



Diversity of *Archaea* and detection of crenarchaeotal *amoA* genes in the rivers Rhine and Têt

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ABSTRACT: Pelagic archaeal phylogenetic diversity and the potential for crenarchaeotal nitrification of Group 1.1a were determined in the rivers Rhine and Têt by 16S rRNA sequencing, catalyzed reported deposition-fluorescence *in situ* hybridization (CARD-FISH) and quantification of 16S rRNA and functional genes. *Euryarchaeota* were, for the first time, detected in temperate river water even though a net predominance of crenarchaeotal phylotypes was found. Differences in phylogenetic distribution were observed between rivers and seasons. Our data suggest that a few archaeal phylotypes (*Euryarchaeota* Groups RC-V and LDS, *Crenarchaeota* Group 1.1a) are widely distributed in pelagic riverine environments whilst others (*Euryarchaeota* Cluster Sagma-1) may only occur seasonally in river water. *Crenarchaeota* Group 1.1a has recently been identified as a major nitrifier in the marine environment and phylotypes of this group were also present in both rivers, where they represented 0.3% of the total pelagic microbial community. Interestingly, a generally higher abundance of *Crenarchaeota* Group 1.1a was found in the Rhine than in the Têt, and crenarchaeotal ammonia monooxygenase gene (*amoA*) was also detected in the Rhine, with higher *amoA* copy numbers measured in February than in September. This suggests that some of the *Crenarchaeota* present in river waters have the ability to oxidize ammonia and that riverine crenarchaeotal nitrification of Group 1.1a may vary seasonally.

KEYWORDS: *Archaea* · River · Diversity · Nitrification

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INTRODUCTION

16S rRNA sequences belonging to non-extremophilic aquatic *Archaea* were first discovered in the oxygenated water column of the Pacific and Atlantic Oceans (DeLong 1992, Fuhrman et al. 1992); however, it is now clear that these organisms are ubiquitously present in marine systems (Massana et al. 2000, Herndl et al. 2005, Wuchter 2006, Wuchter et al. 2006b), lakes (MacGregor et al. 1997, Schleper et al. 1997, Keough et al. 2003) and rivers (Crump & Baross 2000, Abreu et al.

2001, Bouvier & del Giorgio 2002, Galand et al. 2006, 2008, Garneau et al. 2006, Wells et al. 2006). Several studies have focused on the distribution of archaeal assemblages in the water column and sediments along salinity gradients in estuaries (Crump & Baross 2000, Abreu et al. 2001, Bouvier & del Giorgio 2002). These studies revealed that pelagic *Archaea* represent a small proportion (3%) of the riverine prokaryotic community and, in contrast to *Bacteria*, no specific estuarine archaeal community is developed. The estuarine archaeal community consists mainly of allochthonous

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members originating from either the river or the adjacent coastal ocean (Crump & Baross 2000, Bouvier & del Giorgio 2002). Phylogenetic analysis also demonstrated that *Crenarchaeota* were the predominant archaeal group in estuarine sediments (Abreu et al. 2001).

The above findings, derived from temperate rivers, also hold for boreal and polar rivers and estuaries. Recent investigations in the Mackenzie River based on cell counts following fluorescence *in situ* hybridization (FISH) using general archaeal probes showed that pelagic *Archaea* constitute a slightly larger fraction (5 to 9%) of the total polar riverine prokaryotic community (Garneau et al. 2006, Wells et al. 2006). The observation reported by Crump & Baross (2000) that riverine *Archaea* tend to be associated with particles was confirmed by Wells & Deming (2003) and Wells et al. (2006), suggesting that there is a relationship between crenarchaeotal abundance and particle load in river-influenced coastal Arctic shelf systems. Moreover, the relatively higher archaeal abundance in river waters (1.3×10^5 cells ml^{-1}) compared to surface seawater (0.4×10^5 cells ml^{-1}), as well as the relatively high archaeal contribution (15% of prokaryotic abundance) in the nepheloid layer of the adjacent Beaufort Shelf and Franklin Bay, led to the conclusion that a large part of the archaeal assemblage of coastal shelf waters might originate from the adjacent rivers (Wells et al. 2006). Clearly this is not the case, since 2 studies have now shown that the archaeal assemblage of the Mackenzie River had little phylogenetic resemblance to that of the adjacent coastal Beaufort Sea (Galand et al. 2006, 2008).

Remarkably, a significant correlation was found throughout the data set of Wells et al. (2006), which includes the Mackenzie River, between archaeal abundance and concentrations of particulate organic nitrogen, indicating that *Archaea* might play an important role in nitrogen cycling in rivers (Wells et al. 2006). Most physiological studies on non-extremophilic aquatic *Archaea* have been carried out on pelagic marine *Archaea*. Chemoautotrophy, heterotrophy and mixotrophy have all been suggested as possible metabolisms for the 2 main groups of non-extremophilic *Archaea*: the *Crenarchaeota* (Ouverney & Fuhrman 2000, Pearson et al. 2001, Wuchter et al. 2003, Herndl et al. 2005, Ingalls et al. 2006, Teira et al. 2006) and the *Euryarchaeota* (Herndl et al. 2005, Teira et al. 2006). Recently, a potential for phototrophy in *Euryarchaeota* has been indicated by the discovery of genes encoding for proteorhodopsin, a protein that catalyses lightdriven proton transfer across the cell membrane (Frigaard et al. 2006). The ability of at least some marine *Crenarchaeota* to oxidize ammonia to nitrite has also been suggested by several molecular

studies of environmental samples (Venter et al. 2004, Francis et al. 2005, Treusch et al. 2005, Coolen et al. 2007, Herfort et al. 2007, Mincer et al. 2007) and recently confirmed in enriched and pure cultures of *Crenarchaeota* (Könneke et al. 2005, Hallam et al. 2006, Wuchter et al. 2006b).

Generally, more studies have focused on marine rather than riverine *Archaea*. Consequently, much less is known about the physiology and ecology of riverine archaeal communities. The River Rhine was selected for the present study because it is the largest river flowing into the North Sea, where we recently conducted several studies on *Archaea* (Herfort et al. 2006b, 2007, Wuchter 2006). In contrast, the Têt is a small European river located in the South of France where the mountain—the rivers origin—is adjacent to the shore, and therefore we were able to collect water spanning the entire river length, from a mountain lake to the estuary. Using DGGE and sequencing of DGGE bands, we first assessed the phylogenetic diversity of *Archaea* in the surface waters of these 2 rivers, the lower Rhine in February and September 2005 and the Têt in June 2005. Interestingly, sequences related to ammonia-oxidizing marine group I *Crenarchaeota* (Group 1.1a) were among the sequenced DGGE bands, and their abundance or potential role in nitrification within river water remains unexplored. We therefore determined the abundance of *Crenarchaeota* Group 1.1a in both rivers via catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD–FISH) cell counts and quantitative PCR (qPCR) of Group 1.1a 16S rDNA. The potential of *Crenarchaeota* Group 1.1a to oxidize ammonia was also examined by targeting a gene (*amoA*) which encodes the catalytic α subunit of the key enzyme in the first nitrification step (ammonia monooxygenase A, *amoA*).

The results of the present study form the basis for more detailed quantitative studies of additional archaeal groups found in this work, which will include determination of their potential role in rivers.

MATERIALS AND METHODS

Study area. The River Rhine originates in the Gottard Massif in Switzerland from several small Alpine creeks. The river flows through Lake Constance at the border of Germany, Austria and Switzerland and meanders over a distance of 1320 km before entering the southern North Sea through the Nieuwe Waterweg near Rotterdam and the sluices in the Haringvliet, The Netherlands. With an average annual discharge of $2300 \text{ m}^3 \text{ s}^{-1}$ into an estuary fed also by the River Meuse, the River Rhine is the largest river entering the southern North Sea. The River Têt is the largest river of the

Roussillon region in southwestern France; it originates in the Pyrenees and flows for 120 km before entering the Mediterranean Sea near Perpignan, southern France. The Têt is nonetheless a rather minor European river with a catchment area of only about 1400 km² and average water discharge of about 10 m³ s⁻¹.

Study sites. Surface water was collected in the River Rhine in February and September 2005, midway be-

tween each bank at 3 locations in The Netherlands: Millingen (Rhine, at the Dutch–German border), Gorinchem (Boven-Merwede) and Maassluis (Nieuwe Waterweg, estuary of the Meuse/Rhine) (Fig. 1). These 3 sites were primarily selected because regular crossings of the river by ferries made sampling midway between each bank possible. Extremely low salinity values representative of river water were expected at Millingen and Gorinchem, which we viewed as replicate sites. Given its location in the estuary, higher values were anticipated at Maassluis. Salinity values typically range from 0.7 to 6 psu (Herfort et al. 2006a) and are reported in Table 1. Surface water was also collected in the River Têt at Lake Bouillouses, Mont-Louis, Rodès and Villelongue in June 2005 (Fig. 1). As mentioned above, these sites were selected to span the length of this short river. Sampling sites at Lake Bouillouses and Mont-Louis are little affected by human activities, and 90% of the land is covered by natural vegetation. In contrast, Rodès is characterized by intensive agricultural land use, while Villelongue is located downstream of the inflow of treated water of the Perpignan sewage treatment plant (Garcia-Esteves et al. 2007). The exact same sites were used in previous studies investigating the major element and nutrient fluxes in the Têt (Garcia-Esteves et al. 2007) and the transport of organic matter to the ocean via the measurement of crenarchaeotal membrane lipids (Kim et al. 2007). Nutrient and temperature data collected in June 2001 by Garcia-Esteves et al. (2007) are presented in Table 1.

Nucleic acid extraction. River water was stored at 4°C until filtered back in the laboratory less than 12 (Rhine) or 24 h (Têt) after collection. Water (1 l) was filtered onto 0.2 µm

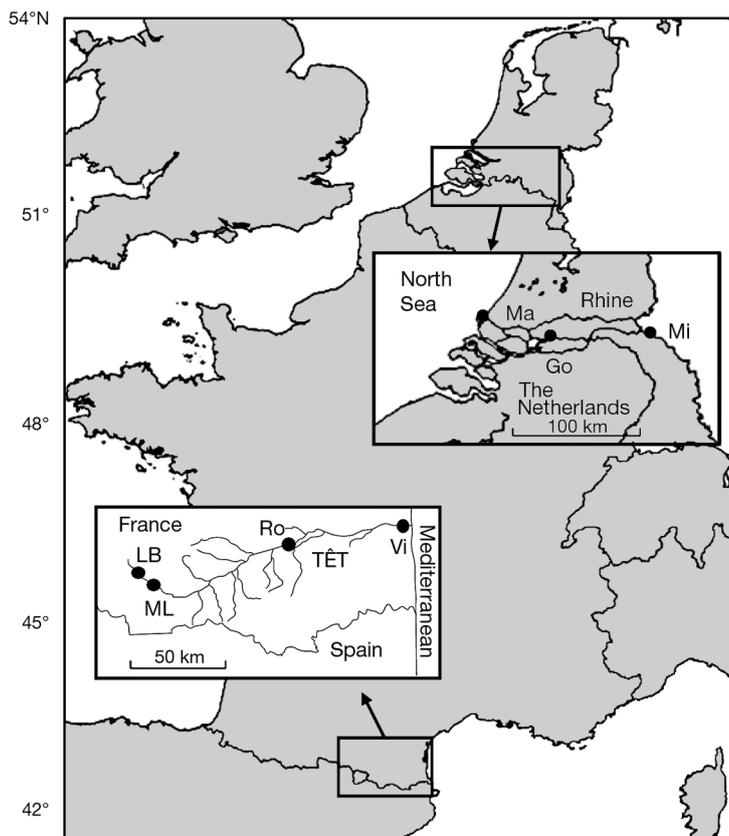


Fig. 1. Study sites in the rivers Rhine and Têt. Ma: Maassluis; Go: Gorinchem; Mi: Millingen; Vi: Villelongue; Ro: Rodès; ML: Mont-Louis; LB: Lake Bouillouses

Table 1. Nutrient concentrations (µM), temperature (°C) and salinity (psu) of water collected in the River Rhine in September and February 2005 at Maassluis (Ma), Gorinchem (Go) and Millingen (Mi) and in the Têt in June 2001 (data from Garcia-Esteves et al. 2007) at Villelongue (Vi), Rodès (Ro) and Mont-Louis (ML). All salinity and temperature data for the February Rhine samples are from Herfort et al. (2006a,b). –: no data

		Phosphate	Ammonium	Nitrate	Nitrite	Temperature	Salinity
Rhine (Feb)	Mi	0.9	10.2	279.4	1.9	4.9	0.7
	Go	1.1	8.0	276.1	1.7	4.9	1.0
	Ma	2.7	9.9	271.4	2.1	5.0	1.9
Rhine (Sep)	Mi	0.7	2.0	161.3	0.2	17.7	1.0
	Go	0.9	3.0	158.0	0.2	19.7	1.0
	Ma	2.9	5.8	128.4	0.7	20.3	6.0
Têt (Jun)	ML	–	–	5.2	–	13.6	–
	Ro	4.6	3.4	24.4	–	16.7	–
	Vi	16.2	150.2	135.4	–	24.2	–

pore-size, 47 mm filter diameter polycarbonate filters (Schleicher & Schuell) and stored at -80°C until extraction. Total DNA was extracted according to Wuchter (2006). Following the addition of 6 ml extraction buffer (10 mM Tris HCl, 25 mM EDTA, 1 vol% SDS, 100 mM NaCl and 0.1 ml zirconium beads) to each filter, DNA was extracted with phenol/chloroform/isoamyl-alcohol and chloroform, precipitated in ice-cold ethanol and resuspended in 100 μl sterile 10 mM Tris-HCl (pH 8.0) buffer.

Archaeal 16S rRNA gene analysis. Partial archaeal 16S rRNA gene fragments (420 bp) were amplified with the iCycler system (BioRad) by using the following universal archaeal PCR primers: Parch 519f (5'-CAGCCGCGCGGTAA-3') (Øvreås et al. 1997) and Arch915r (5'-GTGCTCCCCGC CAATTCCT-3') (Stahl & Amann 1991). This set of primers amplifies part of the variable region V3 of the 16S rRNA gene and the entire V4 region. The reaction mixture (20 μl) contained 2 to 5 ng template DNA, 0.4 μM primers, 1 U Picomaxx High Fidelity DNA polymerase (Stratagene), 2 μl 10 \times Picomaxx PCR buffer (Stratagene), 250 μM of each dNTP and 20 μg μl^{-1} BSA. During PCR, primer annealing occurred according to the following touch-down thermal profile in order to increase the specificity: 9 cycles at 68 to 64 $^{\circ}\text{C}$ for 40 s, followed by 31 cycles at 64 $^{\circ}\text{C}$ for 40 s. All other PCR steps were standard: initial denaturation (95 $^{\circ}\text{C}$, 4 min), several cycles of denaturation (94 $^{\circ}\text{C}$, 30 sec), annealing (as mentioned above) and extension (72 $^{\circ}\text{C}$, 40 s), and final extension (72 $^{\circ}\text{C}$, 10 min). A re-amplification—initial denaturation (95 $^{\circ}\text{C}$, 4 min), 13 cycles of denaturation (94 $^{\circ}\text{C}$, 30 sec), annealing (64 $^{\circ}\text{C}$, 40 s) and extension (72 $^{\circ}\text{C}$, 40 s), and final extension (72 $^{\circ}\text{C}$, 30 min)—was carried out with the same reaction mixture composition but with a 40 bp guanine-cytosine (GC)-rich clamp attached on the 5' end of the reverse primer (Muyzer et al. 1993). These amplicons were separated according to their GC content and secondary structure by DGGE (BioRad) using a linear denaturing gradient of 30 to 60% at 12.5 V cm^{-1} for 5 h according to Coolen et al. (2004). Gels were stained with SYBR Gold for 20 min and documented with a Fluor-S Multi Imager (BioRad). DGGE bands were excised and eluted in 75 μl sterile 10 mM Tris-HCl (pH 8.0) at 4 $^{\circ}\text{C}$ for 24 h. For the sequencing reactions, 1 μl of each eluted 16S rRNA gene fragment was re-amplified using primers without a GC clamp and the above-mentioned PCR conditions, except that only 25 cycles were used.

Sequencing. Following DNA quantification with PicoGreen, re-amplified DGGE fragments were purified using a Qiaquick PCR Purification Spin Kit (Qiagen) as recommended by the manufacturer. Ten ng of DNA was added to the 20 μl cycle sequencing reaction of a Big Dye Sequencing protocol. Nucleotide se-

quences were determined by automated sequencing using an ABI PRISM 310 capillary sequencer (Applied Biosystems). It is important to note that a total of 84 bands were excised and 57 bands were successfully sequenced. All 57 DGGE bands were sequenced in both directions, a consensus was made and all bands were then extensively checked for sequencing errors. Various DGGE bands which melted at the same position in the DGGE were sequenced to verify whether the same sequence appeared in different samples.

Phylogenetic analyses. Sequence data were compiled using ARB software (Ludwig et al. 2004, available at www.arb-home.de/) and aligned with complete length sequences of closest relatives obtained from the NCBI database using the ARB FastAligner utility. Using ARB, phylogenetic trees were first generated with the aligned, almost complete length sequences of closest relatives from the NCBI database using the neighbour-joining method and the Jukes-Cantor correction. Then, the short aligned DGGE sequences were added to the trees using the maximum parsimony option implemented in ARB. Classification was based on that of Schleper et al. (2005) and Galand et al. (2006). Sequences obtained in the present study have all been deposited in NCBI (accession nos. FJ746499 to FJ7464553).

CARD-FISH. River water (15 ml) was fixed with formaldehyde (final concentration 4%) at room temperature for 1 h and then filtered onto 0.2 μm pore-size polycarbonate filters (Millipore, 25 mm filter diameter) using 0.45 μm pore-size cellulose nitrate filters (Millipore, HAWP) as support. *Crenarchaeota* were stained using the improved CARD-FISH protocol described by Teira et al. (2004). Briefly, sections of filters embedded in low-gelling point agarose were incubated with proteinase K to enhance cell wall permeabilization at 37 $^{\circ}\text{C}$ for 1 h. Hybridization was carried out at 35 $^{\circ}\text{C}$ for 12 to 15 h using a horseradish peroxidase (hrp)-labeled oligonucleotide probe specific for *Crenarchaeota*, Cren537 (5'-TGACCACTTGAGGTGCTG-3') (Teira et al. 2004). The specificity of the probe was tested using the Check Probe function implemented in ARB. The probe matched 100% with 1543 of 1700 *Crenarchaeota* Group 1.1a sequences available in the database in July 2007. A total of 1746 hits appeared to have one mismatch, with again most of them related to *Crenarchaeota* Group 1.1a and some to *Crenarchaeota* Group 1.1b. Consequently, the *Crenarchaeota* detected with probe Cren537 are essentially *Crenarchaeota* Group 1.1a, with a few possible *Crenarchaeota* Group 1.1b. With this in mind, those *Crenarchaeota* detected with probe Cren537 will hereafter be referred to as *Crenarchaeota* Group 1.1a. Tyramide-Alexa488 was employed to further amplify the CARD-FISH signal and picoplankton cells were

counterstained with DAPI (4',6'-diamidino-2-phenylindole) (Teira et al. 2004). *Crenarchaeota* Group 1.1a and total DAPI-stainable cells were counted using an epifluorescence microscope (Zeiss Axioplan 2) equipped with a 100 W Hg lamp as well as the appropriate filter sets for DAPI and Alexa488 fluorescence. To determine crenarchaeotal and total cell abundance, a minimum of 200 crenarchaeotal cells and 200 DAPI-stained cells were counted per filter.

Real-time quantitative PCR. Copy numbers of the crenarchaeotal *amoA* gene were quantified using the specific primers amplifying a 256 bp region developed by Wuchter et al. (2006b), Arc-*amoA*-for (5'-CTG AYT GGG CYT GGA CAT C-3') and Arch-*amoA*-rev (5'-TTC TTC TTT GTT GCC CAG TA-3'). For quantification of *Crenarchaeota* Group 1.1a 16S rRNA gene fragments (122 bp), the specific primers MCG-1 391F (5'-AAG GTT ART CCG AGT GRT TTC-3') (Takai et al. 2004) and MCG-1 554R (5'-TGA CCA CTT GAG GTG CTG-3') (Teira et al. 2004) were employed. Note that this reverse primer is identical to the CARD-FISH probe mentioned above. All qPCR were carried out in an iCycler system (BioRad). A total of 40 cycles was run with PCR conditions and reagents as described above, except that the annealing temperature was set at a fixed temperature of 61°C for *Crenarchaeota* Group 1.1a 16S rRNA gene fragments and 58.5°C for archaeal *amoA*. The accumulation of newly amplified double-stranded gene products was measured by the increase in fluorescence due to the binding of the fluorescent dye SYBR Green to the double-stranded amplification products. A 10-fold dilution series of 10^0 to 10^7 copies of PCR-amplified *Crenarchaeota* Group 1.1a 16S rRNA gene- or archaeal *amoA* fragments from the North Sea enrichment culture (Wuchter et al. 2006b) served as standard series to calibrate the samples ($R^2 = 0.99$ and 1.00 , PCR efficiency = 82.9 and 73.1% , respectively). Reactions without the addition of template DNA served as controls for contamination and were also subjected to qPCR. Reactions with 4×10^7 copies of 16S rDNA of *Methanosarcina mazei* were subjected to qPCR as controls for the specificity for crenarchaeotal 16S rDNA qPCR, while genomic DNA from the bacterial ammonia oxidizers *Nitrosomonas europaea* (*Beta-proteobacteria*) and *Nitrosococcus oceani* (*Gamma-proteobacteria*) served as controls for specificity in crenarchaeotal *amoA* quantification. Melting curves were analyzed and aliquots of these qPCR products were run on an agarose gel in order to identify unspecific PCR products such as primer dimers or bands with unexpected fragment lengths.

Nutrients. To determine nutrient concentrations in the river Rhine, duplicate samples of river water (3 ml) passed through a 0.2 μm Acrodisc filter and kept frozen until processed. They were later analyzed col-

orimetrically using a segmented continuous flow analyzer (TRAACS 800 autoanalyzer, Bran & Luebbe). Data presented here are means of duplicate samples. Nutrient samples were not collected from the River Têt.

RESULTS

Archaeal community composition

DGGE fingerprinting of 16S rRNA gene fragments obtained from the surface waters of the rivers Rhine and Têt (including Lake Bouillouses) showed a complex banding pattern (Fig. 2). An additional DGGE gel was also run with diluted concentrations of the archaeal 16S rDNA PCR product, which showed equivalent diversity and relative density of the banding pattern (data not shown). Fifty-five archaeal phylogenotypes were retrieved (Figs. 3 & 4), with a net predominance of *Crenarchaeota* (40) over *Euryarchaeota* (15). Most euryarchaeotal sequences (60%) belonged to the Lake Dago Sediment (LDS) and Rice Cluster (RC-V) clusters, which were represented in both rivers and all seasons sampled. One euryarchaeotal sequence was related to sequences of the Sagma-1 group and was found at all sites in the Têt (E11, E12 and E15; Figs. 2 & 3) but not in the Rhine. *Euryarchaeota* Group III sequences were only detected at 1 site (Gorinchem) in the Rhine in September (E5; Figs. 2 & 3). Crenarchaeotal sequences fell into 5 distinct groups: 1.1a, 1.1b, 1.3a, 1.3b and 2/3c (Fig. 4). Differences in crenarchaeotal phylogenetic distribution between rivers and seasons were also observed. For example, major DGGE bands related to sequences from Group 1.1b were found at all sites in the Rhine in February (e.g. C15 and C16; Figs. 2 & 4), whilst only one faint band was detected in September in the Rhine at Millingen (C33; Figs. 2 & 4) and none was recovered from the Têt. Similarly, Group 1.1a was found in the Rhine at all sites and seasons studied (e.g. C1, C3 and C6; Figs. 2 & 4), but was only detected in the Têt at Mont-Louis (C5; Figs. 2 & 4). Group 1.3a was only retrieved at all sites in the Rhine in September (C12, C13, C36 and C7; Figs. 2 & 4) and in the Têt at Villelongue (C38 and C39; Figs. 2 & 4). It is interesting to note that the most intense DGGE band in Lake Bouillouses (C10; Fig. 2) was related to sequences belonging to *Crenarchaeota* Group 1.3b (Figs. 2 & 4), which was first discovered in freshwater lake sediments (Ochsenreiter et al. 2003).

It is important to note that several DGGE bands were in fact related to bacterial sequences (DGGE bands labeled 'B', Fig. 2). This is indicative of a dominance of bacterial over archaeal template DNA that resulted in non-specific amplification of bacterial 16S rRNA.

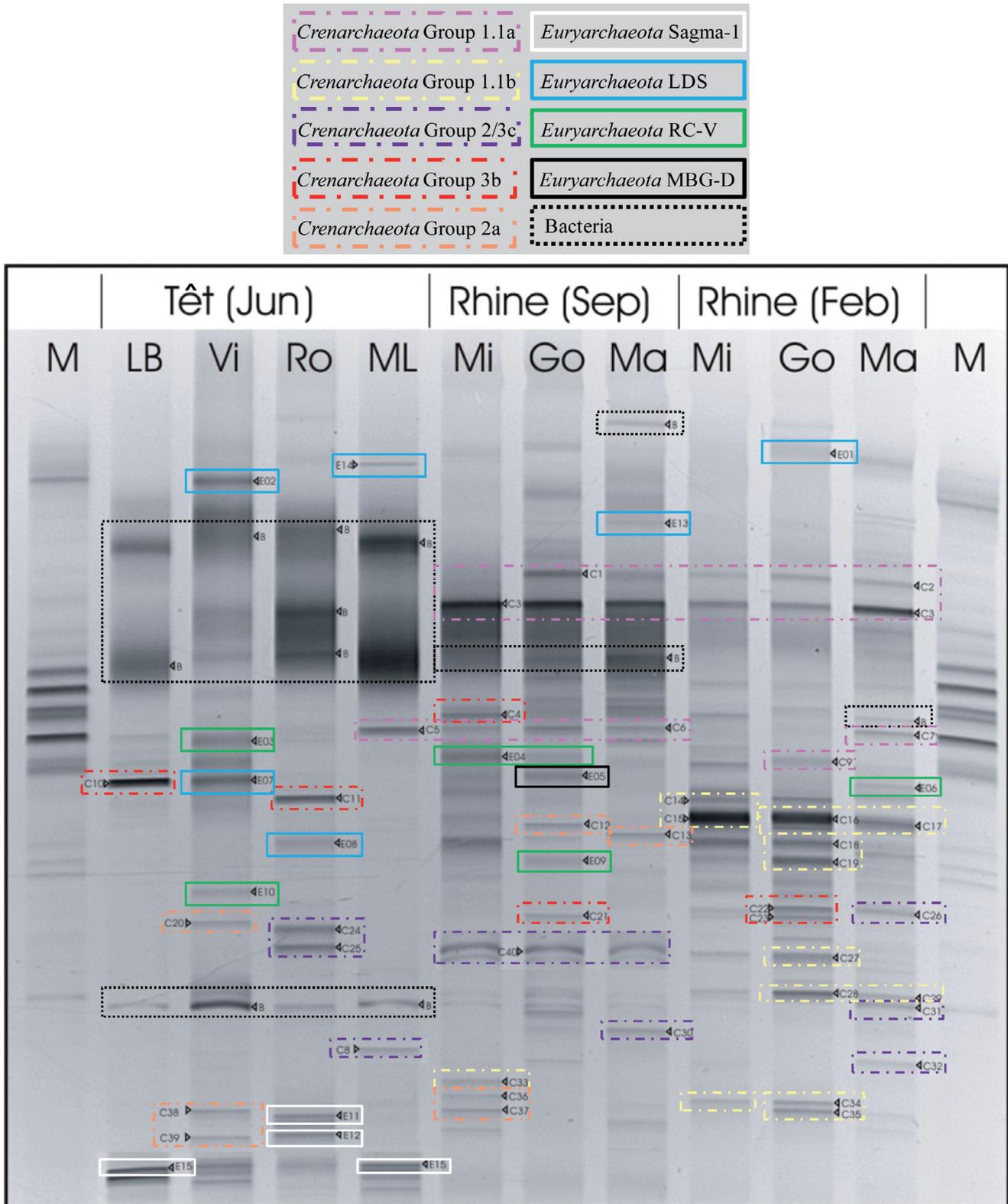


Fig. 2. DGGE banding pattern of archaeal 16S rRNA gene fragments of the rivers Rhine and Têt. Sequenced DGGE bands are numbered. C: *Crenarchaeota*; E: *Euryarchaeota*; B: *Bacteria*; M: a PCR product from seawater sampled at the Frisian Font in the North Sea (Herfort et al. 2007) that served as position marker for DGGE bands. Colored boxes mark sequenced DGGE bands in the same phylogenetic cluster. These boxes also include bands which were not sequenced but which melted at identical horizontal positions in the gel. Station abbreviations are given in Fig. 1

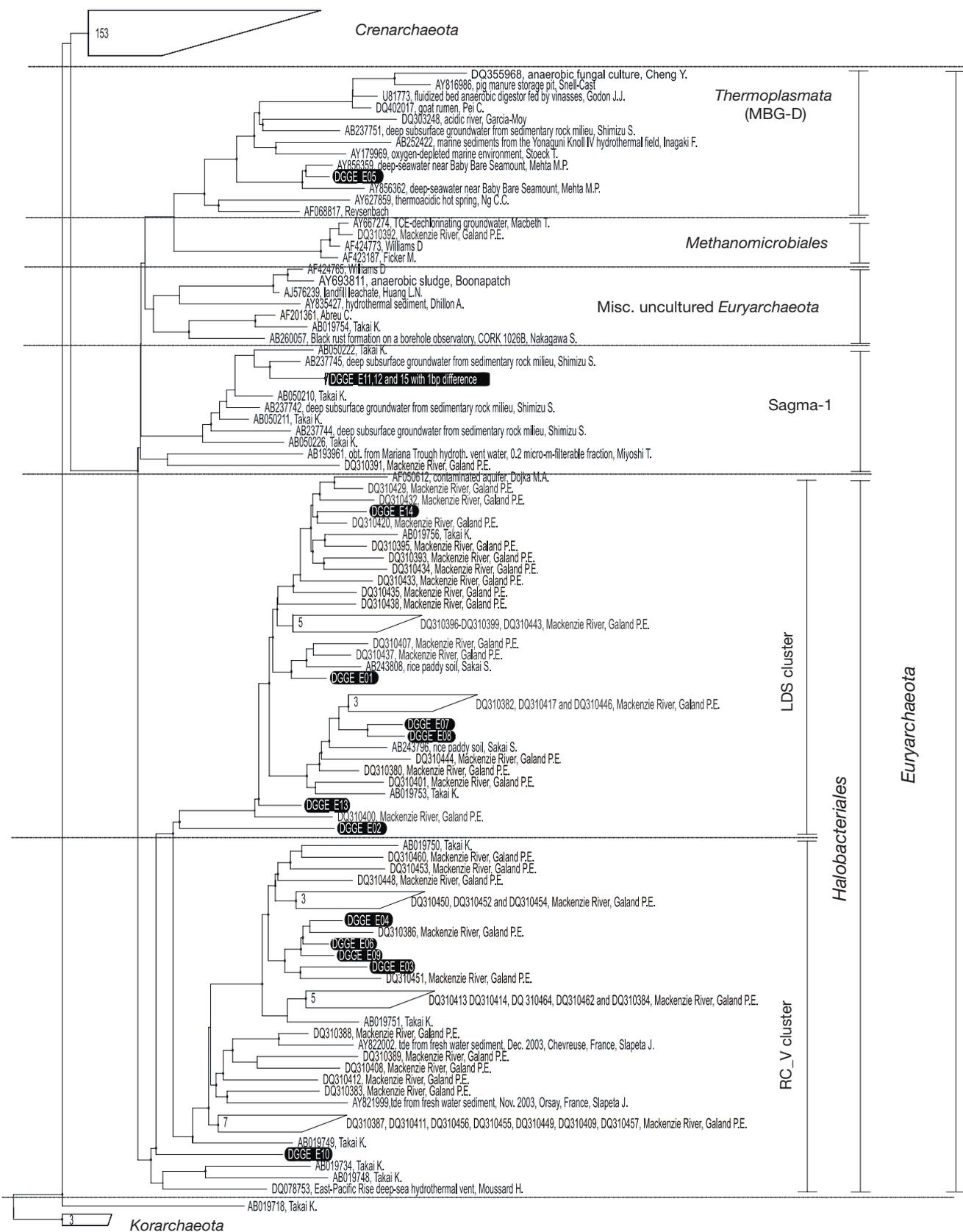


Fig. 3. Neighbor-joining phylogenetic tree showing the affiliation of the euryarchaeotal 16S rRNA gene fragments recovered from the rivers Rhine and Têt to archaeal sequences in the NCBI (National Center for Biotechnology Information) database. Sequences retrieved in the present study are outlined in black and numbered according to the excised DGGE band, while sequences from the NCBI database are identified by accession number, sampling location and first author name. (●): >90% posterior probability values. Scale bar = 10% sequence divergence



Fig. 4. Neighbor-joining phylogenetic tree showing the affiliation of the crenarchaeotal 16S rRNA gene fragments recovered from the rivers Rhine and Têt to archaeal sequences in the NCBI database. Sequences retrieved in the present study are outlined in black and numbered according to the excised DGGE band. (●): >90 % posterior probability values. Scale bar = 10 % sequence divergence

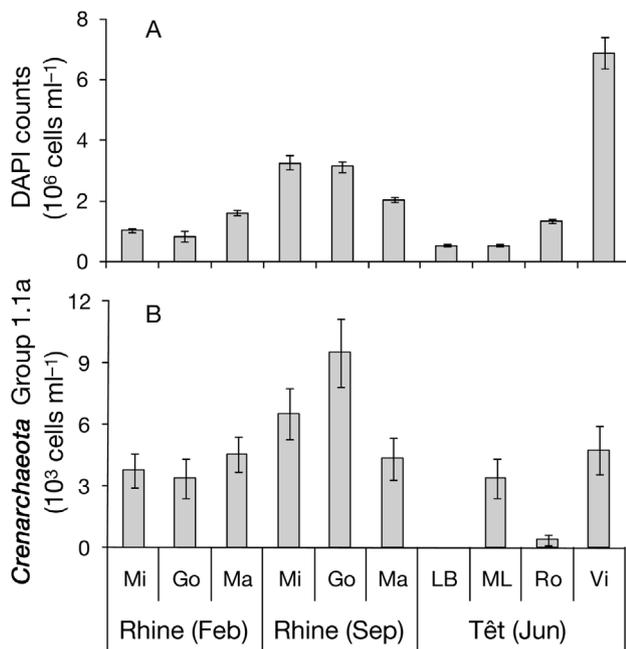


Fig. 5. Abundance of (A) picoplankton and (B) *Crenarchaeota* Group 1.1a at the different sampling sites in the surface waters of the River Rhine in September and February 2005 and the river Têt in June 2005 estimated by DAPI and CARD-FISH (means \pm SD). Station abbreviations are given in Fig. 1

Total and *Crenarchaeota* Group 1.1a cell counts

Total microbial abundance (prokaryotes and pico-eukaryotes) estimated by DAPI counts ranged from 0.5×10^6 to 6.9×10^6 cells ml^{-1} and was, in the River Rhine, on average about twice as high in September than in February (mean \pm SD; $2.8 \pm 0.7 \times 10^6$ and $1.2 \pm 0.4 \times 10^6$ copies ml^{-1} , respectively) (Fig. 5A). CARD-FISH analysis indicated that *Crenarchaeota* Group 1.1a cells were present in the Rhine at all 3 sites and in both seasons (Fig. 5B). In June, *Crenarchaeota* Group 1.1a cells were absent from Lake Bouillouses (Fig. 5B). The contribution of *Crenarchaeota* group 1.1a cell numbers was generally rather low in both rivers, ranging from 0.4×10^3 to 9.5×10^3 cells ml^{-1} (Fig. 5B), constituting only between 0.03 and 0.6% of the total microbial population.

Abundance of *Crenarchaeota* Group 1.1a and crenarchaeotal *amoA* distribution

The abundance of *Crenarchaeota* Group 1.1a as determined by qPCR is shown in Fig. 6 along with the copy numbers of the crenarchaeotal *amoA* gene for the

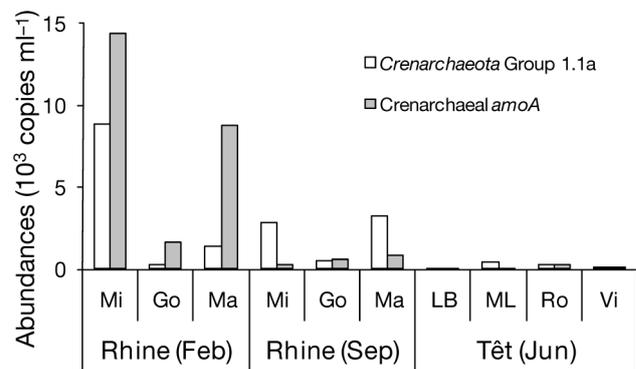


Fig. 6. Abundance of crenarchaeotal *amoA* and 16S rRNA gene copies of *Crenarchaeota* Group 1.1a at the different sampling sites in the surface waters of the River Rhine in September and February 2005 and the River Têt in June 2005 as revealed by qPCR. Station abbreviations are given in Fig. 1

different sampling sites in both rivers. Similar to DGGE and CARD-FISH results, *Crenarchaeota* Group 1.1a were not detected with qPCR in Lake Bouillouses (Fig. 6). The abundance of *Crenarchaeota* Group 1.1a determined by qPCR was on average 1 order of magnitude lower at all sampling sites in the Têt (mean \pm SD; $0.2 \pm 0.2 \times 10^3$ copies ml^{-1}) than in the Rhine ($2.8 \pm 3.1 \times 10^3$ copies ml^{-1}). The relatively large SD reflects important variability between sites in the abundance of *Crenarchaeota* Group 1.1a, but no seasonal pattern could be observed in the Rhine with qPCR (Fig. 6). However, despite considerable variability among the different sampling sites, the copy numbers of crenarchaeotal *amoA* in the Rhine were 14 times higher in February ($8.2 \pm 6.3 \times 10^3$ copies ml^{-1}) than in September ($0.6 \pm 0.3 \times 10^3$ copies ml^{-1}), which corresponds to an average of 5 and 0.5 copies of the crenarchaeotal *amoA* gene for each *Crenarchaeota* Group 1.1a 16S rDNA copy, respectively. The copy numbers of the crenarchaeotal *amoA* gene were extremely low in the Têt, averaging $0.1 \pm 0.1 \times 10^3$ copies ml^{-1} .

Nutrient concentrations

Table 1 gives the dissolved nutrient concentrations of surface water collected in the Rhine in September and February 2005. At 1.6 ± 0.6 and 1.5 ± 0.7 μM (mean \pm SD), the average dissolved phosphate concentrations were similar in September and February 2005. In contrast, dissolved nitrogen concentrations were different between the 2 months, with 2.6-, 1.6- and 5.3-fold higher concentrations of ammonium, nitrate and nitrite, respectively, in February compared to September.

DISCUSSION

Diversity of *Archaea*

The presence of *Euryarchaeota* and *Crenarchaeota* in the surface waters of the rivers Rhine and Têt was established by sequencing 16S rRNA gene fragments (Figs. 2 to 4). *Euryarchaeota* had previously been detected by phylogenetic analyses in sediments of temperate freshwater environments (e.g. MacGregor et al. 1997, Abreu et al. 2001) but not in the water column (Crump & Baross 2000). Recently, using universal archaeal primers developed for soil *Archaea*, Galand et al. (2006) identified 2 novel euryarchaeotal groups, Rice Cluster-V (RC-V) and Lake Dagow Sediment (LDS), in the water of a large Arctic river, the Mackenzie River, during maximum open water conditions (October 2002). RC-V sequences were first detected in anoxic rice paddy soil (Großkopf et al. 1998), whilst LDS were first identified in Lake Dagow sediment (Glissman et al. 2004). These 2 groups represented more than 90% of all clones obtained by Galand et al. (2006). Our DGGE approach indicated that the dominant euryarchaeotal phylotypes in the Rhine, a large European temperate river, also belong to the RC-V and LDS clusters. Furthermore, our data clearly show that these 2 euryarchaeotal groups are not solely found in large rivers, since sequences affiliated with both groups were also retrieved from the Têt, a rather minor European river. This suggests that pelagic *Euryarchaeota* belonging to the RC-V and LDS clusters may be widely distributed in river water. Future studies using CARD-FISH should focus on determining if *Euryarchaeota* from the RC-V and LDS clusters also account for a large proportion of the archaeal cell counts in river water.

Interestingly, in contrast with Galand et al. (2006), >70% of archaeal sequences retrieved from the rivers Rhine and Têt belonged to the *Crenarchaeota*, hence suggesting a higher diversity of pelagic *Crenarchaeota* (40 phylotypes, Fig. 4) over *Euryarchaeota* (15 phylotypes, Fig. 3) in temperate river water. In the only other phylogenetic study that assessed archaeal diversity in temperate river water (Columbia River) using universal archaeal primers, *Euryarchaeota* were found in the estuary but not in the river itself, whilst *Crenarchaeota* were detected in both environments (Crump & Baross 2000). The inconsistency between these 3 studies (Crump & Baross 2000, Galand et al. 2006, and the present study) may be due to methodological differences (e.g. primers used) and/or to location-specific differences (temperate vs. arctic). It is essential to note that, with our primer combination and protocol, we were able to capture without any mismatch 88% of all sequences retrieved by Galand et al. (2006) and >90% of the ~19 000 archaeal sequences available through the

ARB database (release 90), suggesting that the dissimilarity between the data from the present study and that of Galand et al. (2006) are probably not derived from methodological differences. Instead, results from the present study showed that location may be an important determinant of archaeal diversity because, on average, high percentages of crenarchaeotal phylotypes were found in the Rhine in both seasons studied (80% in September and 85% in February), whilst in the Têt more equivalent numbers of *Crenarchaeota* (43%) and *Euryarchaeota* (47%) phylotypes were detected (Figs. 2 to 4). The ratio of *Crenarchaeota* to *Euryarchaeota* phylotypes may thus be determined by location-specific conditions, but more phylogenetic studies on pelagic riverine *Archaea*, preferably combined with data on coinciding physical and chemical growth parameters, are needed to further test this hypothesis.

Most crenarchaeotal groups detected in the present study (Groups 1.1a, 1.3a, 1.3b and 2/3c) were represented in both rivers and all seasons sampled, but *Crenarchaeota* Group 1.1b was only present at all sites in the Rhine in February, while a faint DGGE band appeared in a sample from a single site in the Rhine in September and none in the Têt (Figs. 2 & 4). The presence of *Crenarchaeota* Group 1.1b in the Rhine in February may be linked to increased winter precipitation and the associated runoff because this group has been abundantly detected in soil (Ochsenreiter et al. 2003). However, relative band intensity does not necessarily reflect the actual relative abundance of a given phylotype in environmental samples because of possible PCR bias (Kurata et al. 2004). Nonetheless, given the cosmopolitan distribution of aquatic archaeal phylotypes reported for marine (Massana et al. 2000) and lacustrine environments (Keough et al. 2003), we suggest that a few archaeal phylotypes, such as *Euryarchaeota* Groups RC-V and LDS and *Crenarchaeota* Group 1.1a, are widely distributed in pelagic riverine environments whilst others, such as *Euryarchaeota* Cluster Sagma-1, may only occur seasonally in river water. Our data further highlight the need for more extensive biogeographical studies on pelagic *Archaea* in rivers, especially considering that a recurring temporal pattern has been identified in sedimentary and pelagic bacterial populations of streams and rivers (Crump & Hobbie 2005, Hullar et al. 2006, and that euryarchaeotal and crenarchaeotal seasonality have both been reported in marine environments (Wuchter 2006, Herfort et al. 2007).

Distribution and potential for nitrification of *Crenarchaeota* Group 1.1a

The ability of non-thermophilic *Crenarchaeota* Group 1.1a to oxidize ammonia to nitrite has recently

been demonstrated on the first isolate of this group (Könneke et al. 2005) and in an enrichment culture (Wuchter et al. 2006b). In addition, a number of studies have detected the *amoA* gene encoding the catalytic α subunit of the ammonia monooxygenase, a key enzyme for crenarchaeotal nitrification in aquatic environments (Francis et al. 2005, Hallam et al. 2006, Wuchter et al. 2006b, Coolen et al. 2007, Mincer et al. 2007, Herfort et al. 2007). Beman & Francis (2006) have also identified the presence of ammonia-oxidizing *Archaea* in the sediments of a subtropical estuary.

In the present study, the presence of *Crenarchaeota* Group 1.1a in the river water of the Rhine and Têt was demonstrated by phylogenetic analysis of sequenced DGGE bands and qPCR (Figs. 2, 4 & 6), even if, on average, they only represented a small fraction (0.3%) of the total microbial community as indicated by CARD-FISH (Fig. 5). Although data obtained with qPCR and CARD-FISH agree in that they both showed a lack of apparent seasonality in abundance of *Crenarchaeota* Group 1.1a in the Rhine, these 2 techniques did not show the exact same pattern. For instance, on average, more copy numbers were detected in the Rhine in February than in September, whilst the maximum number of crenarchaeotal cells was found in September (Figs. 5 & 6). This highlights the importance of using multiple approaches for quantifying microbial communities, especially when abundances are as low as those reported here for *Crenarchaeota* Group 1.1a. Nonetheless, in good agreement with the DGGE results, both quantitative approaches clearly indicated a generally higher absolute abundance of *Crenarchaeota* Group 1.1a in the Rhine than in the Têt (Figs. 5B & 6). Indeed, *Crenarchaeota* Group 1.1a 16S rRNA genes (sequenced DGGE bands) were only recovered from the River Têt at Mont-Louis, and a relatively higher number of cells (except for Villelongue) and 16S rRNA genes were also only found at this station. The high counts of *Crenarchaeota* Group 1.1a at Villelongue were counts of cells with an extremely faint CARD-FISH signal compared to those observed elsewhere (including the Rhine). Villelongue is located downstream of the inflow of treated water of the Perpignan sewage treatment plant. Given that high concentrations of organic matter, inorganic nutrients and bacteria have been shown to emanate from such effluents (Servais et al. 1999) and that *amoA* genes have been detected in sewage effluents (Park et al. 2006), the observed peak in *Crenarchaeota* Group 1.1a abundance likely results from local input of allochthonous communities.

The importance of crenarchaeotal nitrification is now a major question in aquatic microbial ecology (Karl 2007). We report here for the first time that pelagic *Crenarchaeota* Group 1.1a of riverine systems also

harbors the *amoA* gene (Fig. 6). To confirm which *amoA* was quantified, we subjected the *amoA* qPCR products to DGGE followed by phylogenetic analysis of sequenced DGGE fragments. This was performed for water collected at one site in the Rhine (data not shown) and revealed that the quantified crenarchaeotal *amoA* sequences were identical to those obtained in the southern North Sea at the South Frisian Front in February (see Herfort et al. 2007 for more details). It is important to note that the presence of *amoA* genes is only an indication of a potential for nitrification that cannot be directly related to actual ammonia oxidation rates. Only process-oriented studies, such as the analysis of *amoA* gene transcripts (i.e. mRNAs of *amoA*), provide definite proof of active crenarchaeotal nitrification at the time of sampling (Treich et al. 2005). Unfortunately, we did not attempt this analysis since our samples were not obtained and stored properly to guarantee the preservation of mRNA. Interestingly, crenarchaeotal *amoA* genes were on average more abundant in the Rhine in February (8.2×10^3 copies ml^{-1}) than in September (0.6×10^3 copies ml^{-1}) (Fig. 6) and the proportion of *Crenarchaeota* Group 1.1a to crenarchaeotal *amoA* as revealed by qPCR varied over time (5.0 ± 2.9 in the Rhine in February and 0.5 ± 0.5 in September); for the River Têt, the ratio was 0.7 ± 1.5 in June. Variation in numbers of *amoA* gene copies per cell has also been reported for the marine environment. Wuchter et al. (2006b) reported a single copy of *amoA* gene per cell of an enriched crenarchaeote from the North Sea, but 2.8 copies per crenarchaeotal cell for water freshly collected in the North Sea. The observed variation in *amoA* copy numbers per *Crenarchaeota* Group 1.1a 16S rRNA gene copy in river water may indicate that the relative contribution of crenarchaeotal nitrification of Group 1.1a to total nitrification activity varies seasonally. Again, it is essential to remember that this is a DNA study, which therefore only suggests a potential for crenarchaeotal nitrification but not activity or rate. Nonetheless, there is an apparent seasonal change in this potential which may indeed be explained by a seasonal variation in crenarchaeotal nitrification. In support of this idea, a winter occurrence of pelagic marine *Crenarchaeota* Group 1.1a and crenarchaeotal *amoA* gene has also been found in the North Sea (Wuchter et al. 2006b, Herfort et al. 2007). In addition, high nutrient concentrations in marine waters have been linked to crenarchaeotal nitrification (Herfort et al. 2007), and in the present study, the higher number of *amoA* copies found in February in the Rhine was also associated with higher concentrations of ammonium, nitrate and nitrite (Table 1). Correlations between microbial processes and biogeochemical parameters have previously been described for the Rhine. For example, Admiraal et al. (1994)

reported that in winter in the lower Rhine, relatively high bacterial production rates were associated with large inputs of organic matter. Seasonal variations in river nitrification rates are not uncommon either, since different nitrification rates have in fact been measured in summer and winter in the River Nervión (Spain) (Iriarte et al. 1997). In temperate regions, similar to marine environments, river pelagic crenarchaeotal nitrification of Group 1.1a may thus primarily take place in the winter when bacterial and phytoplankton abundances are low. Clearly, future studies on riverine *Archaea* should not only focus on determining the biogeography of pelagic *Archaea*, especially comparing temperate and tropical rivers, but also on assessing the seasonal importance of crenarchaeotal nitrification to the nitrogen cycle, in particular that of the other *Crenarchaeota* groups that have been identified in river water in the present study.

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