RESEARCH ARTICLE

International Microbiology (2013) 16:177-189
doi: 10.2436/20.1501.01.192 ISSN 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Phylogenetic characterization and quantification of ammonia-oxidizing archaea and bacteria from Lake Kivu in a long-term microcosm incubation

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Received 30 July 2013 · Accepted 30 September 2013

Summary. A microcosm cultivation-based method was set up to investigate the growth of ammonia-oxidizing archaea (AOA), isolated from a water sample acquired at a depth of 50 m from the northern basin of Lake Kivu. For this purpose, both CARD-FISH and qPCR targeting of archaeal 16S rRNA and *amoA* genes were used. Archaeal cell growth at the end of the 246-day microcosm experiment accounted for 35 % of the SybrGold-stained cells, which corresponded to 6.61×10^6 cells/ml and $1.76 \pm 0.09 \times 10^6$ archaeal 16S rRNA gene copies/ml. Clone libraries and DGGE fingerprinting confirmed the dominance of AOA phylotypes in the archaeal community microcosm. The majority of the identified archaeal 16S rRNA gene sequences in the clone libraries were affiliated with Thaumarchaeota Marine Group 1.1a. Subsequent cultivation of the AOA community on deep-well microtiter plates in medium containing different carbon sources to stimulate archaeal growth failed to show significant differences in archaeal abundance (ANOVA $t_{14} = -1.058$, $P = 0.308$ and ANOVA $t_{14} = 1.584$, $P = 0.135$ for yeast extract and simple organic acids, respectively). The lack of growth stimulation by organic compounds is in concordance with the oligotrophic status of Lake Kivu. Finally, the addition of antibiotics to the growth medium resulted in archaeal cell counts that were significantly lower than those obtained from cultures in antibiotic-free medium (ANOVA $t_{14} = 12.12$, $P < 0.001$). [Int Microbiol 2013; 16(3):177-189]

Keywords: ammonia-oxidizing archaea and bacteria · ammonia monooxygenase alpha subunit (*amoA*) · Lake Kivu · microcosm · multi-color CARD-FISH

Introduction

Following the pioneering work of Könneke and co-workers with *Nitrosopumilus maritimus* [24], the ability to grow ammonia-oxidizing archaea (AOA) under laboratory conditions has made rapid progress. The first isolation of an autotrophic nitrifying archaeon not only was considered a milestone in microbial ecology but it also provided clues to the enrichment or isolation of several new mesophilic nitrifying *Candidatus* (*Ca.*) species from both aquatic and terrestrial habitats [14,20,25,30,44] and one thermophilic species from a terrestrial hot spring [10,18]. *Nitrosopumilus maritimus* was initially identified as the first mesophilic member of the kingdom Crenarchaeota to be isolated in pure culture, but further genome-based studies of that species and its nitrifying relatives, i.e., *Crenarchaeum symbiosum* and "*Ca*. Nitrososphaera gargensis," provided robust genetic data to accommodate

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these archaeal nitrifiers within a new archaeal kingdom, the Thaumarchaeota [41]. Thus far, marine species are affiliated with Marine Group 1.1a cluster (with the exception of "*Ca*. Nitrosoarchaeum koreensis" isolated from soil) whereas archaeal soil nitrifiers belong to either Marine Group 1.1a-associated or to Soil Group 1.1b. The unique thermophilic Thaumarchaeota described to date have been assigned to group ThAOA [17]. Since all cultured members of this new kingdom rely on the oxidation of ammonia for their energy metabolism, this metabolic trait has been considered as a defining characteristic of Thaumarchaeota.

Despite these advances in our knowledge, the cultivation of AOA remains a difficult, time-consuming task and the final isolation of the targeted species in a pure culture is only rarely achieved. Among the alternative strategies to study AOA that have been tested thus far are state-of-the-art genomic techniques [7] and experimentation with highly enriched cultures containing satellite bacterial communities [14,20]. These culture-dependent methods provide highly valuable information allowing the proper interpretation of data obtained by culture-independent techniques in ecological studies focused on AOA [42]. Physiological characterization and determination of the growth requirements of recently described species are also useful to refine cultivation strategies and thereby improve cultivability. For instance, differences in the ammonia oxidation kinetics of AOA and ammonia-oxidizing bacteria (AOB) not only provide an elegant explanation for their niche segregation but can also be used to selectively enrich AOA in samples containing a heterogeneous bacterial and archaeal nitrifying community [29]. Data from such investigations indicate that archaeal nitrifiers from aquatic habitats are similar in their tolerances of oxygen, ammonia concentrations, and pH range whereas soil representatives have greater variability in relation to optimal pH and their tolerance of high ammonia concentrations (e.g., 15 mM for "*Ca*. Nitrosoarchaeum koreensis" [20]). These differences are consistent with the higher ammonia concentrations found in soils than in aquatic environments and they suggest specific adaptations for planktonic and terrestrial archaeal nitrifiers [47]. For example, the capacity to use simple organic compounds including pyruvate has been described for "*Ca*. Nitrososphaera viennensis" [44], and genes involved in organic carbon utilization have been identified in AOA genomes ([7] and references therein).

Recently, we reported the accumulation of AOA in the oxycline of Lake Kivu, as part of a heterogeneous community consisting of members of Marine Group 1.1a and Soil Group 1.1b [28]. The presence of the latter group in the water column is generally thought to be a consequence of water inputs by surface runoff, which are also responsible for the large nutrient loads entering the lake [33,34]. Assuming different growth requirements and physiological constraints for soil and planktonic AOA, the presence of the former in the water column has raised the question whether they are capable of active growth or simply passively accumulate in the lake's rare biosphere. To answer this question and to identify active members of the archaeal community we established an experimental microcosm using water collected at the oxycline of Lake Kivu. Ammonia concentrations and incubation conditions were manipulated to stimulate AOA growth, which was monitored for almost a year using multi-color catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) and qPCR [16S rRNA and the alpha subunit of ammonia monooxygenase (*amoA*) for both AOA and AOB]. The former technique was chosen to avoid potential overestimation of archaeal abundance following the non-specific hybridization of archaeal probe ARC915 to members of the phylum Bacteroidetes. Simultaneous hybridization of the probes ARC915 and CF319a allowed us to identify non-specific targets and to correctly quantify archaeal cells throughout the incubation. Archaeal members of the microbial community growing in the microcosm were identified by means of denaturing gradient gel electrophoresis (DGGE) fingerprinting and clone libraries. In addition, the effect of different organic compounds on archaeal growth was monitored using 96-deep well microtiter plates to elucidate whether some organic compounds stimulate archaeal nitrifiers, as recently demonstrated [44].

Materials and methods

Set-up of the experimental microcosm. Lake Kivu is located between Rwanda and the Democratic Republic of the Congo, 1,463 m above sea level. The lake is a deep (maximum depth: 489 m) meromictic and oligotrophic body of water with steeply increasing temperature and salinity gradients. Water samples were collected during a sampling campaign conducted during the rainy season in March 2007. In a previous study, the maximal abundance of AOA in Lake Kivu was detected at the oxic–anoxic interface (30–50 m depth) of the main basins [28]. Accordingly, in this study we initiated an enrichment culture using water collected at a depth of 50 m from the Lake Kiwu Northern Basin (NB50). The experimental set-up consisted of a sterile 1-l Erlenmeyer flask filled with 500 ml of lake water and capped with a cotton wool stopper to ensure oxic conditions. At day 0, the water was supplemented with NH_4Cl (1 mM), $NaHCO_3$ (2.7 μM), and KH_2PO_4 (1.8 mM) to stimulate the growth of the autochthonous nitrifying community. The enrichment culture was maintained for 246 days at 22 ºC in the dark without shaking. Samples were periodically collected to quantify archaeal and bacterial abundances and to monitor changes in microbial community composition (see below). The samples were coded using a standard label (M50) followed by indicating the time in days at which each sample was collected (e.g., M50_246 was collected on day 246).

Enrichment culture cultivation in microtiter plates. To determine whether the AOA present in the enrichment could be further stimulated by the addition of organic compounds, we used a multi-enrichment system consisting of 96-deep-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA) containing different media (Fig. 1). The plate wells were filled with synthetic freshwater mineral medium for nitrifying Crenarchaeota [24] but with the following modifications: NaCl (17.11 mM, final concentration), NH_4Cl (1 mM), $MgCl_2 \cdot 6H_2O$ (1.97 mM), $CaCl_2 \cdot 2H_2O$ (0.68 mM), and KCl (6.71 mM). Once autoclaved, the basal medium was amended with KH_2PO_4 (0.03 mM), $NaHCO_3$ (17 mM), trace elements solution, and vitamin V7 solution [5]. The medium was supplemented with the following substrate combinations: (i) NH_4Cl (15 mM, final concentration), (ii) a mixture of organic acids (acetate, citrate, formate, α-ketoglutarate, propionate, pyruvate and succinate, 200 mM each), (iii) yeast extract (0.001 % w/v [44]), (iv) mineral medium, and (v) the original microcosm enrichment. The last two treatments were considered as controls. Each medium combination (900 ml total volume) was dispensed into six wells in order to analyze several replicates for each treatment (Fig. 1).

For treatments 2, 3, and 4, an additional row of six wells was filled with the same medium amended with an antibiotic mixture (carbenicillin, an inhibitor of cell wall synthesis [100 mg/l]; streptomycin, an inhibitor of ribosomal protein synthesis [100 mg/l]; polymyxin B, a disruptor of the lipid bilayer [20 mg/l]; and triclosan, an inhibitor of fatty acid synthesis [10 mg/l]) to inhibit bacterial growth, with the aim of enriching archaeal cells. The inhibitors were selected among a larger group of diverse antimicrobial agents after several inhibition tests previously carried out against *Sulfolobus solfataricus* DSM1617, *Bacillus subtilis* CECT 39, *Escherichia coli* CECT 831, and *Pseudomonas aeruginosa* CECT 532 (data not shown). The medium-containing wells were then manually inoculated with 100 µl (10 % inoculum) of the enrichment suspension (M50_246), resulting in a final concentration of 6.6×10^5 cells/ml. Peripheral wells were not inoculated but were instead used as controls for potential contamination during handling. Finally, the plates were covered with a sterile porous adhesive film (Kisker, Steinfurt, Germany) and then incubated at 18 ºC in the dark for two months. The activity of the inhibitor mixture in the enrichment plates throughout incubation was assessed after 7, 30, and 60 days of incubation by susceptibility diffusion tests using 10-µl aliquots withdrawn from the wells and transferred to nutrient agar lawn growth cultures of *B. subtilis* CECT 39 and *P. aeruginosa* CECT 532. No substantial decrease in the antimicrobial activity of the mixture against these strains was evident in any of the performed tests (data not shown).

Multi-color CARD-FISH. Archaeal abundance was periodically monitored in the microcosm and measured at the end of the incubation period in microtiter plate cultures using CARD-FISH [36] and the archaea specific probe ARC915 [28]. Samples of 0.5 and 9 ml were taken from three replicate wells of the microtiter plate cultures and from the microcosm, respectively, fixed with paraformaldehyde (final concentration, 2 % [w/v]) overnight at 4 ºC, and filtered onto 25-mm diameter, white, 0.22-µm-pore-size polycarbonate filters (Millipore, Eschborn, Germany). To overcome non-specific binding of the ARC915 probe to members of the Bacteroidetes (see the Discussion), the multi-color CARD-FISH protocol was optimized to evaluate the extent of the probe bias by the simultaneous hybridization of probe ARC915 and the *Bacteroidetes*-specific probe CF319a [2]. As positive controls, fixed cells of *Sulfolobus solfataricus* DSM 1617 and *Cytophaga fermentans* DSM 9555 were included as independent samples in every set of hybridizations. Immediately after cell permeabilization, a first hybridization step using probe ARC915 (0.85 ng/µl, final concentration) was followed by a washing step and then a signal amplification step with Alexa-Fluor 488 tyramide conjugate as described [28], except that 55 % (v/v) formamide was used. A portion of the filter was cut, air-dried, embedded in antifading solution (Citifluor, London, UK), and then examined using an Axioskop epifluorescence microscope (Zeiss, Jena, Germany) to confirm the correct fluorescence signal of ARC915.

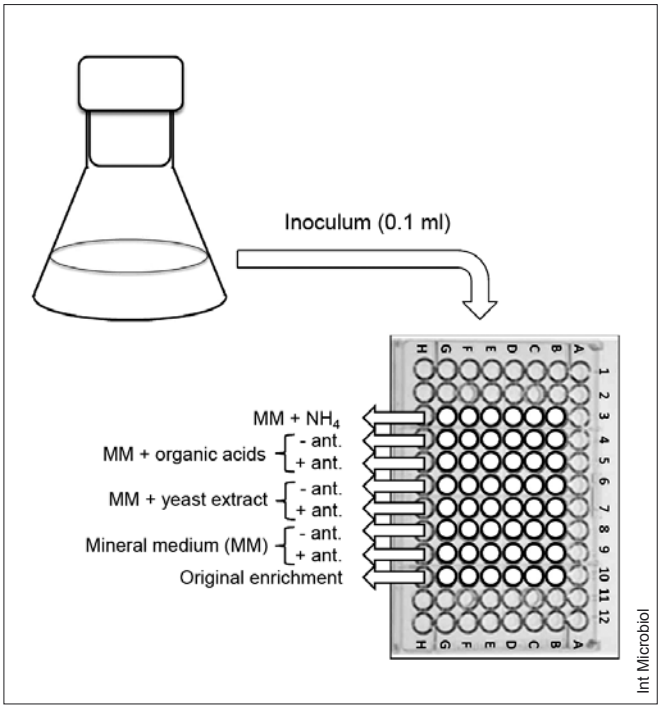

Fig. 1. Schematic view of the microcosm and the microtiter plates used as cultivation systems. Mineral medium (MM) and MM+NH_4Cl contained 1 and 15 mM ammonium chloride, respectively.

Next, the filters were prepared for the second hybridization by inactivating horseradish peroxidases as previously done with cellular peroxidases but in this case extending the incubation time in 1 M HCl to 25 min. The air-dried filters were then submitted to the second hybridization using probe CF319a (0.28 ng/µl, final concentration) followed, after a wash, by a signal amplification step with Alexa-Fluor 546 tyramide conjugate exactly as described for probe ARC915. Finally, the filter sections were air-dried, DAPI (4′,6-diamidino-2-phenylindole) stained [28], embedded in antifading solution (Citifluor, London, UK) and examined using an Axioskop epifluorescence microscope (Zeiss) equipped with a 50-W Hg bulb and appropriate filter sets for Alexa-Fluor 488, Alexa-Fluor 546, and DAPI. To avoid overestimation of archaeal abundance because of the non-specific hybridization of probe ARC915 to members of Bacteroidetes, archaeal cells were defined as those showing positive ARC915 and negative CF319a hybridization signals and positive DAPI staining. For total cell counts, the filters were immersed in SybrGold (diluted 1:10,000 [v/v]) in the dark at room temperature for 20 min and then washed into 30 ml of double-distilled particle-free water and in 30 ml of 70 % cold ethanol. Between 250 and 600 SybrGold-stained cells were counted in ten randomly selected microscopic fields from one filter section using an Axioskop epifluorescence microscope (Zeiss). The results are expressed as the mean cell counts from one filter section; hence, a standard deviation does not apply.

Molecular characterization of the microcosm. DNA from the autochthonous community in Lake Kivu was extracted from water samples collected during the sample campaign using a combination of enzymatic cell lysis and cetyltrimethyl ammonium bromide (CTAB) as previously described [28]. The same protocol was applied to extract DNA from enrichment culture samples (10 ml) collected at different times throughout the 246-day incubation. The DNA concentration was determined spectrophotometrically using NanoDrop (Thermo Scientific, Wilmington, DE, USA) and immediately

stored at –80 °C until use. Amplification of partial archaeal 16S rRNA and *amoA* gene sequences, DGGE fingerprinting, further acrylamide band excision containing DNA melting types, and PCR reamplification of the selected melting types were carried out as previously described [28]. Additionally, the composition of both the autochthonous prokaryotic planktonic community from Lake Kivu and that enriched in the experimental microcosm was assessed by clone libraries using amplicons of the archaeal and bacterial 16S rRNA genes and the primer pairs 21f/958r and 28f/1492r, respectively [11,49]. PCR products were purified using the QIAquick spin kit (QIAGEN, Valencia, CA, USA) and polyadenylated to improve cloning efficiency by adding 26 µl of the clean PCR product to 4.5 µl of a PCR mix containing 0.98× PCR buffer (Invitrogen, Paisley, UK), 0.16 mM of $MgCl_2$ (QIAGEN), 0.26 mM of dATP (Promega, Madison, WI, USA) and 0.61 units of Taq polymerase (QIAGEN). The mix was then incubated for 10 min at 72 ºC in a GeneAmp 2700 thermocycler (Applied Biosystems). The polyadenylated products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Randomly selected clones were grown overnight in LB liquid medium containing ampicillin and then amplified using the M13 primers. Clones showing the correct size insert were sequenced in both directions. Sequencing of the clones and phylotypes recovered from the DGGE bands corresponding to the 16S rRNA and *amoA* genes was carried out externally (Macrogen, Seoul, South Korea).

Quantitative PCR (qPCR). Copy numbers of the 16S rRNA gene of Bacteria, Archaea, and Marine Group 1.1a Thaumarchaeota and of the *amoA* gene from AOA and AOB were quantified by qPCR as previously described, with minor modifications [45]. All qPCR assays were performed in duplicate in a 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using MicroAmp optical 96-well reaction plates covered with optical caps (Applied Biosystems). The reaction mixtures (20 µl) contained 10 µl of Sso-Fast EvaGreen supermix (Bio-Rad, Richmond, CA, USA), 2 µl of template DNA (5 ng), 1 and 0.5 µM of each 16S rRNA and *amoA* primer pair, respectively, 2 µl of bovine serum albumin (10 mg/ml) (Sigma-Aldrich, Steinheim, Germany), and 4 µl of molecular biology-grade water (Sigma). The standard curves were obtained from serial dilutions of linearized plasmids (pGEM-T Easy; Promega) containing standard sequences. The PCR efficiency was 80–90 %, with R^2 values >0.99. The results are expressed as the mean and standard deviation of duplicate qPCR assays. Negative controls resulted in undetectable values in all cases.

Phylogenetic analyses. Consensus sequences from clones or DGGE bands were aligned using the NAST algorithm [12] and analyzed to detect chimeras using the Bellerophon tool available at the GreenGenes website (http://greengenes.lbl.gov). High quality, chimera-free sequences were then aligned in Mothur v1.20.0 [39] using the SILVA archaeal database as the reference alignment. Aligned sequences were imported into ARB [26] loaded with the SILVA 16S rRNA ARB-compatible database (SSURef-104, October 2010) and manually checked to refine the alignment. Clone sequences were then exported and loaded into MOTHUR to assign operational taxonomic units (OTU, defined at a 97 % cutoff) and their representative sequences after calculation of a neighbor-joining (NJ) distance matrix using the Jukes-Cantor algorithm. Rarefaction curves, Good's coverage, and Shannon diversity index were also determined in MOTHUR. OTU assignment for short DGGE sequences (ca. 500 bp) were analyzed separately following the same procedure but using the alignment containing all of the 16S rRNA gene sequences retrieved in this study. A phylogenetic tree was constructed using the representative sequences for each OTU and the reference sequences from the ARB database of at least 900 bp using the maximum likelihood (ML) algorithm with 100 bootstrap replicates. Shorter sequences from DGGE fingerprints were then added to this tree using the ARB "parsimony (quick add marked)" tool and termini filter, thereby maintaining the overall tree topology. A NJ tree with Jukes-Cantor correction with 1000 bootstrap replicates was also generated using the ARB software and resulted in a similar tree topology.

Environmental sequences for archaeal *amoA* genes were obtained from public databases and aligned with those retrieved from DGGE gels using the MAFFT on-line alignment tool and applying the recommended parameters [http://mafft.cbrc.jp/alignment/server/]. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 and manually checked. The ML method was used to generate 1000 bootstrap replicates. The resulting tree topology was compared with the one obtained using the NJ method, applying the Jukes-Cantor algorithm in both cases to calculate phylogenetic distances.

Statistical analyses. Data on archaeal abundance were used to calculate a 5 % trimmed mean for each combination of three wells from the microtiter plate cultures. Data on the archaeal numbers from the microtiter plate cultures were evaluated using Levene's test for homogeneity of variances and Shapiro–Wilk's test for normality. The data were normally distributed and homoscedastic, although a logarithmic transformation was needed in some cases. Archaeal numbers obtained from all seven treatments were compared using analysis of variance (ANOVA) and ANOVA linear contrasts. All tests were performed using SPSS v15.0 (SPSS, Chicago, IL, USA).

Nucleotide sequence accession numbers. The 16S rRNA and *amoA* gene sequences obtained in this study were deposited in the GenBank database under accession numbers KF418394 to KF418540 and KF418541 to KF418542.

Results

Archaeal growth during incubation. The abundance of total archaeal cells increased from 2×10^4 cells/ml in the autochthonous lake community at 50 m depth to 6.61×10^6 cells/ml after 246 days of incubation (Fig. 2A). The percentage of false positives (i.e., cells hybridizing with probes ARC915 and CF319a) rapidly decreased from 63.81 % at the beginning of the incubation period to 0.76 % at the end, suggesting that the enrichment conditions were not optimal for members of Bacteroidetes (Fig. 2B). False-positives comprised only a minor fraction of the SybrGold-stained cells, ranging from 2.87 % at the beginning of the incubation period to less than 0.5 % at the end, thus confirming a specific cell identification. Archaeal growth was further confirmed uing qPCR, by monitoring archaeal 16S rRNA gene copies, which increased from $1.13 \pm 0.08 \times 10^5$ to $1.76 \pm 0.09 \times 10^6$ cells/ml at days 64 and 211, respectively.

Crenarchaeal 16S rRNA gene copies were determined in order to precisely quantify the AOA present in the microcosm. The abundance of Crenarchaeota increased from $6.62 \pm 0.36 \times 10^4$ to $6.68 \pm 0.52 \times 10^5$ gene copies/ml for the first 133 days. Afterwards, archaeal 16S rRNA gene abundances remained almost constant until the end of the incubation period ($5.20 \pm 0.62 \times 10^5$ gene copies/ml at 246 days, Fig. 2C). Overall, Crenarchaeota accounted for between 22.72 ± 0.03 % and

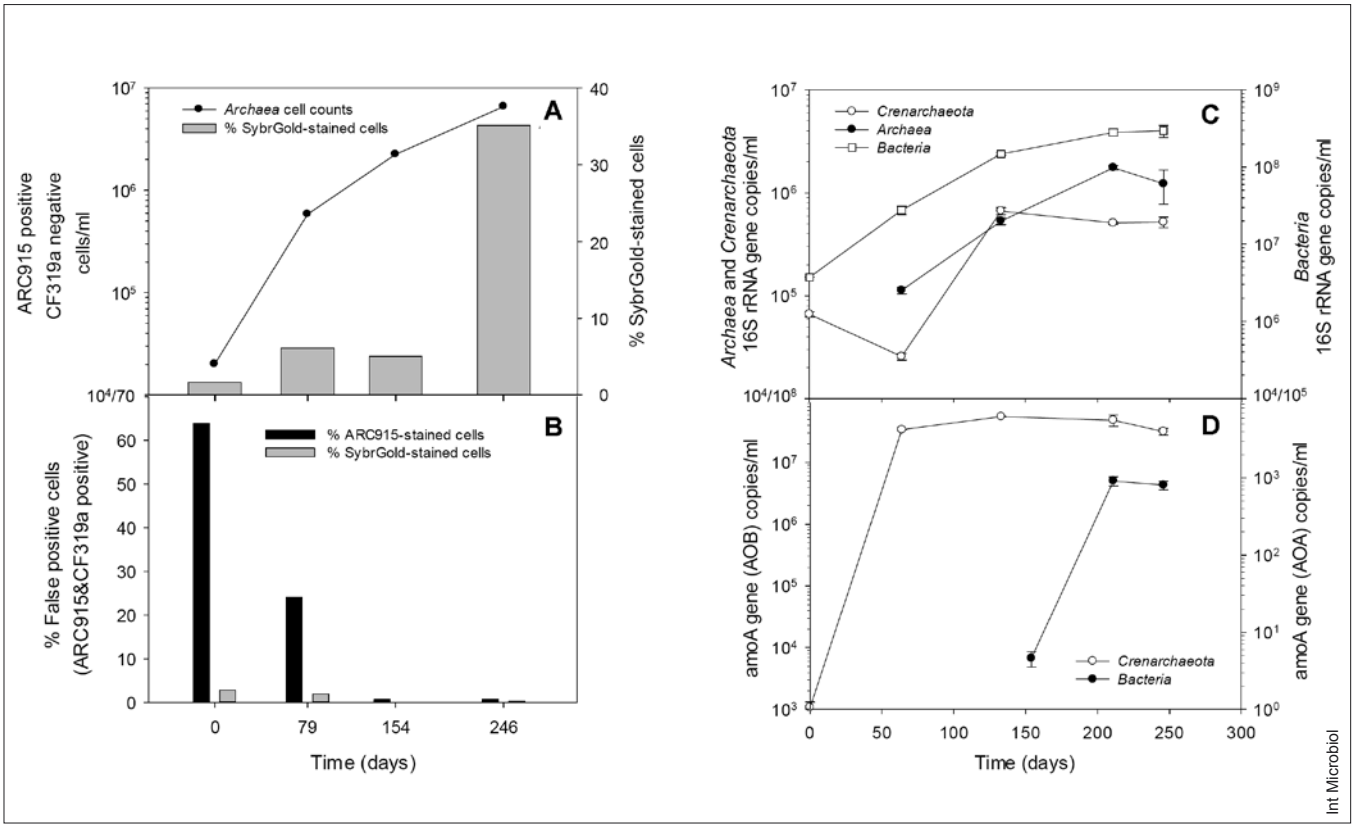

Fig. 2. (**A**) Total archaeal cell numbers and relative abundances of Archaea in the enrichment microcosm throughout the incubation period, as determined with multi-color CARD-FISH. (**B**) Reduction of false-positives for the ARC915 probe in samples collected during the incubation. (**C**) 16S rRNA gene copy numbers for Bacteria, Archaea, and Crenarchaeota and Marine Group 1.1a Thaumarchaeota as determined in the microcosm during the 246-day incubation. (**D**) Gene copy numbers for archaeal and bacterial *amoA* in the enrichment microcosm throughout the incubation.

46.86 ± 17.10 % of the total archaeal 16S rRNA gene copies. Assuming that mesophilic Crenarchaeota harbor a single genomic copy of the 16S rRNA gene and that Euryarchaeota contain at most two to four genomic copies of the 16S rRNA gene [http://rrndb.umms.med.umich.edu], the former would represent a significant fraction of the total archaeal community quantified by qPCR, thus suggesting the positive stimulation of AOA by the enrichment conditions. Nonetheless, bacterial abundances always exceeded those of archaea by one and two orders of magnitude at the beginning and end of the microcosm experiment, respectively, confirming that archaea were a minor component of the microbial community in the microcosm. In fact, bacterial 16S rRNA genes increased during the first 133 days, from $3.73 \pm 0.01 \times 10^6$ copies/ml to $2.96 \pm 0.05 \times 10^8$ copies/ml (Fig. 2C).

Since the cultivation conditions were designed to promote ammonia oxidation, archaeal and bacterial *amoA* gene copy numbers were also monitored to determine the effect of medium conditions on both groups of ammonia-oxidizers. Archaeal *amoA* gene copies rapidly increased during the first 133 days of cultivation, from less than 10±0.17 copies/ml to $6.24 \pm 0.08 \times 10^3$ copies/ml, whereas during the same period the bacterial *amoA* gene was not detected (Fig. 2B). Instead, bacterial *amoA* was detected only later and the copy number consistently increased until the end of the incubation period, when it outnumbered that of the archaeal *amoA* gene by three orders of magnitude (Fig. 2D).

Phylogenetic composition of bacterial and archaeal communities. Two clone libraries were generated from samples obtained at the initial phase of enrichment (64 days) and at the end of the experiment (246 days). The bacterial community, which was initially dominated by Betaproteobacteria (81.7 % of the total clones in the 64-day library), became a progressively more diverse community mainly composed of Alpha- and Betaproteobacteria (65.2 % and 25 % of total clones in the 246-day library, respectively). The major representatives of these proteobacterial classes were members of the Rhizobiales and Burkholderiales, respectively, most of which are well-known nitrogen-fixing

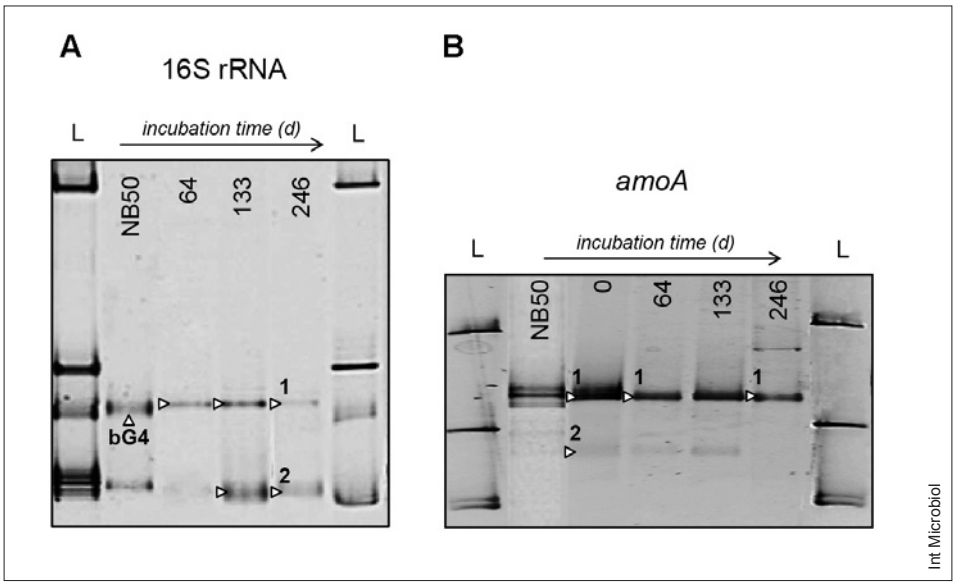

Fig. 3. Negative image of a SybrGold-stained DGGE gel showing: (**A**) archaeal 16S rRNA gene fingerprints obtained from the natural archaeal assemblage used as inoculum (Lake Kivu, Northern Basin, 50 m depth, NB50) and the archaeal community enriched in the experimental microcosm at different sampling intervals (from day 64 to 246), and (**B**) fingerprints for the archaeal *amoA* gene. Band labels correspond to codes used in the phylogenetic tree (DGGE1, DGGE2, Fig. 4; M50_DGGE1 and M50_DGGE2, Fig. 5). L stands for DGGE ladder.

soil bacteria. Note that no sequences of known AOB were identified in the clone library (355 clones in total) although the presence of these bacteria had been deduced by qPCR targeting their *amoA* genes (Fig. 2D). The composition of the archaeal community was assessed by DGGE fingerprinting and by clone libraries. 16S rRNA gene fingerprints obtained at selected dates showed two bands that persisted throughout the incubation and coincided with the band pattern obtained from the planktonic community in Lake Kivu (Fig. 3A). The sequence recovered from band DGGE1 clustered within uncultured crenarchaeota from Marine Group 1.1a, mainly represented by marine AOA. Specifically, DGGE1 exactly matched phylotype aG2 (FJ536698) and was highly similar (>99 %) to bG4 (Fig. 3A); these two phylotypes were previously identified in the Northern Basin of Lake Kivu [28]. In addition, the sequence similarity of DGGE1 to two previously characterized ammonia oxidizers, "*Ca.* Nitrosoarchaeum koreensis" MY1 (HQ331116) [20] and "*Ca.* Nitrosoarchaeum limnia" SFB1 (NZ_CM001158) [7], was 100 % and 99.3 %, respectively (Fig. 4).

The phylotype DGGE1 also showed a sequence similarity of 92.2 % to previously enriched AOA-DW (JQ669391) and AOA-AC5 (JQ669390), two archaeal sequences from freshwater sediment samples of Acton Lake and Delaware Lake (OH, USA) [14]. Phylotype DGGE2 clustered within the Miscellaneous Crenarchaeotic Group (MCG), showing 96 % and 94 % similarity to uncultured MCG from Griffy Lake (IN, USA) and Lake Pavin (France), respectively, and was only distantly related (88.3 %) to aG7, a previously identified MCG phylotype occurring in the same basin of Lake Kivu. Further DGGE analysis targeting archaeal *amoA* gene fragments showed two melting types that agreed with the results obtained with the 16S rRNA genes (Fig. 3B). Only one of these bands was very similar to that from the autochthonous community and it clearly persisted in the enrichment throughout the incubation. The recovered *amoA* sequences grouped in two subclusters, Kivu1 (melting type M50_DGGE1: M50_0, M50_133 and M50_246) and Kivu2 (melting type M50_DGGE2: M50_0) (Fig. 5). The first *amoA* variant was identical to *amoA* sequences previously retrieved from the Lake Kivu oxycline [28] and it grouped within a freshwater clade showing high similarities (>99 %) to the *amoA* sequence from "*Ca.* Nitrosoarchaeum koreensis" MY1 (HQ331117). The second variant occurred as a faint band and was not detected after 133 days (Fig. 3B). This phylotype had not been previously found in Lake Kivu and showed high pairwise similarity (99.72 %) to an *amoA* clone sequence (FJ951737) retrieved from the surface sediments of Lake Taihu (China). Overall, the DGGE fingerprints for the archaeal 16S rRNA and *amoA* genes indicated the persistence of AOA throughout the incubation period and therefore their presence as a significant fraction of the archaeal community in the microcosm.

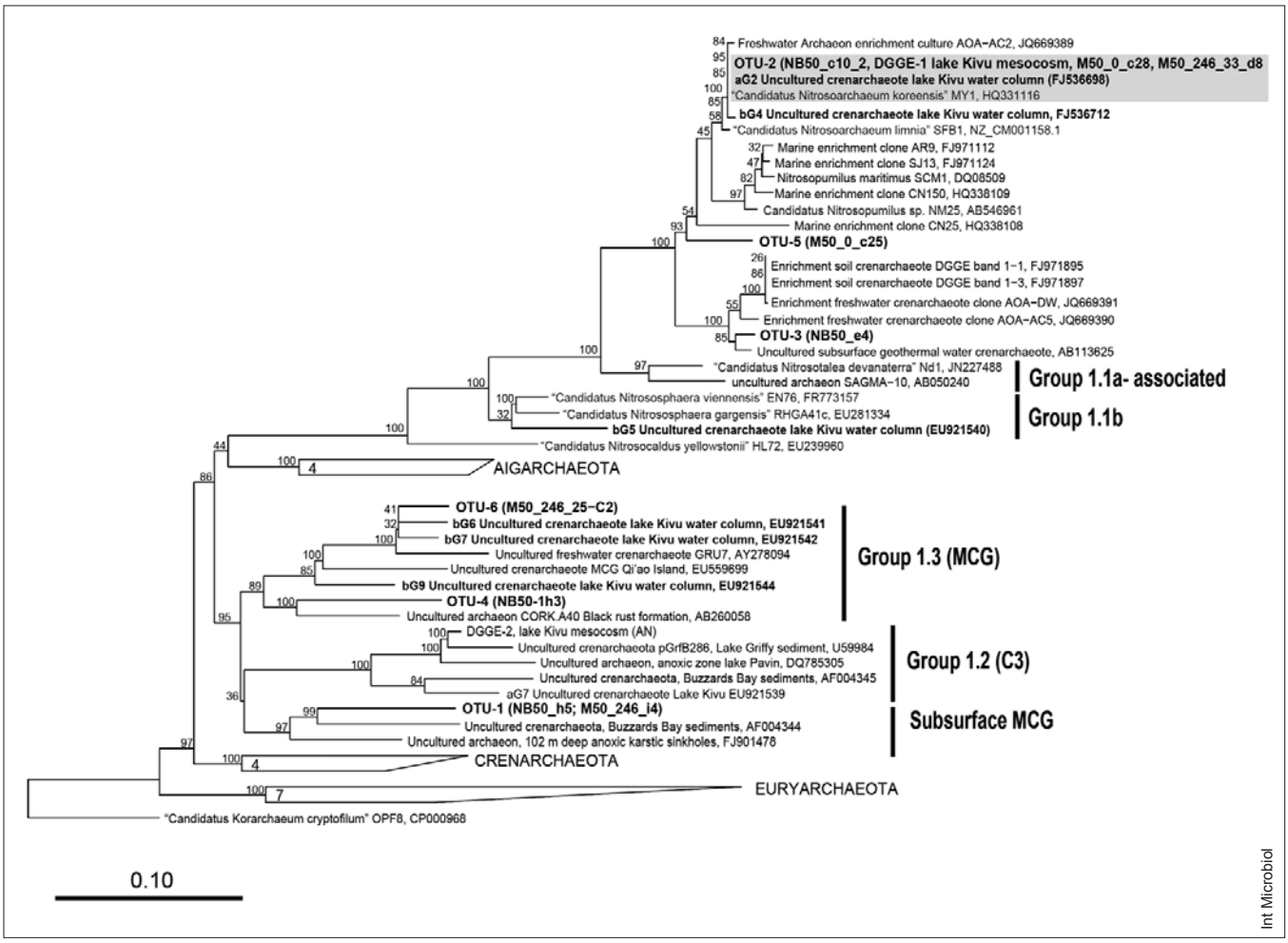

Fig. 4. Maximum-likelihood phylogenetic tree calculated for the full and partial 16S rRNA gene sequences retrieved from Lake Kivu and microcosm samples (in bold). Sequences obtained from the natural community (NB50) after inoculation of the enrichment culture (M50_0) and at the end of the 246-day incubation (M50_246) are specified for each OTU. DGGE band sequences are coded according to the band numbers in Fig. 3. Sequences identical to those of the cultured isolates are shaded in gray. Significant bootstrap values (≥50 %) from 100 replicates are shown at branch nodes. Scale bar indicates an estimated sequence divergence of 10 %.

We analyzed 146 clones, which grouped into six OTUs (defined at 97 % cutoff). Three of these OTUs were affiliated with Marine Group 1.1a Thaumarchaeota and grouped together 130 sequences (89 % of the total). From these AOA sequences, 126 could be assigned to OTU-2 (86 % of the clones), which exactly matched a phylotype previously identified in Lake Kivu (aG2, FJ536698, [28] and showed a 99 % pairwise similarity to "*Ca.* Nitrosoarchaeum limnia" SFB1 (NZ_CM001158) and "*Ca.* Nitrosoarchaeum koreensis" MY1 (HQ331116) [20]. In addition, the representative clone sequence from OTU-2 had a 99 % pairwise similarity with phylotype DGGE-1, previously identified by 16S rRNA DGGE fingerprinting. Phylotype DGGE-2 was not identified in our clone libraries.

Only clone sequences affiliated with OTU-2 were identified in the clone libraries constructed from the natural lake community (NB50) and from the microcosm enrichment (M50_0 and M50_246). Sequences affiliated with OTU-2 were prevalent in all clone libraries analyzed, representing 84 %, 93.5 %, and 85 % of the total sequences in the NB50, M50_0, and M50_246 libraries, respectively). Variations in the relative abundance of OTU-2 sequences caused changes in diversity, as estimated by the Shannon index (data not shown). The remaining OTUs comprised OTU-5 and OTU-6, which contained sequences only identified at the beginning (M50_0) and at the end (M50_246) of the cultivation period, OTU-3 and OTU-4, which grouped together sequences from the autochthonous community in the lake (NB50), and OTU-

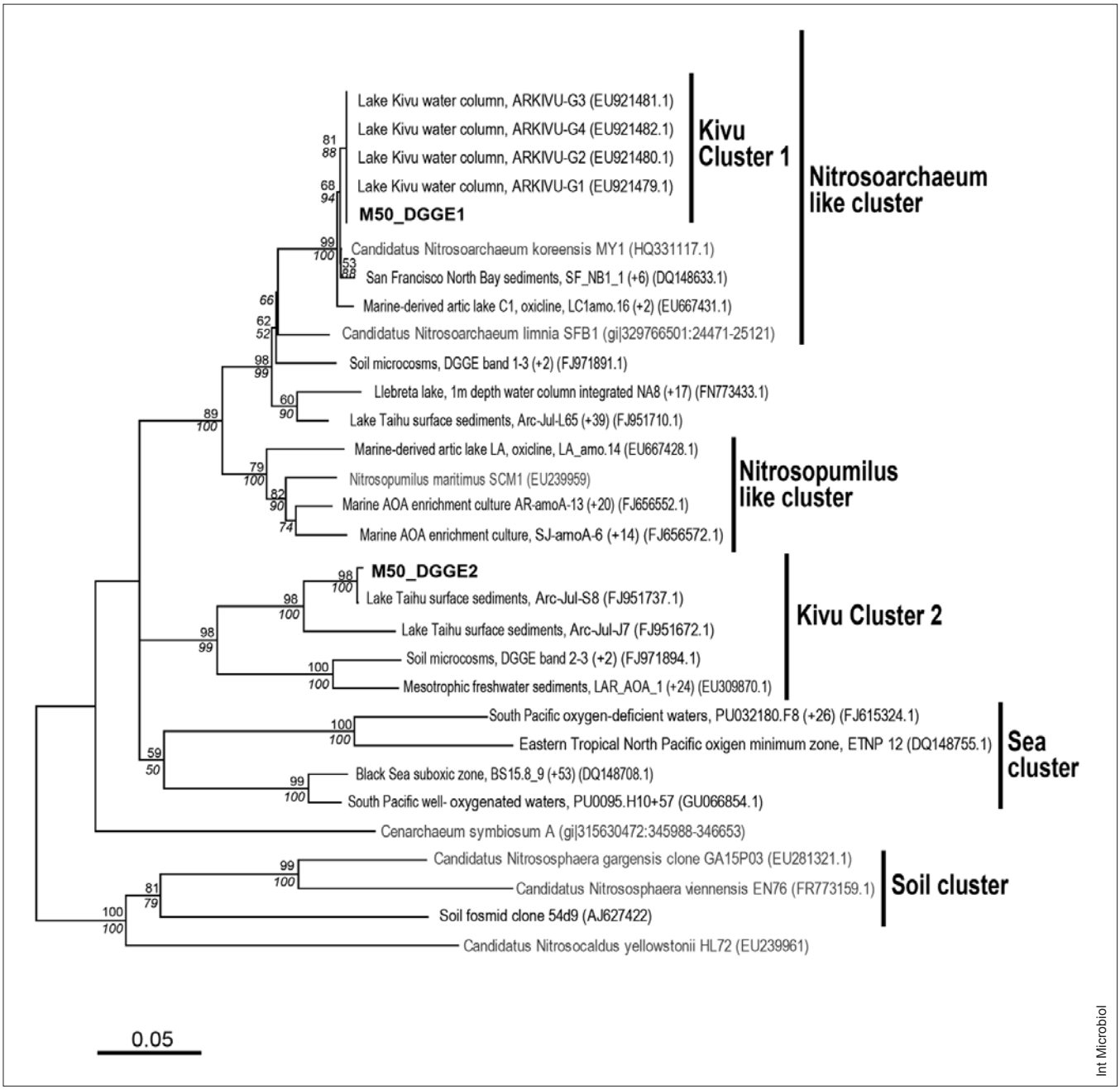

Fig. 5. Maximum-likelihood phylogenetic tree for archaeal *amoA* partial gene sequences retrieved from microcosm samples (in bold). Significant bootstrap values (≥50 %) from 1,000 replicates are shown at branch nodes; those corresponding to maximum likelihood and neighbor joining analyses are indicated above and below the line, respectively. Scale bar indicates an estimated sequence divergence of 5 %.

1, which included sequences recovered from the lake community and from the microcosm at the end of the incubation.

Substrate tests in microtiter plates. The analyses of non-inoculated control wells in the microtiter plates showed no contamination in any of the experiments. The addition of antibiotics to the medium was expected to inhibit the growth of bacteria present in the microcosm, thereby enhancing the growth of archaea. However, a decrease in archaeal abundances was determined by CARD-FISH in all antiobiotic-supplemented media (Fig. 6); therefore, the effect of antibiotic addition on archaeal growth was evaluated. Indeed, the statistical analyses revealed significant differences (ANOVA $t_{14} = 12.12$, $P < 0.001$) in archaeal cell counts from deep-well

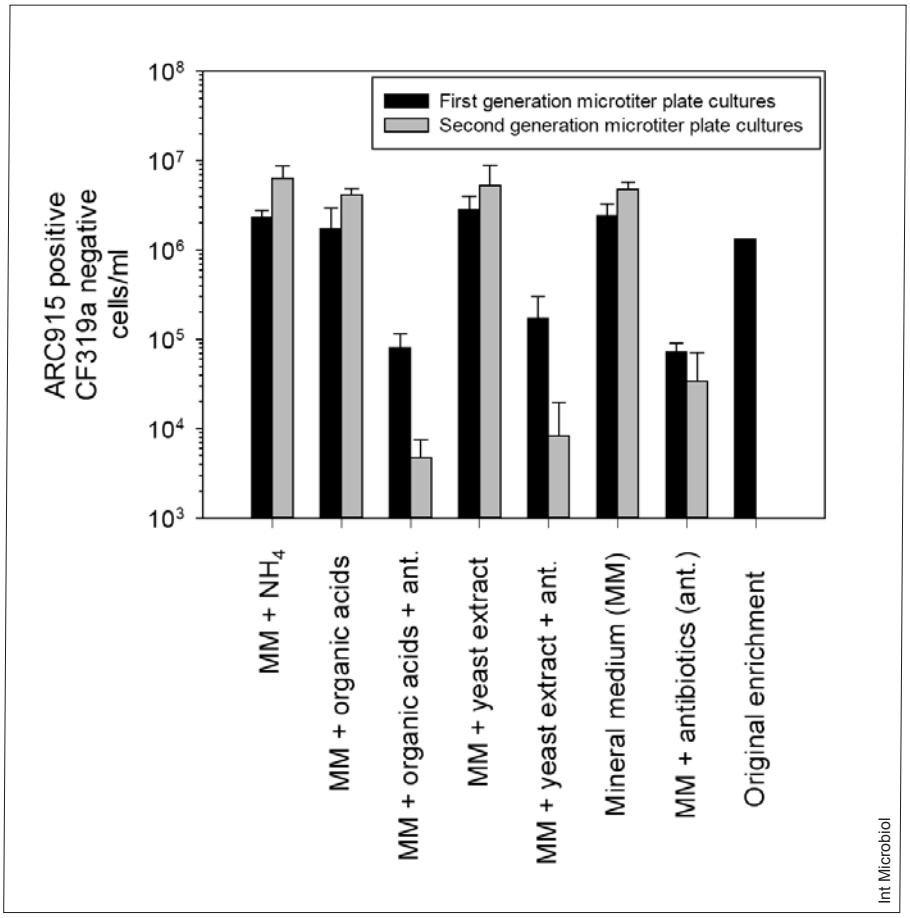

Fig. 6. Total cell numbers of archaea determined in microtiter plate enrichments by means of multi-color CARD-FISH in the first and the second microtiter plate cultivation systems (for details, see Materials and methods).

microtiter plate enrichments differing only in the presence or absence of antibiotics. The addition of a high concentration of ammonia (15 mM) did not significantly increase archaeal cell numbers compared to deep-wells supplemented with low ammonia concentrations (1 mM) (ANOVA $t_{14} = -0.042$, $P = 0.967$). By contrast, amendment of the basal mineral medium with yeast extract yielded the highest increase in archaeal abundance, although the differences were not significant compared to the other supplements (Fig. 6). Additional tests confirmed this observation as they failed to show significant differences in archaeal cell numbers in enrichments consisting of mineral medium with or without added organic compounds (ANOVA $t_{14} = -1.058$, $P = 0.308$ and ANOVA $t_{14} = 1.584$, $P = 0.135$ for yeast extract and simple organic acids, respectively). A second transfer of the enrichment samples from individual plate wells to a new microtiter plate containing fresh medium and incubated under the same conditions also did not result in increased archaeal growth under any of the assayed conditions (Fig. 6).

Discussion

Multi-color CARD-FISH. This technique is the only nucleic acid probing approach that simultaneously allows cell identification and quantitative determination of cell numbers. Although the probe ARC915 is theoretically optimal to specifically identify and count all Archaea [2] by CARD-FISH ([3,27] and references therein), several authors have reported non-specific probe binding to members of the Bacteroidetes (formerly known as the Cytophaga–Flavobacteria–Bacteroides cluster), causing overestimations of archaeal counts in natural samples [6,22,27,32,37]. To avoid this problem and thereby more precisely estimate archaeal cell numbers, we used multi-color CARD-FISH together with probes ARC915 and CF319a. To our knowledge, this is the first study in which this technique has been used to correct potential overestimations of archaeal cell counts be-

cause of non-specific hybridization of the ARC915 probe to members of the Bacteroidetes.

Archaeal cells were correctly quantified by subtracting cells that hybridized with both probes from the total number of ARC915-positive cells. With this method, we detected a decrease in the former over the course of the incubation, such that, finally, only those cells that hybridized with probe ARC915 alone were used to estimate archaeal abundances. Our results therefore question whether previous estimates of archaeal cell numbers based exclusively on probe ARC915 served as reliable indicators of archaeal abundance.

CARD-FISH *vs.* qPCR. Quantification techniques based on cell counts (FISH or CARD-FISH) or gene copy numbers (qPCR) are different approaches to determine bacterial abundances. In microcosm experiments, CARD-FISH is a valuable and reliable monitoring tool for identifying and quantifying slow-growing cells, such as AOA, with an inherently low ribosome content that may not be sufficient to yield a fluorescence signal visible by FISH alone [17,50]. Although qPCR is a very sensitive and specific technique, its use for purposes of quantification is known to have several biases that can result in differences in archaeal abundances compared to the results obtained with CARD-FISH. First, the chosen DNA extraction method strongly affects qPCR-based quantification, as demonstrated in cultures of *Dehalococcoides ethenogenes* ([13,43] and references therein).

Similarly, ten-fold difference between 16S rRNA gene copies and DAPI counts have been reported for pure cultures of *Bacillus cereus* and *Bacillus subtilis* and were attributed to deficient cell lysis [4]. Since the cell wall and membrane structures of archaeal cells differ from those of bacteria, the use of a common extraction method will not assure an equivalent degree of lysis of the two cell types.

Recently, Urakawa and co-workers showed that an improved phenol-chloroform DNA extraction method (similar to our CTAB protocol) enhanced DNA recovery from *Nitrosopumilus maritimus* cells by four-fold and resulted in a similar increase in the qPCR-based quantification of the *amoA* gene of that species [46]. We therefore assume that the limitations of this study included differences in DNA recovery, which in turn would have influenced the qPCR results. Similarly, cell-wall permeabilization by different chemical treatments implies differential effects on cell walls and therefore differences in probe hybridization. Second, mismatches between PCR primers and target DNA can affect duplex stability for PCR amplification. For example, the presence and position of a single internal primer-template mismatch reduce the efficiency of PCR amplification, in turn resulting in an underestimation of gene copy numbers by up to 1,000-fold [8]. Moreover, the authors of a comparative study reported that cell numbers determined by CARD-FISH were always at least one order of magnitude higher than gene copy numbers determined by qPCR, because of the limitations posed by DNA extraction and primer biases [31]. Accordingly, the discrepancies found in this study between the archaeal abundances determined by CARD-FISH vs. by qPCR would reflect differences in DNA recovery and/or primer-template mismatches for some archaeal groups and, therefore, an underestimation of 16S rRNA gene copy numbers. Thus, overall, to properly quantify archaeal abundance CARD-FISH is preferred over qPCR.

Qualitative and quantitative identification of target sequences by PCR-based methods. DGGE is a suitable technique for the rapid comparison of band fingerprinting between samples even though it only detects dominant members of the community (>9 %, [16]) whereas the construction of a clone library may allow the detection of phylotypes with low abundances. Moreover, DGGE allows the direct comparison of band patterns at different dates, thus providing a method to rapidly detect changes in the dominant archaeal phylotypes throughout an incubation and to compare these phylotypes with those in the autochthonous community [28]. Yet, although DGGE fingerprinting is routinously used as a screening technique to detect dominant phylotypes in enrichment cultures [15,16,23], it clearly fails in the proper identification of rare taxa (≤9 % of abundance, [16]).

To overcome this limitation, we constructed clone libraries of archaeal 16S rRNA gene from samples obtained at different dates throughout the incubation period and from the autochthonous planktonic community. The high coverage values calculated for the three clone libraries (>97 %) indicated that most, if not all, of the archaeal richness present in the enrichment was recovered.

In the present study, no AOB members were identified by either DGGE or clone library construction, but the bacterial *amoA* gene was quantified by qPCR. Although biases must be taken into consideration when PCR is used, the absence of any AOB clones in our libraries was surprising. Considering that AOB harbor a mean 2.5 *amoA* gene copies per AOB genome [45] we estimated that the AOB members in our microcosm accounted for 2.70×10^3 and 1.71×10^6 cells/ml at days 154 and 246, respectively. Since it is generally assumed that AOB carry a single 16S rRNA copy per genome [1], the number of 16S rRNA gene copies corresponding to AOB can be inferred

for these dates. Accordingly, at day 246, AOB represented 0.57 % of the total bacterial 16S rRNA gene copies/ml. Consequently, the use of conventional eubacterial primers in PCR would strongly bias amplicon replication in favor of the most abundant target sequences, making it very unlikely that AOB 16S rRNA gene sequences warranting further detection in clone libraries would be amplified. Moreover, the relative abundance of AOB in the first sample used in library construction (M_64) would have been far below 0.57 % of the total 16S rRNA gene copies, since *amoA* was not detected at that date.

Significance of archaeal ammonia oxidizers in Lake Kivu. In a previous study carried out in Lake Kivu, we showed that AOA accumulate at the oxycline (30–50 m depth), forming a community mainly dominated by a single OTU affiliated with Marine Group 1.1a Thaumarchaeota [28]. The same study identified 11 OTUs containing fewer sequences belonging to Soil Group 1.1b Thaumarchaeota, raising the question whether these OTUs were truly planktonic or were introduced into the lake by surface runoff from surrounding crop fields [33,34]. By comparing the autochthonous archaeal community in Lake Kivu with that grown in the enrichment culture, we confirmed the dominance of a single OTU (OTU-2) affiliated with Marine Group 1.1a. In addition, the results from the clone libraries supported those obtained by DGGE fingerprinting and clearly demonstrated that members of Thaumarchaeota Marine Group 1.1a were the major component of the archaeal community that developed in the experimental microcosm. Although this group mainly comprises AOA from aquatic habitats, either marine or freshwater, it also includes species isolated from soils (i.e., "*Ca.* Nitrosoarchaeum koreensis" [20]).

Culture-dependent studies have shown that different species differ in their tolerance of ammonia, consistent with the ammonia concentrations usually found in their respective habitats. For instance, *Nitrosopumilus maritimus* strain SCM1 and probably other marine species are able to cope with the very low ammonia concentrations usually found in the world oceans [29], whereas several AOA from soils can oxidize ammonia at concentrations up to 15–20 mM [20,44]. The optimal and maximum ammonia concentrations for the growth of a new strain of *Nitrosopumilus maritimus* NM25, enriched from sand in an eelgrass zone, are 15 mM and 26 mM, respectively [30]. In Lake Kivu, the average concentration of ammonia at the oxycline is below 0.5 mM [34], at which point planktonic AOA face extreme nutrient limitation. Note that in our study the OTU that contained the most sequences (OTU-2) had a high 16S rRNA gene sequence similarity to "*Ca.* Nitrosoarchaeum koreensis", an archaeal nitrifier isolated from an agricultural soil and able to tolerate ammonium concentrations up to 10 mM [20]. Similarly, several AOA strains enriched from Acton Lake and Delaware Lake (Ohio, USA) were shown to differ in their ammonium tolerances (1–5 mM). One of these strains has 99.6 % 16S rRNA identity to "*Ca.* Nitrosoarchaeaum koreensis" [14]. Consequently, the AOA prevalent in Lake Kivu might have colonized the lake from nearby crop fields and then gradually adapted to a range of ammonia concentrations wider than those in marine environments. This hypothesis merits future investigations considering the increase in nutrient inputs by water runoff over the last several decades [33-35]. However, the identification of AOA prevalent in Lake Kivu leads to the question whether these microorganisms are ecologically relevant given the low concentrations of ammonia present in the lake. It seems more plausible to consider that the AOA were not in an ecological niche appropriate for their growth. The absence of AOB during the first 154 days of the microcosm incubation in the laboratory was in agreement with the low abundances of bacterial *amoA* ($<10^2$ *amoA* gene copies/ml; Llirós M, personal comm.) measured at several sampling sites of Lake Kivu. Remarkably, the rapid growth of AOA in the experimental microcosm (Fig. 2D) was in accordance with the higher ammonia affinities of AOA than AOB [20,29]; however, this trend was reversed during the final stages of the incubation, when AOB clearly outnumbered AOA.

Stimulation of AOA growth by organic compounds and the effect of antibiotics. By using microtiter plates, we were able to examine the effect of different organic compounds on the growth of AOA in a minimal space, thereby optimizing resources and screening efforts. The ability off AOA to grow mixotrophically in oligotrophic habitats, as determined in metagenomic analyses and isotopic studies, could provide them with a selective advantage over their strict autotrophic counterparts [7,44,48]. Findings from experimental studies and metagenomic analysis support this view, by showing that AOA can use simple organic compounds as carbon sources [7,19,48,51]. By contrast, according to our results, the AOA community was neither stimulated nor inhibited by the addition of simple organic compounds to the growth medium (Fig. 6). This finding is in agreement with the high similarity of the most abundant OTU2 to "*Ca.* Nitrosoarchaeum koreensis," which is not stimulated by organic compounds [20], and with the oligotrophic conditions prevalent in Lake Kivu [38]. Previous studies on the enrichment of archaea from rhizospheres of *Licopersinon esculentum* (to-

mato plant) reported a slight stimulation of AOA growth by the addition of root extracts [40,51]. Some AOA might have a mixotrophic and/or heterotrophic metabolism, which would explain their adaptation to eutrophic environments, although AOA species differ in their responses to organic matter, which inhibits the growth of, e.g., *Nitrosopumilus maritimus* [24] while stimulating that of "*Ca*. Nitrososphaera viennensis" [44]. Thus, extreme caution is warranted when drawing conclusions from our stimulation assays, given the relatively few substrates tested and the dominance of the accompanying bacterial community, which probably was able to use the tested organic compounds more efficiently.

The inhibition of archaeal growth by medium containing antibiotics could have been caused either by direct toxicity to AOA or by an indirect effect on the satellite bacterial community. The impossibility to carry out toxicity studies on pure cultures of mesophilic AOA serious limited our ability to properly interpret the results, although our preliminary findings obtained with *Sulfolobus solfataricus* DSM 1617 did not indicate any effects of the inhibitor mixture on growth (data not shown). In addition, direct effects of antibiotics on archaeal growth were reported for *Sulfolobus acidocaldarius*—and especially for methanogens that are pathogenic in humans [9,21]—while studies on the enrichment of mesophilic archaea describe an inhibitory effect of some antibiotics [40]. We were unable to eliminate co-cultured bacteria in the microcosm despite the antibiotic treatment used. Other authors reported the same problem in their attempts to isolate AOA species from enrichment cultures [10,18,20,25]. This difficulty has led to the conclusion that archaea may obtain some benefit from the co-cultured bacteria, either commensally or symbiotically [25]. Thus, the detection in our clone libraries of many bacterial sequences affiliated with potential N_2-fixers (Rhizobiales and Burkholderiales) agrees with previous results obtained following the cultivation of acidophilic AOA from an acid soil. Further work will be needed to determine the possible metabolic implications of a relationship between potential N_2-fixers and AOA in natural environments and in co-cultures.

Aknowledgements. This work was founded by the Spanish Government through projects CRENYC (CGL2006-12058-C02-01), ARKI (CGL2007-29823-E) and ARCANOX (CGL2009-13318-C02-02). A.P.C. and M.F.H. are recipients of pre-doctoral fellowships from the Catalan (2006FI-109) and the Spanish (FPI BES-2010-035225) Governments, respectively. Authors are indebted to Ingrid Cuesta for helping on qPCR quantification of total archaea and Marc Llirós for fruitful discussions along manuscript preparation.

Competing interests. None declared.

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