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Vertical Distribution of Ammonia-Oxidizing Crenarchaeota and Methanogens in the Epipelagic Waters of Lake Kivu (Rwanda-Democratic Republic of the Congo)▽†

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Four stratified basins in Lake Kivu (Rwanda-Democratic Republic of the Congo) were sampled in March 2007 to investigate the abundance, distribution, and potential biogeochemical role of planktonic archaea. We used fluorescence *in situ* hybridization with catalyzed-reported deposition microscopic counts (CARD-FISH), denaturing gradient gel electrophoresis (DGGE) fingerprinting, and quantitative PCR (qPCR) of signature genes for ammonia-oxidizing archaea (16S rRNA for marine *Crenarchaeota* group 1.1a [MCG1] and ammonia monooxygenase subunit A [*amoA*]). Abundance of archaea ranged from 1 to 4.5% of total DAPI (4′,6-diamidino-2-phenylindole) counts with maximal concentrations at the oxic-anoxic transition zone (~50-m depth). Phylogenetic analysis of the archaeal planktonic community revealed a higher level of richness of crenarchaeal 16S rRNA gene sequences (21 of the 28 operational taxonomic units [OTUs] identified [75%]) over euryarchaeotal ones (7 OTUs). Sequences affiliated with the kingdom *Euryarchaeota* were mainly recovered from the anoxic water compartment and mostly grouped into methanogenic lineages (*Methanosarcinales* and *Methanocellales*). In turn, crenarchaeal phylotypes were recovered throughout the sampled epipelagic waters (0- to 100-m depth), with clear phylogenetic segregation along the transition from oxic to anoxic water masses. Thus, whereas in the anoxic hypolimnion crenarchaeotal OTUs were mainly assigned to the miscellaneous crenarchaeotic group, the OTUs from the oxic-anoxic transition and above belonged to *Crenarchaeota* groups 1.1a and 1.1b, two lineages containing most of the ammonia-oxidizing representatives known so far. The concomitant vertical distribution of both nitrite and nitrate maxima and the copy numbers of both MCG1 16S rRNA and *amoA* genes suggest the potential implication of *Crenarchaeota* in nitrification processes occurring in the epilimnetic waters of the lake.

Lake Kivu is a meromictic lake located in the volcanic region between Rwanda and the Democratic Republic of the Congo and is the smallest of the African Great Rift Lakes. The monimolimnion of the lake contains a large amount of dissolved CO_2 and methane (300 km^3 and 60 km^3, respectively) as a result of geological and biological activity (24, 73, 85). This massive accumulation converts Lake Kivu into one of the largest methane reservoirs in the world and into a unique ecosystem for geomicrobiologists interested in the methane cycle and in risk assessment and management (34, 71, 72, 85). Comprehensive studies on the diversity and activity of planktonic populations of both large and small eukaryotes and their trophic interplay operating in the epilimnetic waters of the lake are available (33, 39, 49). Recent surveys have also provided a deeper insight into the seasonal variations of photosynthetic and heterotrophic picoplankton (67, 68), although very few data exist on the composition, diversity, and spatial distribution of bacterial and archaeal communities. In this regard, the studies conducted so far of the bacterial/archaeal ecology in Lake Kivu have been mostly focused on the implications on the methane cycle (34, 73), but none have addressed the presence and distribution of additional archaeal populations in the lake.

During the last few years, microbial ecology studies carried out in a wide variety of habitats have provided compelling evidence of the ubiquity and abundance of mesophilic archaea (4, 10, 13, 19). Moreover, the discovery of genes encoding enzymes related to nitrification and denitrification in archaeal metagenomes from soil and marine waters (29, 86, 88) and the isolation of the first autotrophic archaeal nitrifier (40) demonstrated that some archaeal groups actively participate in the carbon and nitrogen cycles (56, 64, 69). In relation to aquatic environments, genetic markers of ammonia-oxidizing archaea (AOA) of the marine *Crenarchaeota* group 1.1a (MCG1) have consistently been found in water masses of several oceanic regions (6, 14, 17, 26, 28, 30, 37, 42, 51, 52, 89), estuaries (5, 9, 26, 53), coastal aquifers (26, 66), and stratified marine basins (15, 41, 44). Although less information is available for freshwater habitats, recent studies carried out in oligotrophic high-

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mountain and arctic lakes showed an important contribution of AOA in both the planktonic and the neustonic microbial assemblages (4, 61, 89).

The oligotrophic nature of Lake Kivu and the presence of a well-defined redoxcline may provide an optimal niche for the development of autotrophic AOA populations. Unfortunately, no studies of the involvement of microbial planktonic populations in cycling nitrogen in the lake exist, and only data on the distribution of dissolved inorganic nitrogen species in relation to phytoplankton ecology (67, 68) and nutrient loading are available (54, 58). Our goals here were to ascertain whether or not archaeal populations other than methane-related lineages were relevant components of the planktonic microbial community and to determine whether the redox gradient imposed by the oxic-anoxic interphase acts as a threshold for their vertical distribution in epipelagic waters (0- to 100-m depth). To further explore the presence and potential activity of nitrifying archaeal populations in Lake Kivu, samples were analyzed for the abundance and vertical distribution of signature genes for these microorganisms, i.e., the 16S rRNA of MCG1 and the ammonia monooxygenase subunit A (*amoA*) gene by quantitative PCR.

MATERIALS AND METHODS

Study site, sampling, and chemical analysis. Lake Kivu is located between Rwanda and the Democratic Republic of the Congo (2°S, 29°E; Fig. 1) at 1,463 m above sea level. It has a surface area of 2,370 km^2 and a total volume of 580 km^3. The lake is a deep (maximum depth, 489 m) meromictic and oligotrophic body of water with step increases in temperature and salinity gradients. Further details on the hydrology, physicochemistry, and biology of the lake are published elsewhere (33, 67, 68).

Water samples were collected during a sampling campaign conducted during the rainy season (March 2007). The sampling cruise tried to cover the spatial variability within the lake, and four sites were sampled: northern (NB), eastern (EB), and southern (SB) basins and Bukavu Bay (BB) (see Fig. 1 for the exact locations of the sampling sites). Temperature, conductivity, pH, and oxygen were measured *in situ* with a YSI 6600 V2 multiparametric sonde (Yellow Spring Instruments). Water samples for physicochemical and biological analyses were collected using a 5-liter vertical VanDorn bottle and stored in 4-liter plastic containers that were stored at 4°C in a portable icebox until further processing. Ammonia concentrations were determined using the dichloroisocyanurate-salicylate-nitroprussiate colorimetric method (76). Nitrite concentrations were determined by the sulfanilamide coloration method (2). Nitrate concentrations were determined after cadmium reduction to nitrite and quantified under this form following the nitrite determination procedure (2, 35). The detection limits for these methods were 0.3, 0.03, and 0.15 μM for NH_{4^+}, NO_{2^-}, and NO_{3^-}, respectively.

Prokaryotic cell counts. Water samples (100 ml) were fixed *in situ* with paraformaldehyde (PFA) (final concentration, 2% [wt/vol]) and stored overnight at 4°C in the dark. Water samples were passed through white 0.22-μm-pore-size polycarbonate filters (25-mm filter diameter; Millipore, Eschborn, Germany), washed twice with phosphate-buffered saline (PBS) buffer (pH 7.6), dried, and stored at −20°C until analysis. Total cell numbers were determined by epifluorescence counting after staining with 4′,6-diamidino-2-phenylindole (DAPI) as previously described (60). *Bacteria* and *Archaea* were enumerated by fluorescence *in situ* hybridization with catalyzed-reported deposition (CARD-FISH) using specific probes EUB338 and ARCH915 (Table 1), using a modification of the protocol described by Teira and coworkers (82) to improve cell wall permeabilization. Cell losses during permeabilization and filter processing were minimized by dipping the filters in low-gelling-point agarose (0.1% [wt/vol], in Milli-Q water) and drying them upside down on a glass petri dish at 37°C. The filters were subsequently dehydrated in 95% ethanol (vol/vol) and allowed to air dry. To inhibit potentially present intracellular peroxidases, filters were incubated with 0.01 M HCl at room temperature (RT) for 20 min, washed with 1× PBS buffer and Milli-Q water, and dried. For cell wall permeabilization, filters were incubated with lysozyme solution (10 mg ml^{-1} in 0.05 M EDTA, and 0.1 M Tris-HCl at pH 8.0; Fluka) for 30 min at 37°C and, afterwards, gently rinsed with Milli-Q water and absolute ethanol. Filters were then incubated with proteinase K (0.25 U mg^{-1}, concentration, 0.25 mg ml^{-1} in 0.05 M EDTA and 0.1 M Tris-HCl [pH 8.0]; Roche) for 5 min at 50°C and washed with Milli-Q water. Subsequently, the filters were incubated with 4% PFA (wt/vol, final concentration) for 5 min at room temperature, washed with Milli-Q water, dehydrated with absolute ethanol, and air dried. Probe hybridization, washing, signal amplification, and filter preparation were performed as described previously (82), except that 55% (vol/vol) formamide was used for both probes (Table 1). Finally, filter sections were air dried, embedded in Citifluor antifading solution (Citifluor Ltd., London, United Kingdom), and examined under an Axioskop epifluorescence microscope (Zeiss, Germany) equipped with a 50-W Hg bulb and appropriate filter sets for DAPI and Alexa-Fluor 488. Triplicate filters were processed independently for each depth. At least 40 microscopic fields were randomly selected to count DAPI-stained and probe-hybridized cells.

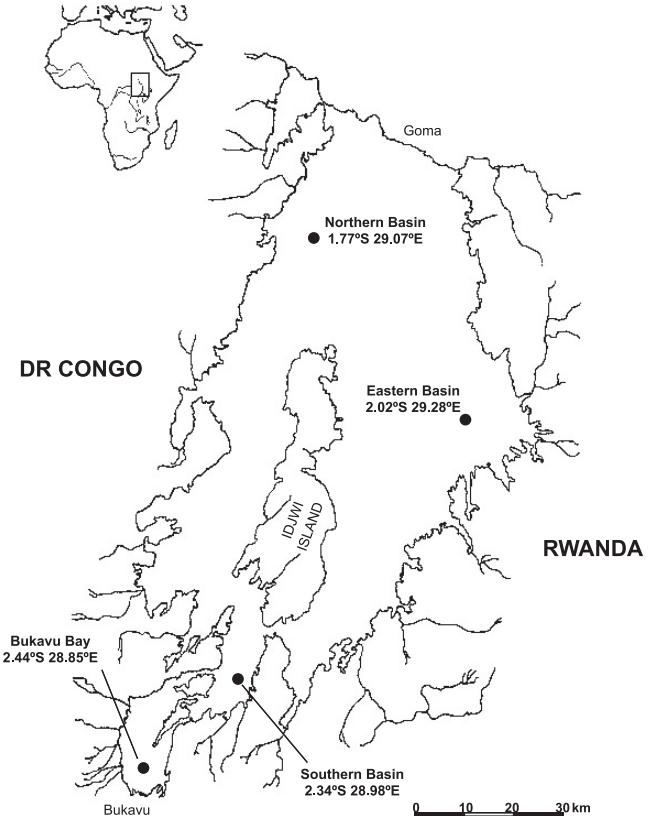

FIG. 1. Geographical location of Lake Kivu and the four sampling stations: northern (NB), southern (SB), eastern (EB) and Bukavu Bay (BB) basins. The geographical GPS (global positioning system) coordinates for each sampling site are indicated. Basins are named as by Sarmento et al. (67, 68).

DNA extraction, PCR amplification, and DGGE fingerprinting. Water samples (0.5 to 1.0 l) for DNA extraction were first passed through 5.0-μm-pore-size, 47-mm-diameter polycarbonate filters (ISOPORE, Millipore, MA) to remove particulate debris as well as large protozoa, which are potential hosts for endosymbiotic archaea (i.e., methanogens) (11). Eluents were then passed through 0.22-μm-pore-size, 47-mm-diameter polycarbonate filters (ISOPORE, Millipore, MA) to retain free-living prokaryotes. Total nucleic acids were extracted using a combination of enzymatic cell lysis and cetyltrimethyl ammonium bromide (CTAB) extraction protocol as previously described (45). Dry DNA pellets were finally rehydrated in 50 μl of 10 mM Tris-HCl buffer (pH 7.4) and further purified using Centricon cleaning columns (Millipore, MA). DNA concentration and purity were then determined in a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop, DE). Purified DNA extracts were stored at −80°C until use.

Amplification of archaeal 16S rRNA gene (ca. 600 bp) was performed using the universal primer combination 21F-958R (19) followed by nested reactions using two primer pair combinations specifically targeting the domain *Archaea* and the kingdom *Crenarchaeota* (Table 1). Amplification of the *amoA* gene,

which encodes the archaeal ammonium monooxygenase subunit A, was performed as described by Francis and coworkers (26). PCR conditions used for amplification of both genes are listed in Table 1. Fingerprinting analyses of the archaea and crenarchaeota planktonic assemblages, as well as of the archaeal *amoA* gene fragments, were carried out by denaturing gradient gel electrophoresis (DGGE) (55) in an INGENY phorU-2 DGGE system (Ingeny International BV, Netherlands). Between 500 and 1,000 ng of PCR product was loaded onto 6.0% polyacrylamide gels and run with 1× TAE buffer using 20 to 80% (16S rRNA) and 20 to 70% (*amoA*) linear gradients of urea and formamide (100% denaturant agent contains 7 M urea and 40% deionized formamide). A DGGE ladder composed of a mixture of known small-subunit (SSU) rRNA gene fragments was loaded in all gels to allow intergel comparison of band migration. Electrophoreses were performed at 60°C and at a constant voltage of 120 V for 17 h. After electrophoresis, gels were stained for 30 min with 1× SYBR gold nucleic acid stain (Molecular Probes Inc.) in 1× TAE buffer, rinsed, and visualized under UV radiation using a GelPrinter system (TDI, Spain). Discrete and clear bands were excised from the gels and rehydrated overnight in 50 μl of 10 mM Tris-HCl buffer (pH 7.4). DNA was eluted after incubation at 65°C for 3 h and amplified using the corresponding primer pairs (without GC clamp) and PCR conditions as cited above (Table 1), but the number of PCR cycles was decreased to 20. PCR products without further treatment were sequenced on both strands using external facilities (Macrogen Inc., Seoul, South Korea).

Gel image analysis. Digital images of acrylamide gels were analyzed using the GELCompar II v.5.1 software package (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Lanes were manually defined, and band positions were identified from corrected intensity plots. Comparison between samples loaded on different DGGE gels was completed using normalized values derived from known standards. A binary matrix showing the presence/absence of identified bands was constructed for all gel lanes. Further, a similarity matrix based on the Dice coefficient was calculated, and samples were clustered according to the unweighted-pair group average linkage method (UPGMA) algorithm using a tolerance position value of 2%.

Phylogenetic analysis. All representative archaeal 16S rRNA sequences from Lake Kivu (84 in total) were analyzed for the presence of chimeras using the Bellerophon tool available at the GreenGenes website (http://greengenes.lbl.gov/) (22). Sequences were then aligned in mothur (http://www.mothur.org) (70) using the SILVA archaeal database as reference alignment. The same program was used to calculate a neighbor-joining (NJ) (65) distance matrix using the Jukes-Cantor (JC) correction, which was then used to assign sequences to operational taxonomic units (OTUs) defined at a 97% cutoff using the furthest-neighbor algorithm. Representative sequences for each OTU were identified using the implemented tool in mothur.

The phylogenetic tree was constructed after importing the alignment into the ARB software package (48) loaded with the SILVA 16S rRNA-ARB-compatible database (SSURef-102, February 2010; http://www.arb-silva.de) and checked manually. An archaeal backbone tree was built with reference sequences of at least 900 bp in length using the NJ algorithm and JC-corrected distances. The aligned sequences from Lake Kivu were then added to this tree using the "parsimony quick add marked" tool, thereby maintaining the overall tree topology. Bootstrap support (1,000 replicates) was calculated in PHYLIP (25) using JC evolutionary distances and the NJ method. Cluster names and grouping used in this study were based on the cluster definitions for *Euryarchaeota* and *Crenarchaeota* proposed by Takai and Horikoshi (77) and DeLong (20), respectively.

Environmental sequences of archaeal *amoA* genes were obtained from public databases and aligned with those retrieved in DGGE gels from Lake Kivu using MEGA4 (80). The phylogenetic analysis was inferred using the NJ method, and evolutionary distances were computed by JC with 1,000 bootstrap replicates.

Quantitative PCR (qPCR). Gene copy numbers of 16S rRNA from MCG1 and the archaeal *amoA* gene were determined by quantitative real-time PCR amplification from DNA extracts obtained from samples collected at the eastern and southern basins of Lake Kivu. All qPCR assays were performed in a 7500 real-time PCR system (Applied Biosystems) using the primers and conditions listed in Table 1. All reactions were carried out in MicroAmp optical 96-well reaction plates covered with optical caps (Applied Biosystems). The reaction mixture (20 μl) contained 10 μl of iQ SYBR green supermix (Bio-Rad, Hercules, CA), 10 μM corresponding primers (Table 1), molecular biology-grade water (Eppendorf), and 9 μl of template DNA (36 ng). Data were collected and analyzed with the 7500 SDS system software version 1.4 (Applied Biosystems). All qPCRs consisted of an initial denaturing step for 4 min at 95°C, followed by 60 cycles of the appropriate quantitative PCR program (Table 1). The fluorescence signal was read in each cycle after the elongation step at 78°C over a period of 32 s to ensure stringent product quantification. All reactions were performed in triplicate, with standard curves spanning from 10^1 to 10^7 and from 10^2 to 10^8

TABLE 1. PCR and qPCR conditions, archaeal primers, and CARD-FISH probes used in this work

Process	Target	Primer pair[a]	CARD-FISH probe	PCR conditions[b]								Final probe concn (ng/μl)	Reference(s)
				Cycles	Denaturation		Annealing		Elongation				
					°C	min	°C	min	°C	min			
Endpoint PCR	16S rRNA gene												
	First round												
	Universal *Archaea*	21f/958r		30	94	1	56	1	72	2			19
	Second round (nested)												
	General *Archaea*	PAIR*a* (ARC344f/ARC915r)		16 + 10	94	1	68°/60	1	72	1.5			11
	Freshwater *Crenarchaeota*	PAIR*b* (ARC337f/ARC915r)		25	94	1	58	1	72	1.5			45
	Functional gene												
	Archaeal *amoA*	Arch amoAf/amoAr		35	94	0.75	55	1	72	1			26
qPCR	16S rRNA gene												
	Marine *Crenarchaeota* group 1	MCGI-391f/MCGI-554r		60	94	0.5	61	0.66	72	0.66			79
	Functional gene												
	Archaeal *amoA*	AOA-amoA-f/AOA-amoA-r		60	94	0.5	59	0.66	72	0.66			89
CARD-FISH	*Eubacteria*		HRP-EUB338									0.28	1, 16
	Archaea		HRP-ARC915									0.84	75

[a] A GC-rich clamp was attached to the 5′ end of each forward primer used in all amplification reactions used to generate amplicons for DGGE analysis.

[b] For endpoint PCR, the temperature was held at 94°C for 4 min before each run of cycles and kept at 72°C for 30 min after all cycles were completed to allow final template elongation. The CARD-FISH conditions were 55% formamide and 3 mM NaCl.

[c] The program consisted of a touchdown protocol where the initial annealing temperature decreased 0.5°C each cycle during the first 16 cycles.

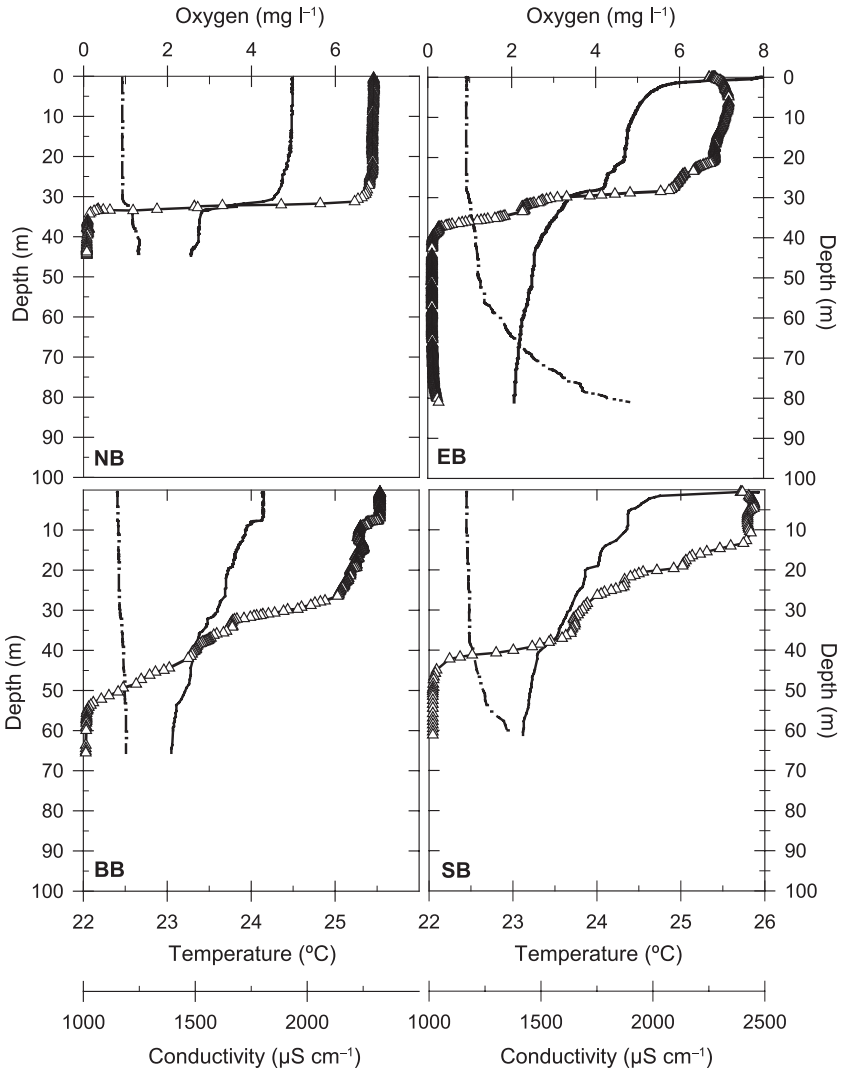

FIG. 2. Depth profiles of temperature (solid line), conductivity (dashed line), and dissolved oxygen (open triangles) in the different basins on the day of sampling.

for 16S rRNA and *amoA* genes, respectively. Standard curves were generated from serial dilutions of previously titrated suspensions of the desired genes (16S rRNA and *amoA*) amplified by conventional PCR from environmental clones (FN691587 for 16S rRNA and FN773417 for crenarchaeal *amoA*), purified (QIAquick; Qiagen), and quantified. Overall, average efficiencies for all quantification reactions ranged from 84% for MCG1 to 88% for archaeal *amoA* with R^2 values of >0.99. The specificity of reactions was confirmed by melting curve analyses and by separating the obtained amplicons by agarose gel electrophoresis to identify unspecific PCR products such as primer dimers or gene fragments of unexpected length (data not shown).

Nucleotide sequence accession numbers. The 16S rRNA sequences obtained in this study were deposited in the GenBank database under accession numbers EU921487 to EU921548 and FJ536696 to FJ536719. The *amoA* gene sequences were deposited under accession numbers EU921473 to EU921486 and FJ536694 to FJ536695.

RESULTS

Physicochemical characterization of the sampling stations. The physicochemical depth profiles of the four sampled stations (Fig. 2) showed downward, stepwise oxic and thermal stratification patterns characteristic of the rainy season (67). The low-level mixing conditions during this season allowed the establishment of a temporary stratification in the epilimnion, which expands from the surface to a 35- to 65-m depth depending on the station. Below a 65-m depth, permanently anoxic waters extend to the bottom of the lake (489 m at its maximal depth). Two well-defined oxycline patterns can be clearly distinguished between stations located at the main basin of the lake (northern [NB] and eastern [EB] basins) and those located at the southern, more wind-protected, side of the lake (southern basin [SB] and Bukavu Bay [BB]). Whereas the former basins (NB and EB) showed a steep oxycline between 30 and 40 m depth, the oxygen concentration profiles in the latter basins (SB and BB) decreased more smoothly, with an oxycline extending from 10 to 50 to 60 m depth. The fact that deep waters of BB are isolated from SB by the presence of a shallow sill (Fig. 1) and the lack of any chemical stratification and its shallower depth (90 m) lead to less gas accumulation in Bukavu Bay than in other basins (81).

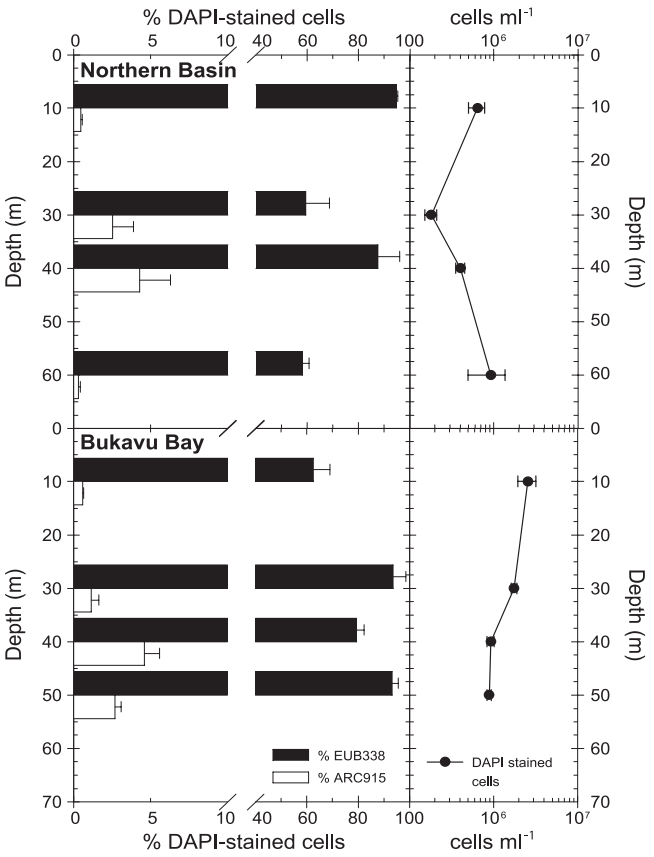

FIG. 3. Relative abundances of *Bacteria* and *Archaea* obtained after CARD-FISH counts (left) and total cell numbers (DAPI staining, right) in northern basin (NB) and Bukavu Bay (BB). Error bars show the standard deviations from results for triplicate filters (see Materials and Methods for details).

Prokaryotic community composition determined by CARD-FISH. Total cell numbers determined by DAPI staining ranged from $(1.80 \pm 0.39) \times 10^5$ to $(9.26 \pm 5.80) \times 10^5$ cells ml^{-1} and from $(8.90 \pm 0.58) \times 10^5$ to $(2.57 \pm 0.77) \times 10^6$ cells ml^{-1} in NB and BB, respectively (Fig. 3, right panels). These abundances are within the same range of those previously reported in the lake by Sarmento and coworkers using flow cytometry (68).

The specific contribution of members of the *Bacteria* and the *Archaea* to the microbial planktonic community was estimated using specific CARD-FISH probes for both domains (Table 1). The sum of abundances of positive hybridized cells (*Bacteria* plus *Archaea*) ranged from 52% to 96% and from 64% to 94% of total cell numbers determined by DAPI staining in NB and BB, respectively. These values agree with those reported for other environments (3, 82). In both basins, *Bacteria* were dominant, with relative abundances ranging from 51.4 ± 15.8% to 95.7 ± 3.5% and from 63.1 ± 10.1% to 93.2 ± 9.5% of total DAPI-stained cells in NB and BB, respectively (Fig. 3, left panels). The relative contribution of archaea to total cell numbers ranged from 0.3 ± 0.1% to 4.3 ± 2.2% and from 0.6 ± 0.1% to 4.5 ± 1.7% in NB and BB, respectively, with higher values at the oxic-anoxic boundary layer (~40-m depth).

Richness and phylogenetic diversity of the archaeal planktonic community. To recover the maximal archaeal richness, two primer combinations that allowed amplification of members of the domain *Archaea* (PAIR*a*) and the kingdom *Crenarchaeota* (PAIR*b*) were applied (Table 1). Despite the selectivity of both primer combinations and the use of previously optimized PCR protocols (45), amplicons suitable to further DGGE analyses were obtained only after nested reactions. DGGE fingerprints of these PCR products showed great similarities for all basins regardless of the primer pair applied (see Fig. S1 and S2 in the supplemental material). The use of an internal DGGE ladder allowed the correction of minor differences in band migration and the proper comparison of fingerprints from the different basins.

The phylogenetic identification of the recovered sequences revealed remarkable aspects of the structure of the planktonic archaeal assemblage. Most of the sequences retrieved from all basins and sampling depths were detected using both primer combinations. This result indicated that no substantial biases were introduced by the primer pair used and that the differences in the archaeal assemblage above and below the oxic-anoxic transition were consistent throughout the lake. Some bands that halted at different positions in the gels resulted in almost identical sequences (≥98% similarity, e.g., *a*K1 [EU921507] [see Table S1 in the supplemental material for band codes and accession numbers], *a*K2 [EU921487], and *a*K9 [EU921491] in Fig. S1 in the supplemental material or *b*K1 [EU921497], *b*K2 [EU921512], *b*K3 [EU921498] and *b*K4 [EU921499] in Fig. S2 in the supplemental material), and they were accordingly ascribed to the same OTU (based on a 97% cutoff). This anomaly has previously been observed by other authors when profiling microbial communities by DGGE and is related to either variable melting behaviors or multiple ribosomal gene copies in a single organism (12).

From all samples, we recovered 28 unique OTUs from 84 16S rRNA gene sequences. Five out of the seven OTUs aligned to *Euryarchaeota* were assigned to methanogenic lineages (*Methanosarcinales* [OTUs 1 to 4] and *Methanocellales* [OTU-5]) (Fig. 4). Some sequences within these OTUs were unexpectedly recovered from oxic layers (see Fig. S1 and Table S1 in the supplemental material). The remaining two OTUs were assigned to *Thermoplasmata* (OTU-6) and to deep hydrothermal vent euryarchaeotic group II (DHVE-5) (OTU-7) (77).

OTUs belonging to *Crenarchaeota* (21 in total) were assigned to different lineages with a clear segregation above and below the oxic-anoxic transition. Sequences recovered from the epilimnion and the oxic-anoxic interphase mainly belonged to *Crenarchaeota* group 1.1a and grouped into a single OTU (OTU-8). The 37 sequences within this OTU showed similarities ranging from 94.9% to 96.2% to "*Candidatus* Nitrosopumilus maritimus," the unique member of marine AOA isolated to date (40). The rest of the sequences recovered from the oxic water compartment affiliated with *Crenarchaeota* group 1.1.b and were distributed into 11 OTUs (OTUs 9 to 19). The remaining sequences belonged to crenarchaeal lineages containing mesophilic representatives from diverse origins, such as group 1.2 (also named C3 by DeLong and Pace [21]; OTU-20 and -21), group 1.3 (also referred to as the miscellaneous crenarchaeotic group [MCG] by Inagaki and coworkers

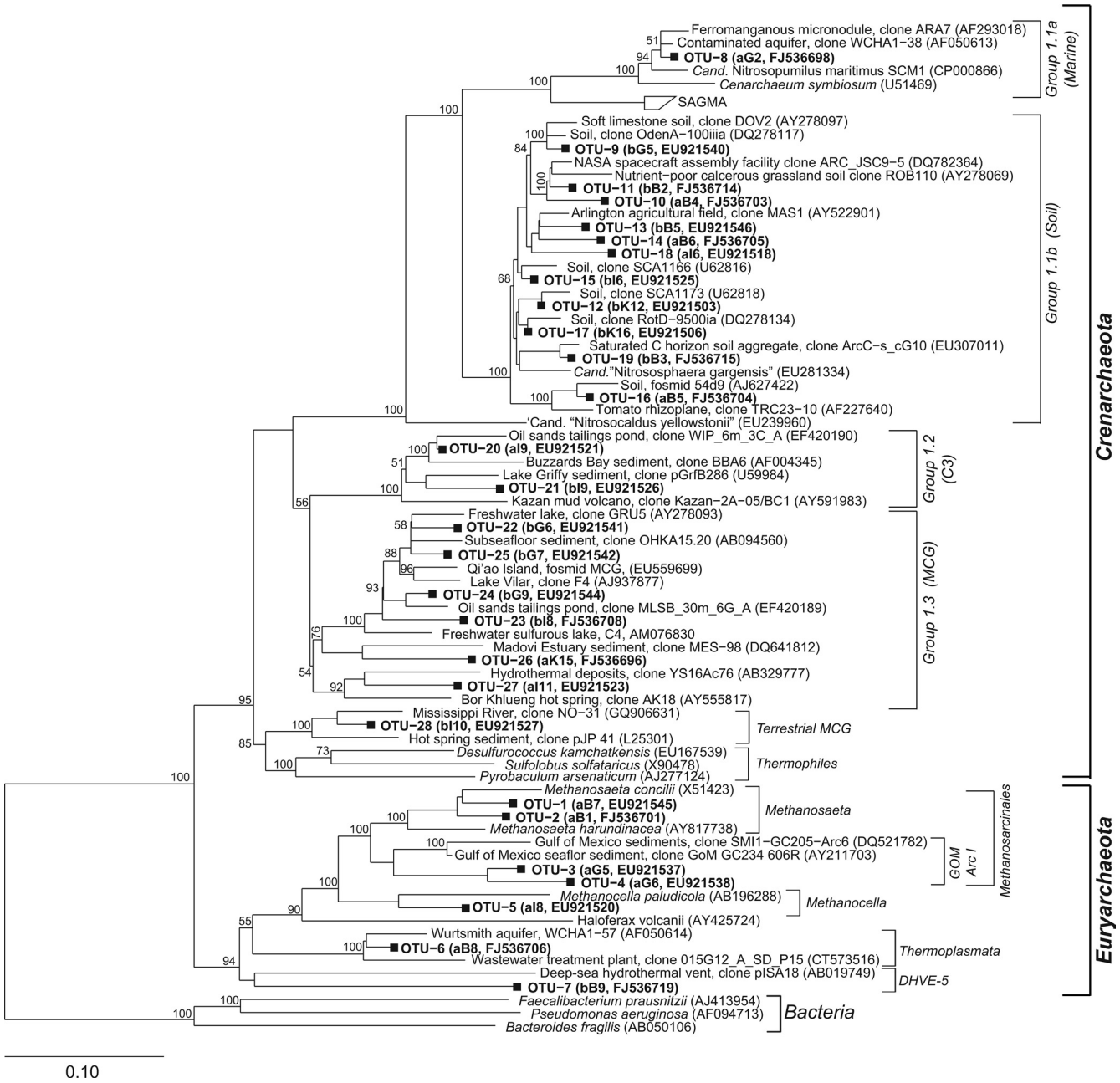

FIG. 4. Neighbor-joining phylogenetic tree generated by the ARB software package showing the affiliation of the OTUs retrieved from Lake Kivu (in bold). Bootstrap support values of >50% (1,000 replicates) are shown. The scale bar indicates 10% estimated sequence divergence. OTU identification numbers (OTU ID) correspond to the same numbers shown in Tables 1 and 2 in the supplemental material. Sequences after OTU ID correspond to the representative sequence for each OTU obtained with mothur (see Materials and Methods for details). Sequences are named according to the primer used (*a* or *b* for primer PAIR*a* or PAIR*b*, respectively), the basin (K for Kibuye [eastern basin], I for Ishungu [southern basin], G for Goma [northern basin], and B for Bukavu [Bukavu Bay]), and the band number for each basin (see Fig. S1 and S2 in the supplemental material).

[32]; OTUs 22 to 27), and the terrestrial MCG (OTU-28 [78]) (Fig. 4 and Tables S1 and S2 in the supplemental material).

Vertical distribution and abundance of ammonia-oxidizing crenarchaeota and diversity of *amoA* genes. DGGE fingerprinting of the archaeal *amoA* gene was carried out to resolve its phylogenetic richness along the vertical profile of the sampled stations. Identical fingerprints were obtained for samples from all basins; these fingerprints revealed a four-band pattern that was especially intense at those depths where distinct maxima of nitrite, nitrate, MCG1 16S rRNA, and *amoA* genes were measured (Fig. 5). The comparison of *amoA* nucleotide sequences obtained from all bands revealed a high similarity between them (99.9 to 100% of sequence identity). Similar banding patterns and similarity indices for *amoA* archaeal se-

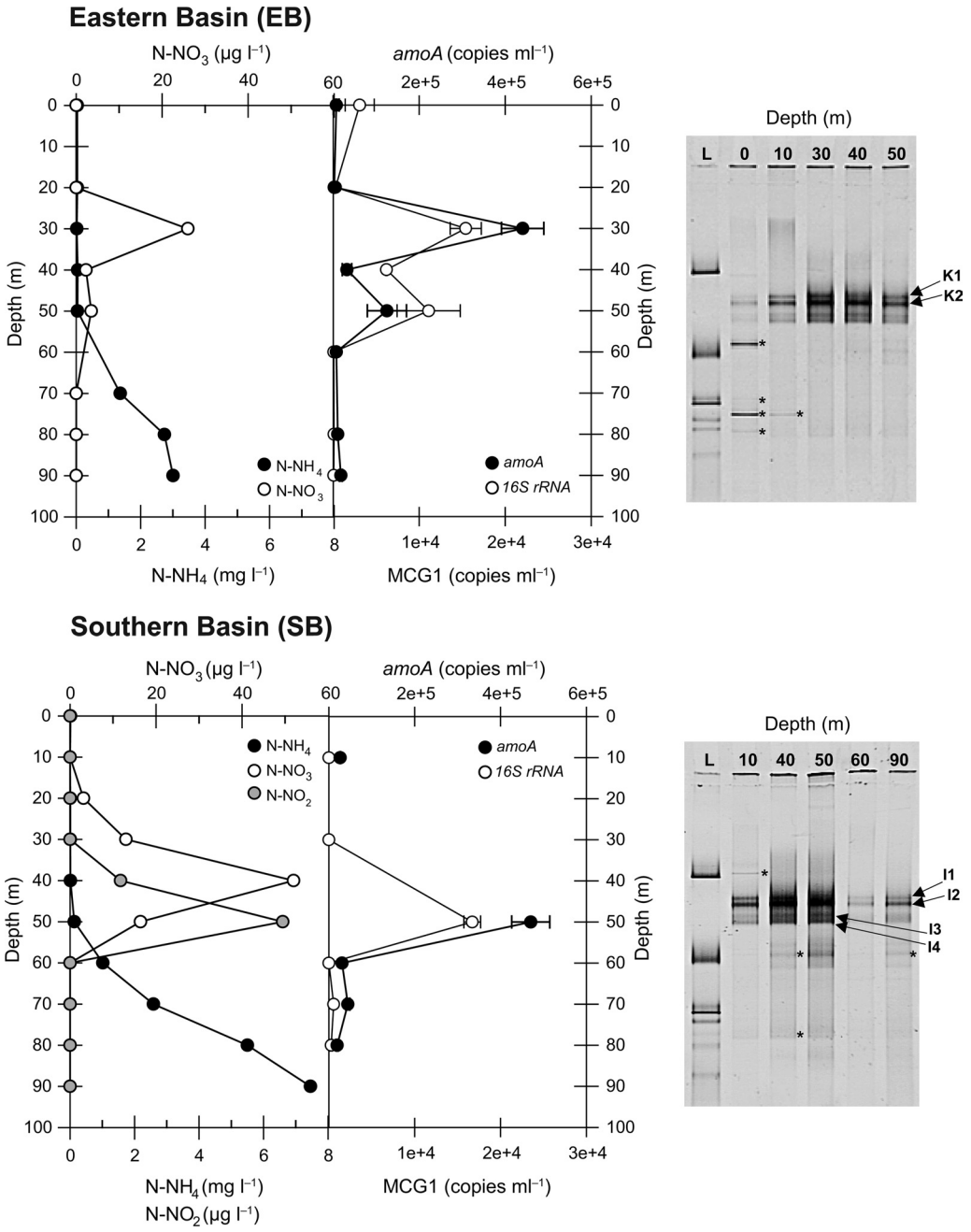

FIG. 5. Vertical distribution of ammonium, nitrate, and nitrite (left panels), gene abundance (MCG1 16S rRNA and crenarchaeal *amoA*) (middle panels), and DGGE fingerprints of *amoA* gene fragments (right panels) in the water column of eastern (EB, upper) and southern (SB, bottom) basins of Lake Kivu. Identical *amoA* fingerprints were obtained with samples collected from NB and BB stations (data not shown). Bands were named using the code for the corresponding basin (K for Kibuye [eastern basin, EB] and I for Ishungu [southern basin, SB]) and numbered sequentially from top to bottom of the gradient. *, unspecific products. Nitrite was undetectable in all depths from the eastern basin.

quences were found by Herfort et al. (30) in the North Sea. Further comparison with *amoA* genes from cultivated AOA representatives gave similarity values that ranged from 71% (for both "*Candidatus* Nitrososphaera gargensis" and "*Candidatus* Nitrosocaldus yellowstonii") to 88.3% ("*Candidatus* Nitrosopumilus maritimus"). The phylogenetic analysis carried out using the *amoA* sequences from Lake Kivu and others from public databases grouped the former in a distinct subcluster within the freshwater clade previously defined by Francis and coworkers (26) and clearly separated the Lake Kivu samples from sequences from both marine and terrestrial environments (Fig. 6).

After the identification of signature genes for AOA in DGGE profiles, qPCR was used to determine gene copy numbers of 16S rRNA-MCG1 and archaeal *amoA* to resolve their vertical distribution and abundance in relation to nitrite and

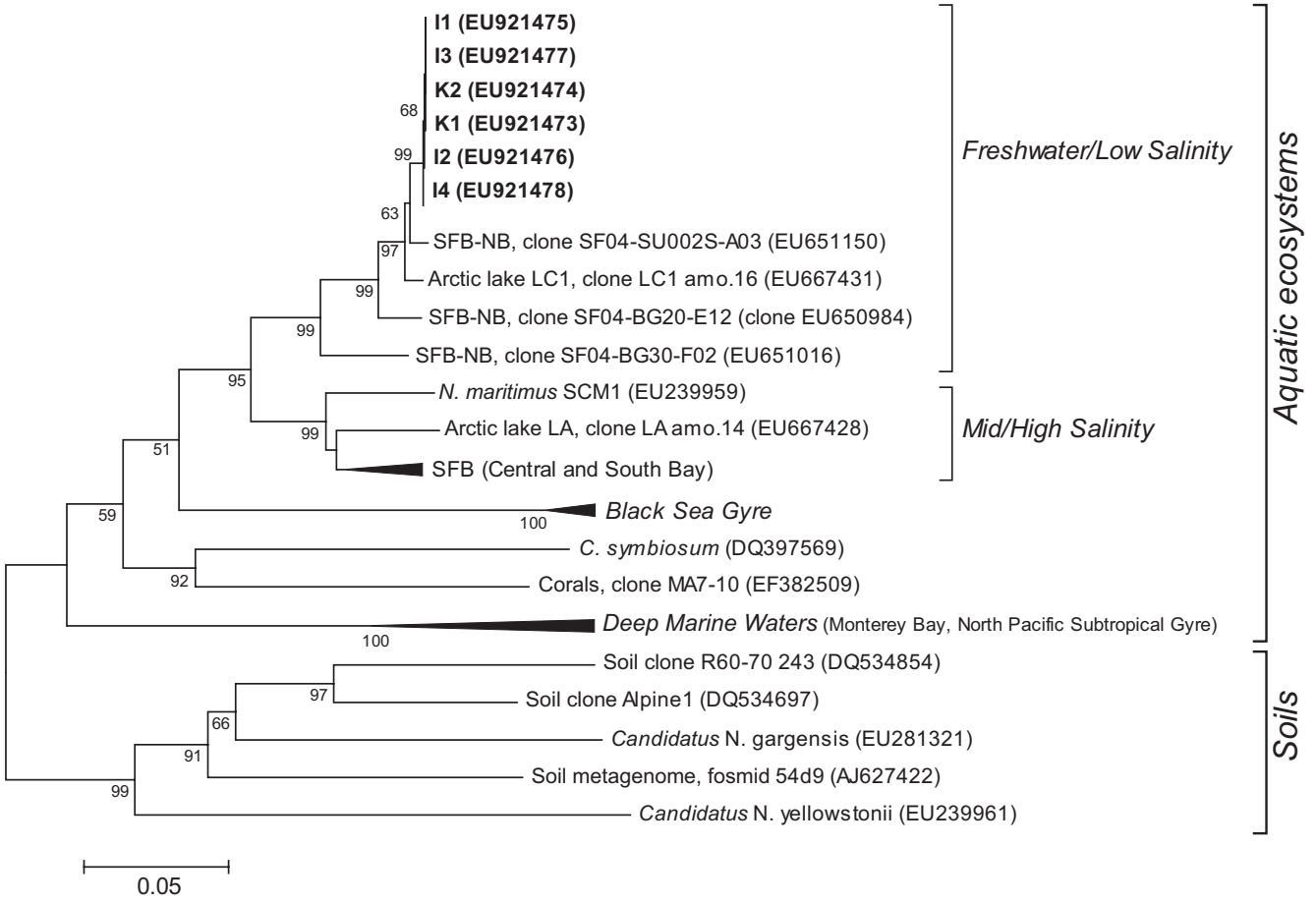

FIG. 6. Neighbor-joining phylogenetic tree for *amoA* sequences constructed using Tamura-Nei-corrected distances with a bootstrap value of 1,000. Bootstrap values higher than 50% are shown. Wedge sizes are proportional to sequences condensed on them. Brackets highlight environmental clusters. The scale bar indicates a 5% sequence dissimilarity.

nitrate profiles available for stations EB and SB. The quantitative distribution of both genes varied with depth and with highest copy numbers in the oxycline (from 30 to 50 m in station EB and from 40 to 50 m in station SB) (Fig. 5). This distribution was concomitant with that found for nitrite and nitrate, which showed distinct maxima at these depths (Fig. 5).

DISCUSSION

The contribution of archaea to planktonic microbial assemblages in stratified freshwater lakes is variable, but reported values are usually lower than those measured for marine environments (10). Although obtained by a different methodological approach, archaeal abundance in neighboring Lake Victoria reached 5.9% of the total nucleic acids (38). Similar values (between 1 and 7% of DAPI-stained cells) were obtained by FISH and CARD-FISH in different freshwater lakes (36, 59), although recent studies carried out in high mountain lakes reported abundances of up to 22% in Crater Lake (87) and 37% in Lake Llebreta (4). In stratified marine environments such as the Cariaco Basin and the Black Sea, the archaeal planktonic fraction ranged from 1% to 9% and from 10% to 30% of total DAPI counts, respectively, showing maximal abundances at the redoxcline coinciding with depth maxima of nitrite and nitrate (15, 41, 44). Similar distribution patterns have also been reported by Pouliot and coworkers (61) in two meromictic arctic lakes. Based on this prior research, the distribution and relative abundance (0.3% to 4.5% of total DAPI counts) of planktonic archaea in Lake Kivu agree with data available for other freshwater environments. It should be noted, however, that extensive studies are needed to ascertain if variations in environmental conditions between the rainy and dry seasons may affect the distribution and abundance of the planktonic archaeal community, as has been described for other microbial populations thriving in Lake Kivu (23, 67, 68).

The phylogenetic structure of the archaeal assemblage in Lake Kivu was fairly homogeneous in all sampling basins, with a clear phylogenetic segregation imposed by the oxic-anoxic transition (see Fig. S3 in the supplemental material). Sequences from the anoxic water compartment mainly affiliated with the highly diverse miscellaneous crenarchaeotic group (MCG) (32) and with methanogenic lineages. The MCG archaea are considered cosmopolitan (83) but are frequently found in anoxic habitats such as deep subsurface marine sediments (7) and hypolimnetic waters of sulfurous mesotrophic lakes (45). Current evidence suggests that some members of

the MCG lineage may obtain energy from the anaerobic oxidation of methane (7, 83), a hypothesis that fits with the prevalent physicochemical conditions in the monimolimnion of Lake Kivu. OTUs assigned to methanogenic lineages grouped with either acetoclastic (identity values of 97.4% and 95.8% with *Methanosaeta concilii* [OTU-1 and OTU-2, respectively] and uncultured *Methanosarcinales* [OTU-3 and OUT-4]) or hydrogenotrophic (95.9% identity of OTU-5 to *Methanocellula paludicola*) representatives, agreeing both with the biological origin of methane in the lake and with the methanogenic archaeal groups commonly found in other stratified lakes (43). The recovery of a few sequences related to methanogens from oxygenated water layers (bands *a*K3, *a*I3, and *a*B1 in Fig. S1 in the supplemental material) is, however, not in accordance with the strictly anaerobic metabolism assumed for these microorganisms. The oxygen tolerance of some members of the *Methanosaeta* cluster (31) or the occurrence of water-mixing processes that transported microorganisms from the upper part of the monimolimnion to shallow depths might explain these findings.

At the oxic-anoxic interphase and above, almost all the recovered sequences grouped within archaeal lineages containing ammonia-oxidizing representatives, i.e., *Crenarchaeota* group 1.1a and group 1.1b (64). Whereas all sequences affiliated to the former were assigned to a single OTU (OTU-8) related to the nitrifying marine archaeon "*Candidatus* Nitrosopumilus maritimus" (Fig. 4), those related to group 1.1b were distributed in 11 OTUs. Since this lineage is mainly composed of crenarchaeal phylotypes recovered from soil (8, 57), the high level of richness found in Lake Kivu for group 1.1b raises the question of whether these phylotypes were indigenous from the plankton or were introduced to the lake by surface runoff. The fact that most of the group 1.1b-related sequences were recovered from the southern basin and particularly Bukavu Bay, which are basins partly isolated from the main lake by sills of different depths (18, 81) and receive high water inflows by rivers and subaquatic sources (18, 54, 72), points to a terrestrial origin of the detected phylotypes. On the other hand, the close affiliation of some of the OTUs within *Crenarchaeota* group 1.1b with archaeal sequences potentially involved in ammonia oxidation in soils (e.g., 98.8% identity of OTU-16 to fosmid soil clone 54d9 [86]) is relevant. In any case, all archaeal *amoA* sequences recovered from Lake Kivu composed a homogeneous subcluster within the freshwater clade (Fig. 6) (26) and clearly separated from marine and terrestrial sequences. This result suggests either that the phylotypes assigned to group 1.1b recovered from Lake Kivu are actually not able to oxidize ammonia or that the *amoA* primers used (see Table 1) (26) present some bias toward marine *amoA* sequences. The use of different primer pairs for *amoA* fingerprinting (26) and qPCR (89) and the different sensitivity of both techniques toward less abundant phylotypes hinder the proper comparison of the data. Thus, further investigation is needed to resolve the actual nitrification capacity of the soil-related phylotypes found in the water column of Lake Kivu, especially considering that the most frequent phylotypes retrieved affiliated with the marine clade.

The increase in archaeal cell numbers at the oxycline (30 to 50 m depth) and the concomitant vertical distribution of molecular signatures of marine ammonia-oxidizing crenarchaeota (MCG1 16S rRNA and *amoA* genes) and nitrate and nitrite maxima at these depths agree with results found in other aquatic environments (15, 41, 42, 52). Unfortunately, logistic problems during field sampling did not permit the preservation of the samples in such a way to allow further analysis of *amoA* transcripts. Caution must therefore be exercised when considering the potential role of nitrifying crenarchaeota in Lake Kivu. Recent studies demonstrate that "*Candidatus* Nitrosopumilus maritimus" strain SCM1 is adapted to extreme nutrient limitation (50). According to these authors, *Nitrosopumilus*-like AOA may benefit from this adaptation to compete for nitrogen sources with ammonia-oxidizing bacteria, heterotrophic bacterioplankton, and phytoplankton. Although it is far from being resolved whether all the marine AOA are specialized oligophiles like strain SCM1, the low ammonia concentrations found in the epilimnetic waters of Lake Kivu (<0.1 μM) (58) may provide an optimal niche for their growth. In this regard, the low level of diversity of archaeal phylotypes in the oxycline and the low level of richness of *amoA* genes found suggest that freshwater members of the *Crenarchaeota* group 1.1a compose a distinct population at these depths. The identity values between the phylotype found in Lake Kivu samples and the reference strain "*Candidatus* Nitrosopumilus maritimus" (94.9% to 96.2%) point to a weak phylogenetic relation probably linked to its freshwater origin. The homogeneous clustering of *amoA* sequences recovered from Lake Kivu samples into the same freshwater clade provides further support to this hypothesis and to the idea of sequence clustering according to habitat (6, 26, 61). In this regard, several authors have recently shown that salinity is a major driver affecting archaeal distribution either at a local or at a global scale (4, 47, 53).

As stated above, the actual role of nitrifying crenarchaeota in the nitrogen cycle of Lake Kivu is, however, far from being resolved. Further activity and expression measurements are needed to confirm the significance of autotrophic archaeal nitrification in the lake and to determine the specific contribution of *Crenarchaeota* group 1.1a and 1.1b in comparison to bacterial nitrifiers. This topic is of special interest, especially considering the small contribution of archaeal cells in the total microbial planktonic assemblage measured in this work. Although recent reports on the deep marine subsurface biosphere highlighted that some important microbial activities can be performed by a small, but very active, subset of community members (27), further studies covering spatial and temporal variations of AOA populations in Lake Kivu should be addressed to ascertain their dynamics and seasonal abundance. Finally, the oligotrophic nature of neighboring lakes Tanganyika and Malawi offers potential habitats for the development of AOA. The finding of crenarchaeotal membrane lipids in sediments from these and other African lakes (62, 63, 74, 84) supports this assumption. Further surveys focused on these aspects will provide a better picture of the processes and players beneath the microbial cycling of nitrogen in large oligotrophic African lakes.

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