51	0.10	0.60	0.37	395.28
Airoto	1.5 - 5 cm		2.00	0.81	14.25	3.74	0.12	0.04	59.83
Aixeus			1.58	0.63	62.44	0.02	0.12	0.07	47.41
Anglas			3.13	1.21	60.98	0.36	1.52	0.15	209.36
Baiau Superior			1.41	0.81	60.84	0.38	0.05	0.24	71.94
Montoliu			1.87	9.51	55.45	0.12	0.20	0.48	556.64
Pica Palomera			1.79	6.39	36.53	0.06	0.63	0.39	326.43
Coefficient of variation			61.4	147.3	44.5	145.2	139.5	70.2	114.3
Biological severe effect threshold *			-	-	-	-	2	-	250

* From OMOE, 1992; Delvalls *et al.*, 1998.

References to Supplementary Table S1

Delvalls, T.A. and Chapman, P.M. (1998) Site-specific quality values for the gulf of Cádiz (Spain) and San Francisco Bay (USA). using the sediment quality triad and multivariate analysis. *Ciencias Mar* **24**: 313–336.

OMOE, O.M. of E. (1992) Guidelines for the protection and management for aquatic sediment quality.

Supplementary table S2. Physico-chemical variables of the studied systems. Samples are sorted in basis of sediment depth and metal pollution source (low levels, L; atmospheric depositions, A; local sources of pollution, H).

			µM		µM			µg L-1	-	ºC
System			**Cl**	**SO_4^{2-}**	**NO_3^-**	**PO_4^{3-}**	**NH_4**	**Chl *a***	**pH**	**Temp**
Compte	0 - 1.5 cm	L	10.30	13.00	0.05	5.82	2.98	7.53	7.55	7.90
Estelat			3.38	6.74	0.05	19.90	6.20	6.20	7.27	14.00
Gran del Pessó			2.90	1.19	9.07	0.06	2.34	0.77	6.49	4.00
Plan			1.41	1.44	0.05	0.50	1.94	1.18	6.71	13.70
Romedo de Dalt			1.32	1.51	0.05	0.06	0.99	0.56	5.91	4.00
Síscar			2.81	10.43	0.05	3.94	3.45	5.80	7.02	11.50
Compte	1.5 - 5 cm		1.90	3.35	0.05	3.97	4.81	7.53	7.55	7.90
Estelat			0.73	1.91	0.05	3.45	4.57	6.20	7.27	14.00
Gran del Pessó			1.76	1.03	1.83	0.06	2.09	0.77	6.49	4.00
Plan			0.68	0.98	0.29	0.06	2.19	1.18	6.71	13.70
Romedo de Dalt			1.18	0.88	0.05	0.19	0.84	0.56	5.91	4.00
Síscar			2.57	3.62	0.05	2.33	2.85	5.80	7.02	11.50
Aubé	0 - 1.5 cm	A	1.14	2.41	0.05	0.06	2.16	1.42	5.79	4.00
Bersau			1.82	3.31	0.05	0.40	1.75	0.57	6.62	4.00
Eriste			8.57	3.13	0.05	0.20	3.92	1.11	6.73	4.80
Lloses			1.11	1.23	1.50	0.06	1.93	2.57	6.39	4.00
Mariola			4.94	1.57	0.05	0.06	2.02	0.45	6.32	4.00
Monges			4.39	3.14	0.05	0.06	1.42	1.12	5.92	4.00
Aubé	1.5 - 5 cm		0.79	0.85	0.05	0.08	2.28	1.42	5.79	4.00
Bersau			1.31	0.69	0.05	5.82	2.46	0.57	6.62	4.00
Eriste			1.06	0.69	0.32	0.20	7.17	1.11	6.73	4.80
Lloses			2.97	0.87	0.37	0.07	3.58	2.57	6.39	4.00
Mariola			6.33	1.07	2.15	0.06	1.62	0.45	6.32	4.00
Monges			1.21	2.11	0.05	0.06	1.66	1.12	5.92	4.00
Airoto	0 - 1.5 cm	H	0.70	0.63	2.38	1.13	3.26	1.49	6.46	4.00
Aixeus			0.95	35.80	1.99	0.06	0.01	0.46	4.88	11.10
Anglas			1.50	3.74	0.05	0.29	1.97	0.16	7.41	9.70
Baiau Superior			6.11	56.10	0.51	0.06	1.68	0.20	4.91	11.60
Montoliu			7.31	3.15	0.14	0.06	1.09	3.79	7.07	7.80
Pica Palomera			1.00	5.06	0.05	0.06	2.73	2.14	4.45	14.40
Airoto	1.5 - 5 cm		0.73	0.39	0.89	0.25	5.10	1.49	6.46	4.00
Aixeus			1.65	7.27	0.05	0.19	0.36	0.46	4.88	11.10
Anglas			0.94	1.02	0.05	0.21	6.18	0.16	7.41	9.70
Baiau Superior			10.71	29.60	0.40	0.06	2.50	0.20	4.91	11.60
Montoliu			1.00	0.70	0.33	0.06	1.70	3.79	7.07	7.80
Pica Palomera			1.16	0.52	0.05	0.06	3.73	2.14	4.45	14.40
Coefficient of variation			99.1	195.0	246.4	257.0	60.6	106.3	13.8	52.3

Supplementary table S3. Geomorphological traits of the studied systems. Samples are sorted in basis of sediment depth and metal pollution source (low levels, L; atmospheric depositions, A; local sources of pollution, H).

		m		ha	UTM	
System		**Depth**	**Altitude**	**Area**	**Long**	**Lat**
Compte	L	4.00	1726	3.40	1.79193	42.632956
Estelat		4.00	2002	4.40	2.2122	42.645229
Gran del Pessó		35.00	2452	9.00	0.913754	42.511748
Plan		8.50	2190	11.00	0.929246	42.621328
Romedo de Dalt		33.00	2132	11.00	1.323295	42.704862
Síscar		4.00	2189	4.50	1.744766	42.601083
Aubé	A	46.00	2094	8.30	1.336427	42.744144
Bersau		33.00	2083	12.00	-0.49623	42.839252
Eriste		20.00	2410	3.80	0.46679	42.645198
Lloses		29.00	2475	3.90	0.653343	42.616465
Mariola		43.00	2273	17.50	1.222701	42.715741
Monges		45.00	2422	15.00	0.87513	42.621802
Airoto	H	40.00	2210	16.50	1.038272	42.701945
Aixeus		14.50	2370	3.40	1.370609	42.609983
Anglas		8.00	2068	2.70	-0.324976	42.931361
Baiau Superior		22.00	2480	7.90	1.430583	42.594986
Montoliu		14.00	2363	10.70	0.92401	42.783465
Pica Palomera		9.00	2308	4.90	0.867233	42.792503
Coefficient of variation		65.7	8.8	56.8	65.3	0.2

Supplementary table S4. Estimators for the taxonomic compositional (number of OTUs) and structural (Shannon Index) and for the phylogenetic compositional (Faith's PD) and structural (Allen index) alpha diversities. Samples are arranged by sediment depth and levels of metal pollution.

System	Depth	Level of metal pollution	Observed OTUs	Shannon index	Faith's PD	Allen index
Gran del pessó	0 - 1.5 cm	L	66	2.6	7.3	2.2
Plan			175	3.4	13.3	3.1
Romedo de dalt			148	3.6	12.1	2.8
Síscar			126	2.0	11.0	1.7
Compte			115	2.4	10.4	2.5
Estelat			186	3.4	13.9	3.1
Bersau		A	180	3.5	13.2	3.5
Eriste			87	2.2	8.7	2.2
Lloses			90	3.5	8.9	3.9
Monges			43	2.0	5.7	2.0
Mariola			189	3.8	13.6	3.2
Aubé			194	4.2	14.0	3.7
Pica Palomera		H	170	3.7	13.0	2.6
Montoliu			151	2.9	12.2	2.3
Airoto			135	3.7	11.5	3.2
Aixeus			32	0.8	4.7	1.3
Baiau			40	2.3	5.4	1.4
Anglas			129	3.6	11.1	2.9
Gran del pessó	1.5 - 5 cm	L	66	1.8	7.8	1.4
Plan			138	3.4	11.5	3.2
R de dalt			34	2.5	5.0	1.9
Síscar			181	2.9	13.6	2.6
Compte			180	3.2	13.5	2.8
Estelat			203	3.3	14.6	3.4
Bersau		A	196	3.8	14.1	3.7
Eriste			79	2.1	8.2	2.0
Mariola			118	3.1	10.4	2.4
Aubé			125	3.6	11.0	3.2
Pica Palomera		H	139	3.7	11.5	2.7
Montoliu			114	2.8	10.4	2.1
Airoto			129	3.6	10.9	3.3
Aixeus			137	3.7	11.1	3.0
Baiau			43	2.1	5.7	1.3
Anglas			206	3.6	14.6	2.4

Supplementary table S5. Number of 16S rRNA gene copies (normalized by g of sediment) for the 6 quantified groups. Samples are ordered in basis of the sediment depth (cm) and metal pollution source (low levels, L; atmospheric depositions, A; local sources of pollution, H).

System			Archaea	Bathy.	*Thermo.*	MBG-D	*Methanoma.*	*mcrA* transcrits	Meth. 16S rRNA
Compte	0 - 1.5 cm	L	1.71E+10	1.25E+07	2.86E+08	3.46E+05	5.75E+07	3.33E+06	9.46E+06
Estelat			3.26E+10	9.75E+06	3.41E+08	1.45E+07	2.42E+07	4.83E+07	1.02E+08
Gran del Pessó			2.50E+09	1.91E+07	3.52E+07	4.90E+07	1.27E+07	2.71E+08	7.06E+08
Plan			1.54E+10	2.20E+07	5.24E+08	8.37E+08	6.47E+06	1.09E+06	3.15E+06
Romedo de Dalt			1.69E+09	1.60E+07	1.88E+07	2.84E+07	6.32E+06	1.07E+08	1.90E+08
Síscar			1.39E+10	3.29E+07	2.79E+08	2.72E+06	1.70E+07	8.01E+06	1.47E+07
Compte	1.5 - 5 cm		3.32E+10	3.24E+07	5.06E+08	8.28E+06	1.06E+08	1.90E+07	4.69E+07
Estelat			3.81E+10	2.60E+07	1.02E+09	1.44E+09	2.21E+08	2.34E+09	6.32E+09
Gran del Pessó			4.41E+09	2.15E+07	3.28E+07	4.16E+07	2.01E+07	1.87E+08	4.50E+08
Plan			5.78E+09	2.12E+06	7.71E+07	3.09E+08	7.88E+04	3.58E+09	6.18E+09
Romedo de Dalt			7.19E+08	6.31E+06	3.22E+06	3.61E+06	1.27E+06	1.51E+07	3.99E+07
Síscar			2.71E+10	6.74E+07	6.45E+08	4.72E+08	1.25E+08	4.18E+08	7.37E+08
Aubé	0 - 1.5 cm	A	9.72E+08	8.49E+03	5.27E+04	2.55E+05	6.50E+03	4.33E+04	1.06E+05
Bersau			2.12E+10	2.46E+06	1.29E+08	1.44E+07	2.41E+06	1.36E+07	3.65E+07
Eriste			1.25E+09	3.30E+06	5.89E+06	1.12E+07	4.11E+06	8.03E+07	3.55E+08
Lloses			6.28E+09	6.23E+05	4.88E+07	8.91E+04	2.15E+06	8.35E+05	4.12E+06
Mariola			4.63E+08	5.35E+05	1.02E+07	4.32E+05	4.34E+05	2.15E+06	3.08E+06
Monges			9.49E+08	1.48E+06	2.37E+06	2.40E+07	9.11E+03	4.63E+07	1.07E+08
Aubé	1.5 - 5 cm		6.51E+08	5.91E+05	1.40E+06	2.20E+06	4.09E+04	7.28E+06	1.51E+07
Bersau			2.44E+10	1.50E+07	3.16E+08	2.52E+08	2.19E+07	1.97E+08	2.91E+08
Eriste			1.90E+08	6.94E+05	4.48E+05	2.41E+06	5.77E+05	7.00E+06	1.97E+07
Lloses			3.49E+09	1.48E+07	5.13E+07	2.61E+07	9.20E+07	2.22E+08	5.61E+08
Mariola			1.55E+09	9.70E+06	1.61E+07	1.22E+07	6.10E+06	3.23E+07	1.18E+08
Monges			3.75E+08	3.96E+07	2.48E+07	9.72E+05	8.37E+06	2.02E+06	6.68E+06
Airoto	0 - 1.5 cm	H	7.33E+09	1.02E+07	8.77E+07	1.31E+08	1.03E+07	6.36E+08	1.11E+09
Aixeus			3.72E+08	5.67E+03	2.08E+05	2.28E+05	7.87E+03	1.82E+07	2.64E+07
Anglas			1.22E+10	1.61E+07	2.33E+08	1.46E+05	1.37E+07	8.09E+05	2.77E+06
Baiau Superior			1.03E+08	3.88E+05	1.14E+05	4.77E+04	5.39E+05	3.74E+05	1.15E+06
Montoliu			2.69E+08	1.64E+06	8.28E+05	3.82E+05	1.76E+05	1.39E+07	3.89E+07
Pica Palomera			1.81E+09	4.71E+06	7.40E+07	1.07E+06	2.05E+05	4.17E+06	9.92E+06
Airoto	1.5 - 5 cm		5.35E+09	5.96E+06	6.20E+07	6.96E+07	6.25E+06	1.84E+09	3.89E+09
xeus			1.81E+08	2.82E+05	6.85E+06	5.36E+06	1.55E+04	1.33E+06	3.75E+06
Anglas			8.09E+09	9.89E+06	1.61E+08	1.08E+05	1.28E+06	1.53E+06	3.64E+06
Baiau Superior			7.87E+06	7.48E+05	1.70E+05	1.08E+05	9.92E+05	2.06E+05	9.24E+06
Montoliu			5.28E+08	5.47E+06	3.90E+06	3.45E+06	8.32E+05	4.26E+06	5.31E+06
Pica Palomera			1.35E+09	7.27E+06	5.13E+07	3.00E+06	4.17E+06	5.80E+06	2.03E+07

Suppl Table S6. Primers and conditions for quantitative PCR (qPCR) quantifications.

Target lineage	Primer	Sequence (5' - 3')	Reference	Annealing temperature	Efficiencies (%)
Archaea	806F 915R	CACAGCGTTTACACCTAG GTGCTCCCCCGCCAATTCCT	Takai *et al.*, 2000 Stahl *et al.*, 1991	64 °C	83.25 - 85.2
Bathyarchaeota	242dF 678R	TDACCGGTDCGGGCCGTG AGAACGCGCCCGACGGTG	Fillol *et al.*, 2014	68 °C	92.35 -101
Thermoplasmata	Thrm-f Thrm-r	GGTAAGACGGGTGGC GTATCTAATCCCGTTTGC	Compte-Port *et al.*, 2017	60 °C	88.3 -89
Marine Benthic Group D	345F 490R	ATATCTGAGACACGATATCRGG CACCACTTGAGCTGCAGGTA	Vetriani *et al.*, 2014		93.15 - 96
Methanomassiliicoccales	AS1 AS2	CAGCAGTCGCGAAAACTTC AACAACTTCTCTCCGGCAC	Mihjalovsky *et al.*, 2010	58 °C	89.35 - 92.5
Methanogen 16S rRNA	Met 630F Met 803R	GGATTAGATACCCSGGTAGT GTTGARTCCAATTAAACCGCA	Hook *et al.*, 2009	60 °C	91 - 99
mcrA	ME3MF ME3r'	ATGTCNGGTGGHGTMGGSTTYAC TCATBGCRTAGTTDGGRTAGT	Nunoura *et al.*, 2008 Hales *et al.*, 1996	54 °C	84 - 97

References to Supplementary Table S6

Compte-Port, S., Subirats, J., Fillol, M., Sànchez-Melsió, A., Marcé, R., Rivas-Ruiz, P., et al. (2017) Abundance and Co-Distribution of Widespread Marine Archaeal Lineages in Surface Sediments of Freshwater Water Bodies across the Iberian Peninsula. *Microb. Ecol.*

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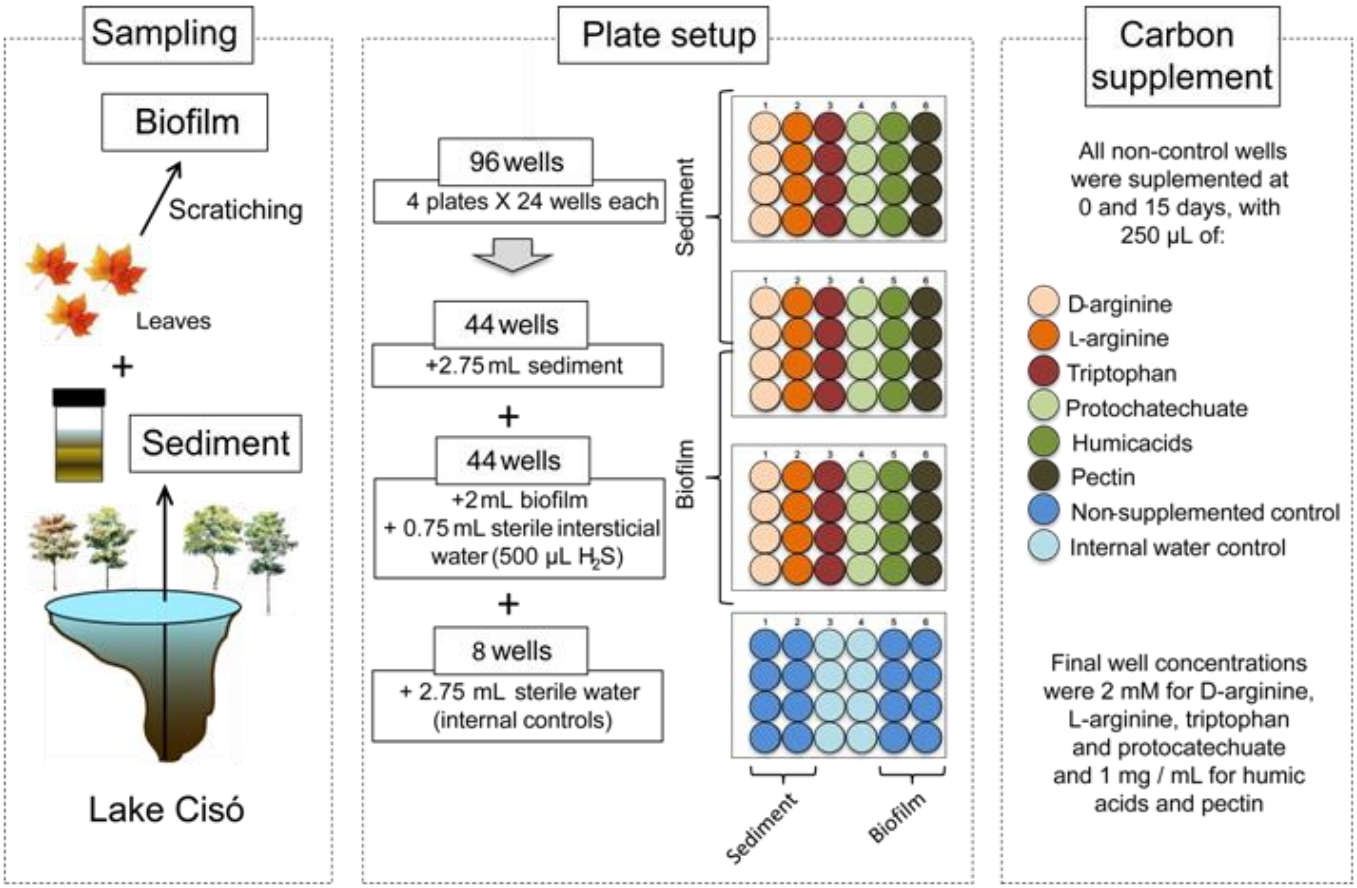
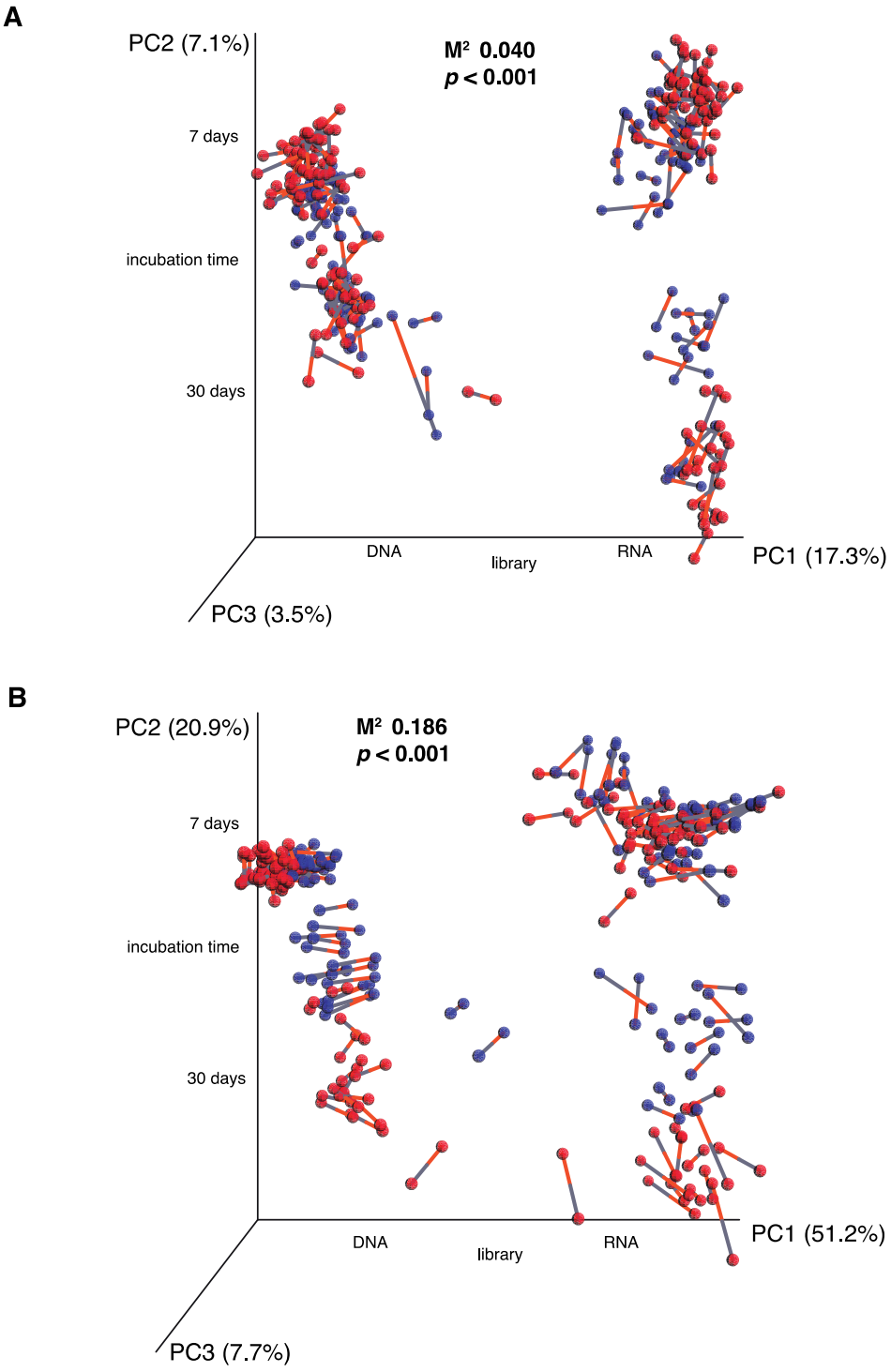
Table S7. Variables included in multivariate analysis and stepwise multiple linear regressions after identification of co-linear variables according to Spearman correlations (R > 0.7; p<0.05).

Full analysis (n=24)			Full analysis (n=8)			Full analysis (n=5)	
Group name	Variable used in the analysis	Correlated variables (R > 0.7)	Group name	Variable used in the analysis	Correlated variables (R > 0.7)	Group name	Variable used in the analysis
Ti	Ti	Ba	**SO_4^{2-}**	SO_4^{2-}	-	**Altitude**	Altitude
Mn	Mn	Mg	**NO_3^{2-}**	NO_3^{2-}	-	**Area**	Area
Cu	Cu	Fe, Ni, Co, Al	**PO_4^{3+}**	PO_4^{3+}	-	**Depth**	Depth
As	As	-	**NH_4**	NH_4	-	**Longitude**	Longitude
Sr	Sr	-	**Chl*a***	Chl*a*	-	**Latitude**	Latitude
Mo	Mo	-	**pH**	pH	-		
Ag	Ag	-	**Temperature**	Temperature	-		
Cd	Cd	-					
Sn	Sn	-					
W	W	-					
Hg	Hg	Zn, Se					
Tl	Tl	-					
Pb	Pb	Sb, V, Cr					
	nº variables = 13			nº variables = 8			nº variables = 5

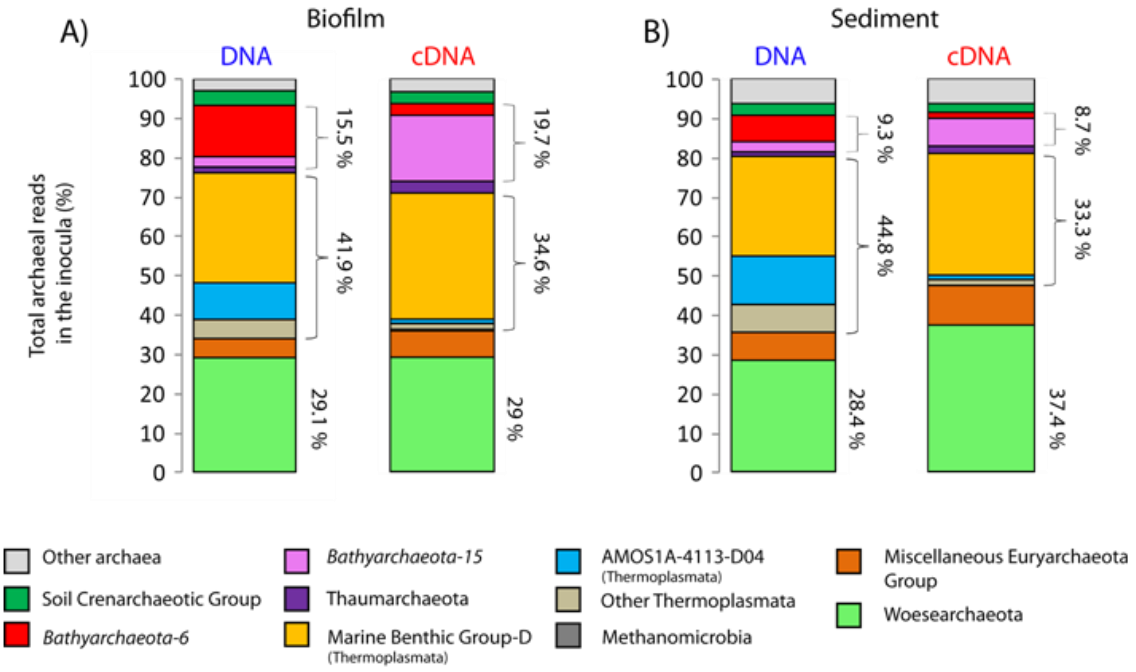
SUPPLEMENTARY INFORMATION **CHAPTER 3**



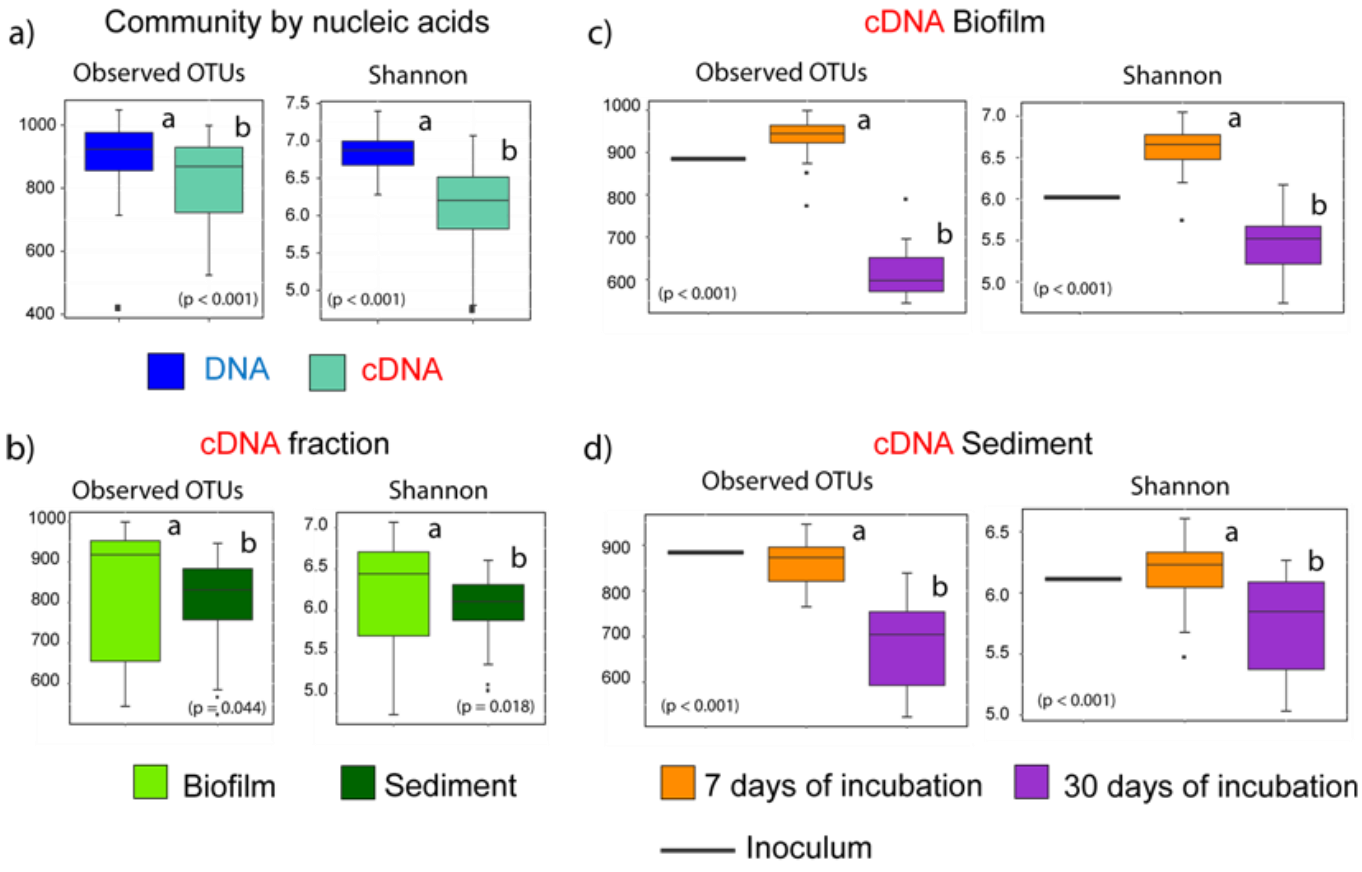
Suppl. Figure S1: Scheme of the sampling procedures (left) experimental setup (centre) and list of the carbon compounds (right).



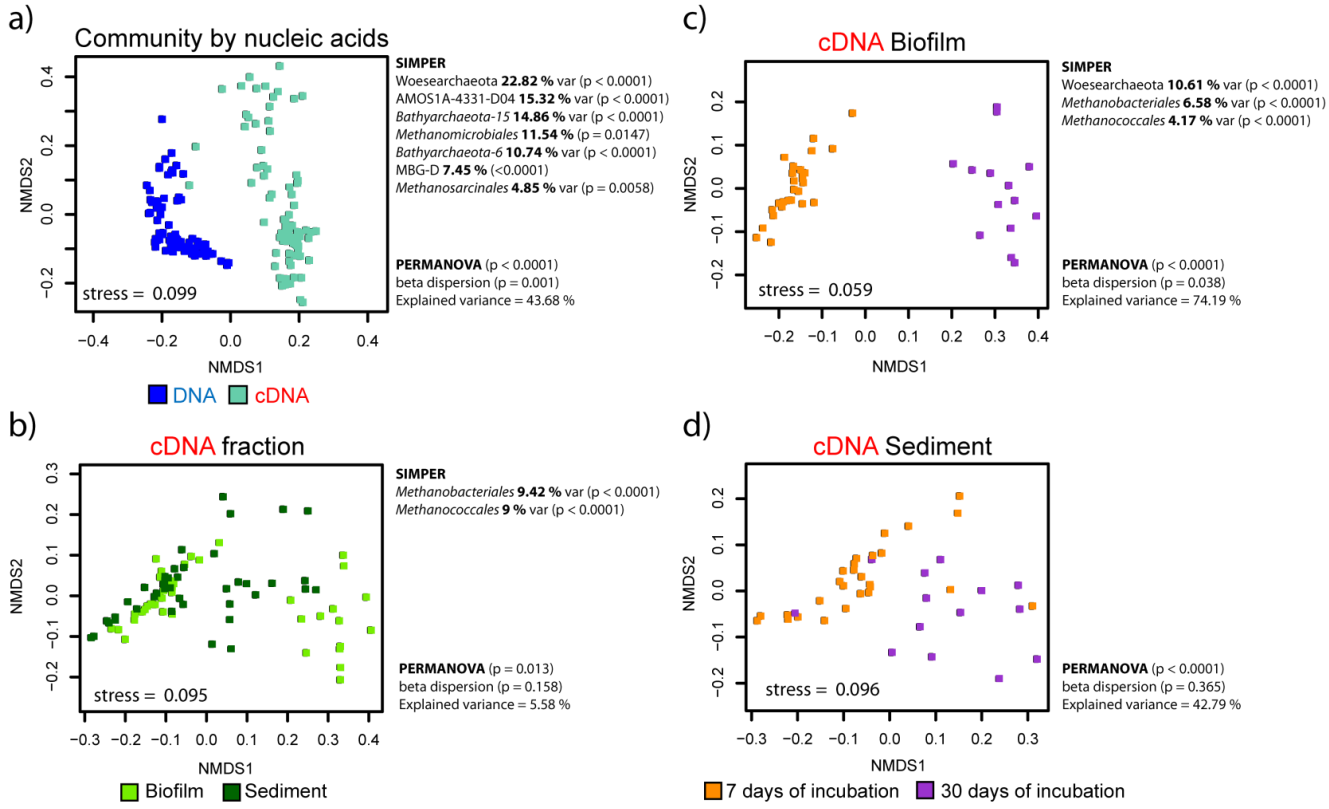
Suppl. Figure S2: PCoA Ordination of samples according to unweighted (A) and weighted (B) UniFrac distance using forward (5') and reverse (3') sequences. Samples are coloured according to substrate, biofilm (red) and sediment (blue). Samples derived from forward and reverse sequencing are linked with a bar: in every case, the distance between the 5′ and 3′ reads of the same samples is much smaller than the distance between samples. Results from the Procrustes analysis are also shown for each case (10.000 Monte Carlo simulations).



Suppl. Figure 3: Composition of archaeal communities in (A) biofilm and (B) sediment samples used as inocula for the experimental microcosms. The relative abundance of each taxon is depicted as the percentage of total reads.



Suppl. Figure S4. Comparison of alpha diversity estimators (number of OTUs and Shannon index) of biofilm and sediment archaeal communities according to (a) nucleic acid (DNA and cDNA); (b) across habitats (only the archaeal community from the cDNA fraction); and incubation time for the cDNA community in biofilms (c) or the bare sediment (d). Significant differences for all comparisons have been assessed by Mann-Whitney test and indicated by alphanumerical indexes and correspondent *p* values.



Suppl. Figure S5. Non-metric multidimensional scaling ordination of samples according to community composition (Bray-Curtis distance). Comparison has been done according to: nucleic acid (a); habitat (only active fraction of the community, (b); incubation time for active communities in biofilm (c) and sediment (d). Significant differences for all comparisons have been assessed by PERMANOVA and beta dispersion analyses (9999 permutations). *p* values of both tests are shown at the bottom right of each plot, together with the amount of variance (%) explained by each grouping. Prominent archaeal lineages contributing to such differences have been identified through a SIMPER analysis (9999 permutations) and results are shown in the top right of each plot when appropriate.

Supplementary Table S1. Organic substrates used for the amendments with the final concentration (in the plate wells) of the different compounds.

	Compound	Commercial name	Final concentration	Reference
Aminoacids	D-Arginine	Arginine (D)	2 mM	1109 (Fluka)
	L-Arginine	Arginine (L) BioChemika Ultra, ≥ 99.5%		11015 (Fluka)
	L-Tryptophan	Tryptophan (L)		1,08374,0010 (Merck)
Plant-derived	Protocatechuate	3,4-Dihidroxybenzoi acid		37580 (Sigma)
	Pectin	Pectin from citrus peel (Poly-D-galacturonic acid methyl ester)	1 mg / mL	76289 (Fluka)
	Humic Acid	Humic acid		53680 (Fluka)

Supplementary Table S2. Primer pairs and conditions used for the quantitative PCR. In all cases denaturalization stages of 95ºC for 20 seconds and annealing steps of 60 seconds were performed. Efficiencies and R^2, of the standard curves are displayed as intervals.

Primer	Target group	Sequence (5' - 3')	Nº cycles	Annealing Temperature (ºC)	Reference*	Efficiency (%)	R^2
1048 F	Bacteria	GTGSTGCAYGGYTGTCGTCA	35	60	[1]	98.4 - 99.8	0.991 - 0.999
1194 R		ACGTCRTCCMCACCTTCCTC					
806 F	Archaea	CACAGCGTTTACACCTAG	40	60	[2] [3]	97.1 - 99.3	0-993 - 1
915 R		GTGCTCCCCCGCCAATTCCT					
242d F	*Bathyarchaeota*	TDACCGGTDCGGGCCGTG	40	68	[4]	93.7 - 97.4	0.997 - 1
678 R		AGAACGCGCCCGACGGTG					
Thrm-f	*Thermoplasmata*	GGTAAGACGGGTGGC	40	60	[5]	89.5 - 100	0.997 - 1
Thrm-r		GTATCTAATCCCGTTTGC					

*[1] Maeda *et al.*, 2003 [2] Takai *et al.*, 2000 [3] Stahl DA, 1991 [4] Fillol *et al.*, 2015 [5] Compte-Port *et al.*, 2017

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Compte-Port, S., Subirats, J., Fillol, M., Sànchez-Melsió, A., Marcé, R., Rivas-Ruiz, P., et al. (2017) Abundance and Co-Distribution of Widespread Marine Archaeal Lineages in Surface Sediments of Freshwater Water Bodies across the Iberian Peninsula. *Microb. Ecol.* **74**: 776–787.

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Supplementary table S3. Copy numbers of the 16S rRNA gene (normalized by dry weight) for Archaea, Bathyarchaeota and Thermoplasmata. Values are displayed as the average ± standard deviation of biological replicates (n=4 for 7 days and n=2 for 30 days of incubation) which share same levels across experimental factors.

		BIOFILM				SEDIMENT				
		DNA fraction		cDNA fraction		DNA fraction		cDNA fraction		
		Average	Standard deviation	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation	
7 days	Control	2.14×10^{10}	1.12×10^{10}	1.02×10^{11}	7.37×10^{10}	5.49×10^{9}	1.54×10^{9}	2.48×10^{10}	9.65×10^{9}	ARCHAEA
	D-Arginine	1.87×10^{10}	9.72×10^{9}	1.45×10^{11}	2.98×10^{10}	4.92×10^{9}	2.22×10^{9}	6.36×10^{9}	2.01×10^{9}	
	L-Arginine	1.78×10^{10}	6.56×10^{9}	1.40×10^{11}	2.18×10^{10}	5.00×10^{9}	1.36×10^{9}	1.50×10^{10}	1.07×10^{10}	
	Tryptophan	1.98×10^{10}	8.78×10^{9}	1.01×10^{11}	3.44×10^{10}	6.13×10^{9}	1.81×10^{9}	1.73×10^{10}	2.88×10^{9}	
	Protocatechuate	2.06×10^{10}	1.15×10^{10}	1.31×10^{11}	2.49×10^{10}	4.69×10^{9}	$1.49E \times 10^{9}$	1.93×10^{10}	1.91×10^{10}	
	Humic acids	2.60×10^{10}	3.03×10^{9}	1.51×10^{11}	4.09×10^{10}	6.28×10^{9}	8.66×10^{8}	2.07×10^{10}	4.79×10^{9}	
	Pectin	3.27×10^{10}	6.47×10^{9}	1.58×10^{11}	6.72×10^{10}	7.98×10^{9}	4.48×10^{9}	3.66×10^{10}	1.29×10^{10}	
30 days	Control	1.70×10^{11}	2.31×10^{10}	3.00×10^{11}	3.38×10^{10}	2.12×10^{10}	1.44×10^{9}	3.75×10^{10}	5.16×10^{9}	
	D-Arginine	2.12×10^{11}	4.25×10^{10}	3.24×10^{11}	3.59×10^{10}	1.11×10^{10}	5.67×10^{9}	3.72×10^{10}	3.68×10^{8}	
	L-Arginine	1.42×10^{11}	2.74×10^{9}	1.89×10^{11}	2.01×10^{10}	1.92×10^{10}	2.53×10^{9}	3.74×10^{10}	9.74×10^{9}	
	Tryptophan	1.26×10^{11}	5.73×10^{10}	2.75×10^{11}	7.61×10^{10}	1.26×10^{10}	8.49×10^{8}	2.83×10^{10}	9.66×10^{9}	
	Protocatechuate	1.52×10^{11}	1.30×10^{10}	3.31×10^{11}	5.16×10^{10}	1.66×10^{10}	4.97×10^{9}	2.66×10^{10}	6.15×10^{9}	
	Humic acids	9.81×10^{11}	4.31×10^{9}	2.89×10^{11}	5.65×10^{10}	1.88×10^{10}	3.90×10^{9}	1.90×10^{10}	1.74×10^{10}	
	Pectin	1.28×10^{11}	5.40×10^{10}	2.30×10^{11}	3.41×10^{10}	1.94×10^{10}	6.19×10^{8}	3.39×10^{10}	1.97×10^{9}	
7 days	Control	7.43×10^{8}	4.33×10^{8}	7.94×10^{8}	6.49×10^{8}	2.19×10^{8}	6.39×10^{7}	5.02×10^{8}	2.01×10^{8}	BATHYARCHAEOTA
	D-Arginine	1.65×10^{9}	9.30×10^{8}	8.97×10^{8}	9.98×10^{7}	2.32×10^{8}	1.49×10^{8}	1.17×10^{8}	8.05×10^{7}	
	L-Arginine	1.86×10^{9}	1.01×10^{9}	1.42×10^{9}	2.33×10^{8}	2.46×10^{8}	7.58×10^{7}	3.43×10^{8}	2.94×10^{8}	
	Tryptophan	1.29×10^{9}	7.43×10^{8}	8.03×10^{8}	2.42×10^{8}	2.42×10^{8}	7.38×10^{7}	3.81×10^{8}	1.31×10^{8}	
	Protocatechuate	7.34×10^{8}	4.79×10^{8}	9.57×10^{8}	4.31×10^{8}	1.79×10^{8}	6.07×10^{7}	4.37×10^{8}	4.61×10^{8}	
	Humic acids	8.97×10^{8}	1.31×10^{8}	1.34×10^{9}	4.11×10^{8}	2.23×10^{8}	3.38×10^{7}	4.26×10^{8}	1.85×10^{8}	
	Pectin	9.48×10^{8}	1.52×10^{8}	8.68×10^{8}	2.99×10^{8}	1.81×10^{8}	1.12×10^{8}	3.94×10^{8}	1.99×10^{8}	
30 days	Control	9.30×10^{9}	8.90×10^{8}	2.97×10^{9}	1.06×10^{9}	2.28×10^{9}	1.17×10^{8}	4.85×10^{8}	7.25×10^{7}	
	D-Arginine	8.15×10^{9}	5.00×10^{8}	3.63×10^{9}	4.67×10^{8}	1.12×10^{9}	7.28×10^{8}	3.96×10^{8}	6.54×10^{7}	
	L-Arginine	1.17×10^{10}	2.03×10^{9}	3.26×10^{9}	6.48×10^{7}	1.26×10^{9}	1.99×10^{8}	4.42×10^{8}	6.45×10^{7}	
	Tryptophan	5.52×10^{9}	3.57×10^{8}	3.06×10^{9}	4.63×10^{8}	7.97×10^{8}	3.02×10^{7}	6.46×10^{8}	2.58×10^{8}	
	Protocatechuate	6.88×10^{9}	1.22×10^{9}	1.92×10^{9}	1.77×10^{8}	2.22×10^{9}	1.10×10^{9}	3.74×10^{8}	4.04×10^{7}	
	Humic acids	5.66×10^{9}	1.49×10^{9}	2.71×10^{9}	3.14×10^{8}	1.66×10^{9}	2.40×10^{8}	3.82×10^{8}	3.92×10^{8}	
	Pectin	6.27×10^{9}	1.78×10^{9}	1.64×10^{9}	1.12×10^{8}	1.08×10^{9}	1.54×10^{8}	3.90×10^{8}	2.74×10^{6}	
7 days	Control	1.97×10^{9}	1.22×10^{9}	3.88×10^{8}	3.04×10^{8}	4.19×10^{8}	1.09×10^{8}	9.80×10^{7}	4.95×10^{7}	THERMOPLASMATA
	D-Arginine	1.79×10^{9}	9.41×10^{8}	7.73×10^{8}	1.28×10^{8}	4.24×10^{8}	1.96×10^{8}	2.08×10^{7}	6.17×10^{6}	
	L-Arginine	1.61×10^{9}	7.36×10^{8}	5.98×10^{8}	1.18×10^{8}	4.57×10^{8}	1.26×10^{8}	7.91×10^{7}	8.34×10^{7}	
	Tryptophan	2.06×10^{9}	1.36×10^{9}	4.41×10^{8}	1.65×10^{8}	4.65×10^{8}	9.66×10^{7}	1.11×10^{8}	9.08×10^{7}	
	Protocatechuate	2.03×10^{9}	1.41×10^{9}	4.97×10^{8}	1.32×10^{8}	3.38×10^{8}	1.19×10^{8}	9.33×10^{7}	1.00×10^{8}	
	Humic acids	2.42×10^{9}	2.27×10^{8}	7.15×10^{8}	2.84×10^{8}	4.53×10^{8}	6.29×10^{7}	8.70×10^{7}	8.34×10^{6}	
	Pectin	3.04×10^{9}	5.58×10^{8}	4.39×10^{8}	2.19×10^{8}	3.92×10^{8}	2.47×10^{8}	1.09×10^{8}	4.15×10^{7}	
30 days	Control	2.18×10^{9}	2.40×10^{8}	1.11×10^{9}	4.00×10^{8}	3.57×10^{8}	7.01×10^{7}	1.60×10^{8}	1.66×10^{7}	
	D-Arginine	1.70×10^{9}	2.40×10^{7}	1.11×10^{9}	1.19×10^{8}	2.11×10^{8}	9.12×10^{7}	1.42×10^{8}	2.59×10^{7}	
	L-Arginine	1.87×10^{9}	3.24×10^{8}	7.75×10^{8}	1.02×10^{8}	3.58×10^{8}	2.65×10^{7}	1.52×10^{8}	3.71×10^{7}	
	Tryptophan	1.12×10^{9}	9.97×10^{7}	9.41×10^{8}	1.27×10^{8}	2.29×10^{8}	4.47×10^{6}	2.04×10^{8}	9.70×10^{7}	
	Protocatechuate	1.65×10^{9}	1.74×10^{8}	6.44×10^{8}	4.00×10^{7}	3.72×10^{8}	1.26×10^{8}	1.07×10^{8}	1.60×10^{7}	
	Humic acids	1.19×10^{9}	1.05×10^{8}	7.77×10^{8}	1.17×10^{8}	3.45×10^{8}	4.09×10^{7}	9.32×10^{7}	9.81×10^{7}	
	Pectin	1.40×10^{9}	4.99×10^{8}	8.96×10^{8}	1.83×10^{8}	3.02×10^{8}	4.65×10^{7}	2.18×10^{8}	2.12×10^{7}	