

Brief report

Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika)

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Summary

Here we provide the first direct evidence for the anammox process (anaerobic ammonium oxidation) in a lacustrine system, Lake Tanganyika, the second largest lake in the world. Incubations with ¹⁵N labelled nitrate showed that anammox occurred in the suboxic water layer at 100–110 m water depth. Anammox rates up to 10 nM N₂ h⁻¹ are comparable to those reported for the marine water column. Up to ~13% of produced N₂ could be attributed to the anammox process whereas the remainder was related to denitrification. Typical lipid biomarkers characteristic of anammox bacteria were found in filtered water from the depths where anammox occurred, thus supporting the presence of anammox bacteria. Further evidence is provided by fluorescence *in situ* hybridization (FISH), revealing up to 13 000 anammox bacteria cells per ml or 1.4% of all DAPI (4'-6-Diamidino-2-phenylindole)-stained cells. Phylogenetic analyses of partial 16S rRNA genes indicated the presence of sequences most closely related to the known anammox bacterium *Candidatus 'Scalindua brodae'* (95.7% similarity). Using the incubation results, a total loss of 0.2 Tg N₂ per year linked to anammox was estimated for the Northern basin of Lake Tanganyika.

Introduction

In aquatic systems, microbial nitrogen fixation, a process that converts atmospheric nitrogen into fixed inorganic nitrogen, i.e. a form available for biological production, is the major source term in the euphotic zone (Capone and Carpenter, 1982); while bacterial denitrification had been recognized globally as the only quantitatively important process converting fixed nitrogen into atmospheric N₂ (Schlesinger, 1997; Jaffe, 2000). However, an overlooked process called 'anammox' (anaerobic ammonium oxidation) has recently been recognized as an important sink for fixed inorganic nitrogen in aquatic environments (Thamdrup and Dalsgaard, 2002; Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2003; 2005).

The anammox process was first described in bioreactors of wastewater treatment plants (van de Graaf *et al.*, 1995; Mulder *et al.*, 1995) where ammonium reacts with nitrite to produce dinitrogen gas. The anaerobic oxidation of ammonium to N₂ has recently been found in various environments (see Dalsgaard *et al.*, 2005). All these studies demonstrated the anammox process through the accumulation of ¹⁵N-labelled N₂ in incubations with ¹⁵N-labelled nitrate or ammonium. Although there is now growing evidence for the widespread occurrence of anammox in marine/brackish environments, evidence from natural freshwater environments until now has included only a trace of the process at the head of the Thames estuary (Trimmer *et al.*, 2003), and the detection of anammox organisms in a Ugandan wetland (Jetten *et al.*, 2003). Thus, no investigation so far has, to our knowledge, shown a contribution of anammox to nitrogen removal from lacustrine systems. In the following we describe the rate at which the anammox process occurs in Lake Tanganyika and the responsible microorganisms involved.

Results and discussion

Oxygen concentrations varied around 200 µM at the surface, decreased sharply to 35 µM at 80 m and were near detection limit (< 1 µM) at 90 m (Fig. 1). The oxygen gradient, confined to a 10- to 20-m-thick layer, was steeper than observed in 2001 and 2002 (B. Wehrli, unpubl.

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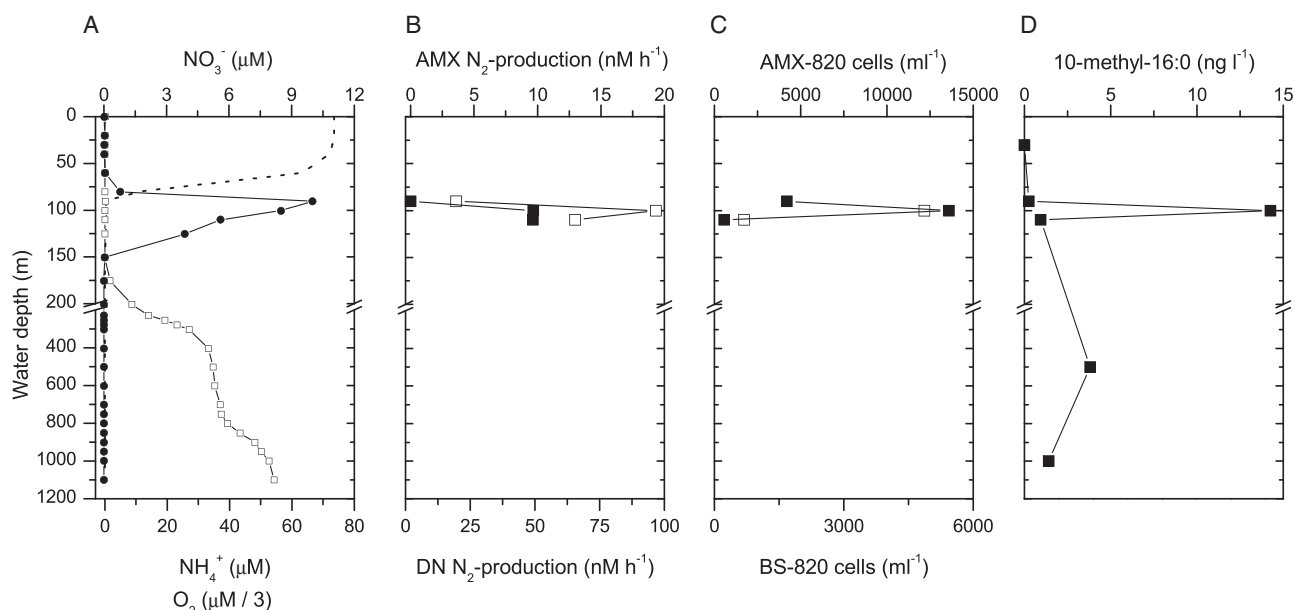


Fig. 1. A. Oxygen (dotted line), nitrate (bold circles) and ammonium (open squares) concentrations in μM (note the change in scale at 200 m). B. N_2 production rates from tracer experiments in nM h^{-1} , anammox-related production (AMX, bold squares) and denitrification-related production rates (DN, open squares). N_2 production was not detected at 80 m, and measurements at 500 and 1000 m are not included as they represent potential rates. C. Cell numbers of anammox bacteria that hybridized with probe Amx-820 (bold squares) and BS-820 (open squares). D. Concentrations of the 10-methyl-16:0 fatty acid methyl ester (ng l^{-1}) indicative of planctomycetes.

results) and in earlier studies (Degens *et al.*, 1971; Edmond *et al.*, 1993). The reason for this rather well-mixed surface layer down to approximately 80 m lies in the storms that occurred before and during our sampling campaign linked to the winter trade wind season (April–September).

Concentrations of nitrate plus nitrite were near the detection limit ($< 0.1 \mu\text{M}$) at the lake surface, presumably due to the vigorous nutrient uptake by phytoplankton (Fig. 1). We did not measure nitrite on our 2003 cruise but expected the contribution of nitrite to the combined measurement to be insignificant, as on our earlier cruises nitrite concentrations were below detection (B. Wehrli, unpubl. results). In addition, Edmond and colleagues (1993) showed extremely low concentrations ($\leq 0.01 \mu\text{M}$) except for sharp peaks of $\leq 0.3 \mu\text{M}$ in the transition zone where nitrate and ammonium are low. In general, nutrient concentrations presented by these authors agree well with our values measured on annual cruises between 2001 and 2004 (B. Wehrli, unpubl. data). Nitrate increased abruptly at the oxic/anoxic interface to $\sim 10 \mu\text{M}$ at 90 m and had disappeared again at 150 m. Ammonium was not detected in the suboxic, nitrate-containing layer between 90 and 150 m, but concentrations increased below reaching $1.6 \mu\text{M}$ at 175 m and $27 \mu\text{M}$ at 300 m (Fig. 1). The nutrient profiles resembled those from the anoxic bay Golfo Dulce, Costa Rica, where a tight coupling of ammonium liberation during denitrification-based mineralization

and further oxidation of this ammonium by anammox was demonstrated (Dalsgaard *et al.*, 2003).

To reveal whether the anammox process occurs in the water column of Lake Tanganyika, incubations were performed under *in situ* conditions using isotopically labelled $^{15}\text{NO}_3^-$. Production of ^{15}N -labelled N_2 was detected at all anoxic depths in our anoxic incubations with $^{15}\text{NO}_3^-$, but not at 80 m where oxygen was present *in situ*. Results from 110 m are shown in Fig. 2A as an example. N_2 production included both masses 29 and 30 ($^{14}\text{N}^{15}\text{N}$ and $^{15}\text{N}^{15}\text{N}$, hereafter abbreviated $^{29}\text{N}_2$ and $^{30}\text{N}_2$ respectively). The two masses were produced at a constant ratio (Fig. 2B), which indicated that the results were not affected by dilution of ^{15}N from nitrate into a nitrite pool originally present in the samples.

While $^{30}\text{N}_2$ was the major product, indicating active denitrification, the production of N_2 with a mass of 29 at 100 and 110 m exceeded that expected from denitrification with random isotope pairing, strongly indicating that anammox bacteria were active in the incubated samples. The rate of N_2 production through anammox was $\sim 10 \text{ nM h}^{-1}$ at both 100 and 110 m, corresponding to 9% and 13% of total N_2 production respectively (Fig. 1B). The relative contributions of anammox to N_2 production of 9–13% fall within the wide range of results from sediments (from below detection to 67%; for a recent compilation see Dalsgaard *et al.*, 2005) and water columns. Rates of anammox in Lake Tanganyika were similar to those mea-

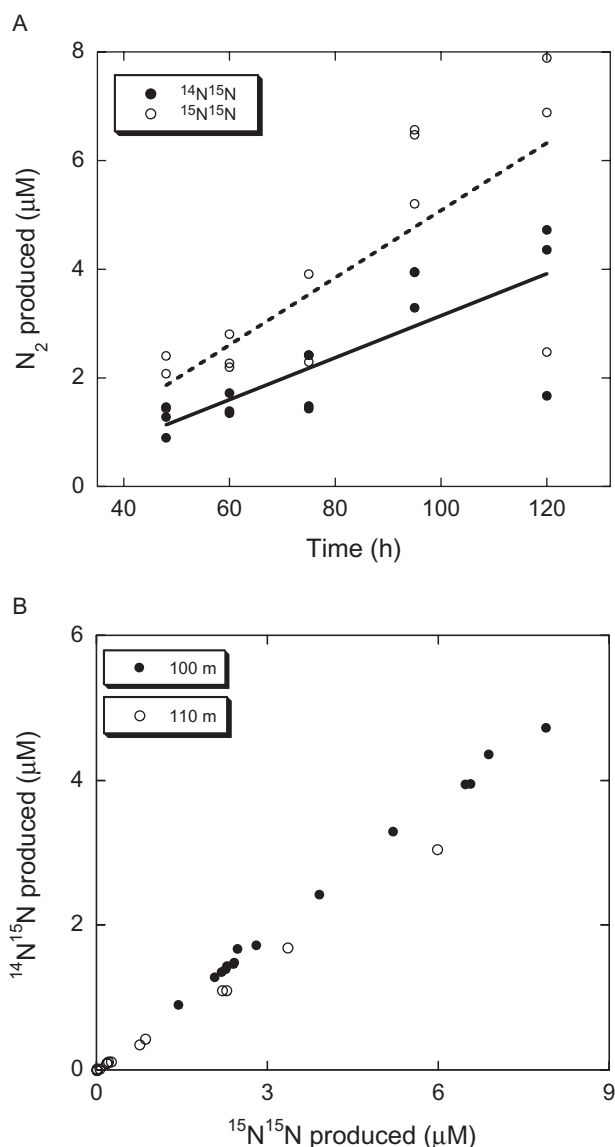


Fig. 2. A. Production of $^{14}\text{N}^{15}\text{N}$ (solid circles) and $^{15}\text{N}^{15}\text{N}$ (open circles) over time of incubated water samples from 110 m water depth. B. The ratio of the produced $^{14}\text{N}^{15}\text{N}$ versus $^{15}\text{N}^{15}\text{N}$ in incubated water samples from 100 m (closed circles) and 110 m (open circles) water depth. Each point represents an individual Exetainer incubation.

sured in Golfo Dulce (1–20 nM $\text{N}_2 \text{ h}^{-1}$; Dalsgaard *et al.*, 2003) and the Benguela upwelling system (0.4–7 nM $\text{N}_2 \text{ h}^{-1}$; Kuypers *et al.*, 2005), while lower rates have been found in the oxygen-deficient waters of the eastern South Pacific ($\leq 0.7 \text{ nM h}^{-1}$; Thamdrup *et al.*, 2006). These are the only other experimentally determined rates of anammox reported so far for pelagic systems.

The lowest salinity at which anammox activity has so far been reported in natural waters is 2 psu in muds of the Thames estuary (Trimmer *et al.*, 2003). In that study, however, the contribution of anammox to total N_2 production was only ~1%, while it increased to 8% further down the

estuary, as salinity reached 10 psu. Thus, our research is the first report of substantial anammox activity taking place in a natural freshwater system.

Further evidence that anammox organisms were present in the chemocline of Lake Tanganyika came from lipid investigations. Anammox bacteria contain unique lipids (Sinninghe Damsté *et al.*, 2002; Schmid *et al.*, 2003) that can be used to trace anammox cells in the environment (Kuypers *et al.*, 2003; 2005). We were able to detect 10-methyl-hexadecanoic acid, a membrane lipid typical for planctomycetes, which occurs in high concentrations in anammox organisms (Sinninghe Damsté *et al.*, 2002; 2004; Schouten *et al.*, 2004). Highest concentrations of 10-methyl-hexadecanoic acid were found at 100 m water depth (up to 14 ng l^{-1} filtered water), whereas at 90 and 110 m water depth a concentration of 0.3 and 1 ng l^{-1} , respectively, and at 500 and 1000 m concentrations of 4 and 1 ng l^{-1} were detected (Fig. 1D). The absence of this specific lipid above 90 m may indicate that no or only negligible amounts of planctomycetes are living in the uppermost water layer, whereas the lower amounts detected below the water layer where the anammox process takes place might be related to relicts from degrading bacteria from above.

To confirm the presence of anammox bacteria in the chemocline of Lake Tanganyika, fluorescently labelled oligonucleotide probes specific for anammox bacteria were applied to filtered water samples. In a microbial community with total cell abundance of $0.4\text{--}1.9 \times 10^6 \text{ ml}^{-1}$ [4'-6-Diamidino-2-phenylindole (DAPI) counts], 600–13 000 cells ml^{-1} or 0.1–1.4% of all DAPI-stained cells could be identified as anammox bacteria (Fig. 1C), exactly at the water depths where incubations revealed anaerobic ammonium oxidation. Both the relative and the absolute anammox bacterial abundances are comparable to the abundances in the Black Sea (0.75%, ~1900 cells ml^{-1} ; Kuypers *et al.*, 2003) and in the Benguela upwelling region (0.5–1.3%, ~4000–22 000 cells ml^{-1} ; Kuypers *et al.*, 2005). Hence, based on cell counts and process rates (see above), anammox bacteria appear to play a similar role in nitrogen cycling in Lake Tanganyika as compared with their marine pelagic counterparts.

The identity of anammox bacteria at 100 and 110 m depth was verified by phylogenetic analyses of partial 16S rRNA genes. Separate clone libraries were constructed for the 16S rRNA genes amplified with primers specific for *Planctomycetales* and anammox bacteria, each for 100 and 110 m water depth. Out of the 400 clones screened in the four clone libraries, 49 unique *Planctomycetales* sequences or operational taxonomic units (OTUs; < 97% similarity) were obtained and one fell into the cluster with known anammox sequences (Fig. 3). The closest relatives to this sequence were *Candidatus* 'Scalindua brodae' (95.7% similarity) found in a wastewa-

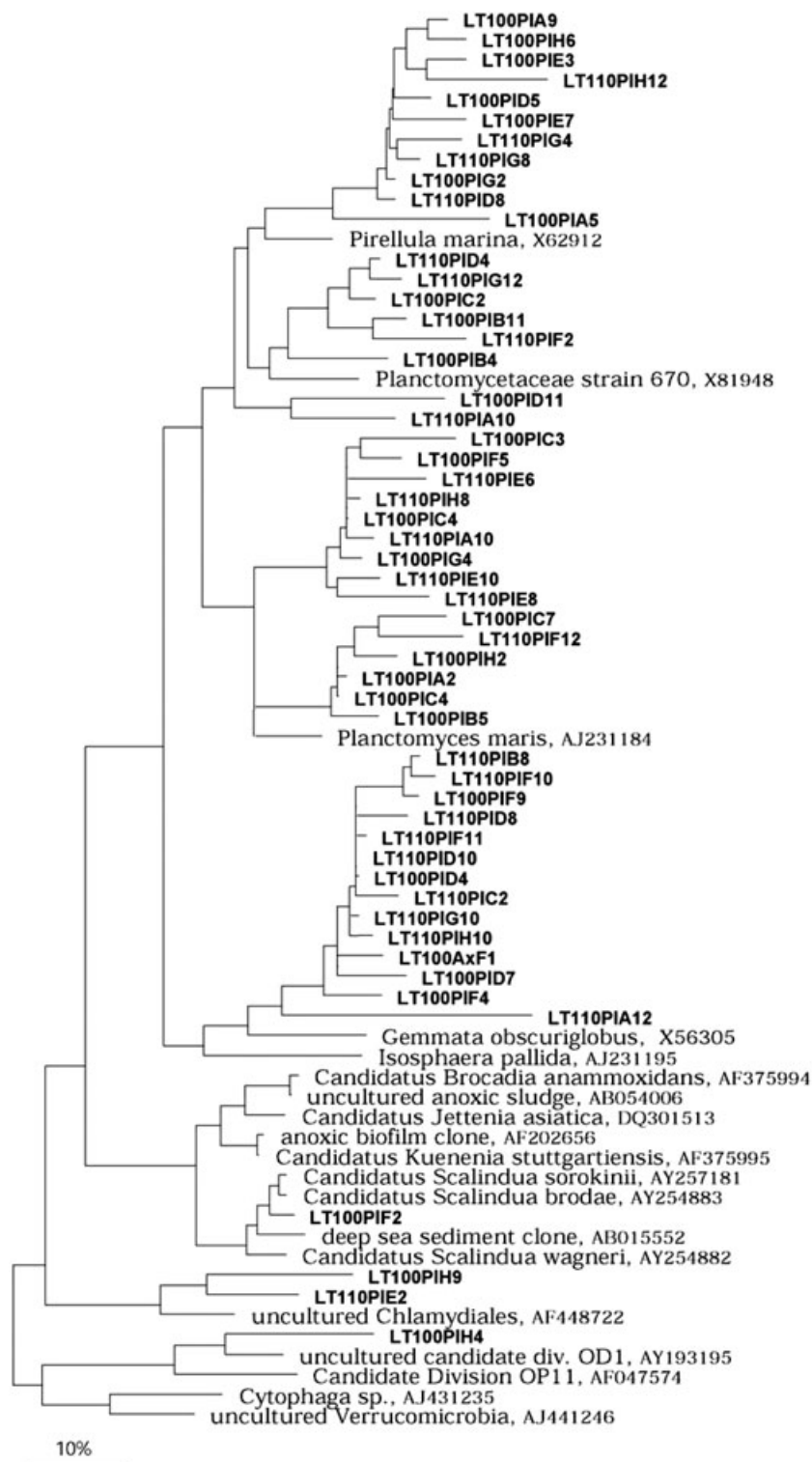


Fig. 3. Phylogenetic tree showing the affiliations of partial 16S rRNA gene sequences retrieved from Lake Tanganyika (in bold) in the order Planctomycetales, with particular reference to clusters of known anammox bacteria, the genera *Pirellula*, *Planctomyces*, *Gemmata* and *Isosphaera*, as well as some related non-planctomycetales sequences. This is a maximum likelihood tree, with the topology verified by maximum parsimony (bootstrapped 100%) and distance matrix (bootstrapped 1000%) methods. The tree branching shown here has been supported by at least 50% of the bootstrap values of maximum parsimony and distance matrix methods, and only sequences over 1000 bp long are included in the analyses. The scale bar represents 10% estimated sequence divergence.

ter treatment plant for landfill leachate (Schmid *et al.*, 2003) and *Candidatus* 'Scalindua sorokinii' (95.6% similarity) found in the Black Sea water column (Kuypers *et al.*, 2003). Although this sequence has two mismatches with the oligonucleotide probe Amx-820 used in

fluorescence *in situ* hybridization (FISH), positive FISH signals are possible if the hybridization stringency is not at the maximum. Results from Probe Match, a service tool in the Ribosomal Database Project II (Cole *et al.*, 2005), showed that even up to three mismatches of this

probe would exclusively bind to anammox-related sequences. Interestingly, while most marine anammox environmental sequences obtained thus far are almost identical ($\geq 98\%$ similarity) to *Candidatus* 'Scalindua sorokinii' found in the Black Sea, the sequence obtained in Lake Tanganyika only shares a 95.6% similarity. Other acquired *Planctomycetales* sequences clustered with cultured and uncultured representatives of *Planctomyces*, *Pirellula*, *Gemmata*, *Isosphaera* and other unclassified planctomycetes. Similar sequences are often obtained from other anoxic/suboxic environments yet their ecological functions remain unknown.

Using the maximum anammox cell abundance of 13 000 cells ml^{-1} and a maximum anammox rate of 10 nM h^{-1} , a specific rate of 18 fmol ammonium per anammox cell per day was calculated for the transition zone of Lake Tanganyika. This is comparable to anammox rates (2–20 fmol $\text{cell}^{-1} \text{ day}^{-1}$; Strous *et al.*, 1999) found in bioreactors. Compared with other natural environments, i.e. the Benguela upwelling and the Black Sea where rates of 4.5 and 3–4 fmol $\text{cell}^{-1} \text{ day}^{-1}$, respectively, were estimated, rates in Lake Tanganyika are approximately four times higher. One explanation could be the difference in ambient water temperatures leading to different enzyme activities. Whereas the aforementioned environments showed water temperatures of 8°C, Lake Tanganyika water temperatures at the depth where the anammox process was observed are much higher (24.6°C). Assuming an Arrhenius-type temperature dependence with exponentially increasing rates with increasing temperatures, a daily cell rate of 18 fmol therefore seems a reasonable number for anammox organisms in a tropical water column.

Although our findings only represent one measurement at a specific time of the year and any extrapolation has to be performed with caution, we try to place our results in a greater context. Using the results from our incubation experiments, the depth integrated rates for the 100–110 m depth interval for the loss of fixed nitrogen via anammox are 2.4 $\text{mmol m}^{-2} \text{ day}^{-1}$. We estimated the area of the northern basin where our study has been performed at approximately 100 m water depth to occupy 40% of the total lake area (32 600 km^2 , Stoffers and Hecky, 1978). This leads to a fixed nitrogen loss per year in the northern basin of Lake Tanganyika due to anammox activity of approximately 0.2 Tg.

In conclusion, our results demonstrate for the first time that anammox bacteria are not only abundant in waste water treatment plants and marine systems but may also play a significant role in the transformation of fixed nitrogen in limnic systems. Future research should focus on this relatively new process in other natural freshwater systems to better constrain its role in the global nitrogen cycle.

Experimental procedures

Sampling

Lake Tanganyika is the largest of the Rift Valley lakes of East Africa, and the second largest freshwater body in the world after Lake Baikal (Hutchinson, 1957; Falkner *et al.*, 1991) with a total water volume of $\sim 18\,940 \text{ km}^3$. Lake Tanganyika is 650 km long and on average 50 km wide and is divided into three basins (Degens *et al.*, 1971). It is meromictic with a sharp chemocline all year round. Thermohaline stratification and a high productivity of about 360 $\text{gC m}^{-2} \text{ a}^{-1}$ (Hecky and Fee, 1981) lead to anoxia below 100–130 m (Degens *et al.*, 1971; Edmond *et al.*, 1993), which makes Lake Tanganyika the largest anoxic freshwater body in the world.

The here presented research was part of a sampling campaign that took place over a period of 4 years from 2001 to 2004. Water samples were recovered with a Niskin bottle connected to a conductivity/temperature/density probe (CTD) system from a station (5°05'S, 29°31'E, 1100 m water depth) close to the town Kigoma in the northern basin of Lake Tanganyika in July 2003. Sampling included a sample every 20 m (down to 100 m), 25 m (down to 300 m) and 100 m (below 300 m). Six additional water depths (80, 90, 100, 110, 500 and 1000 m) were specifically sampled for the here presented research on the anammox process.

Concentration measurements

Filtered aliquots of water were analysed for $\text{NO}_3^- + \text{NO}_2^-$ and NH_4^+ . Nitrate plus nitrite was analysed by standard colorimetric methods after cadmium reduction on board with a 'Procon' autoanalyser (VEB, 1971). Ammonium was analysed using the phenol hypochlorite method on a 'Merck SQ300' photometer (VEB, 1971). Oxygen concentrations were measured with a SBE oxygen sensor (attached to a SBE CTD) that has been calibrated using Winkler titration. The errors of nitrate plus nitrite and ammonium measurements are between 2% and 4%.

Incubations

Denitrification and anaerobic ammonium oxidation were quantified through the production of ^{15}N -labelled N_2 from $^{15}\text{NO}_3^-$ during anoxic incubations using the technique of Dalsgaard and colleagues (2003).

Isotope analysis

Concentrations of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ were determined at the National Environmental Research Institute, Silkeborg, Denmark, by isotope ratio mass spectrometry of the headspace in the sample vials (Robo-Prep-G in line with TracerMass; Europa Scientific, Crewe, UK) and calculated as excess above their natural abundances.

Calculations

Rates of production of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ were determined from the slopes of linear regressions of excess $^{29}\text{N}_2$ and $^{30}\text{N}_2$ con-

centrations over time. These rates were then partitioned between denitrification and anaerobic ammonium oxidation and used to calculate the total rates of these processes (Thamdrup and Dalsgaard, 2002). In summary, consistent with the results from previous studies, we assumed random pairing of ^{14}N and ^{15}N in N_2 produced from nitrate during denitrification (Hauck *et al.*, 1958; Nielsen, 1992), while N_2 produced through anaerobic ammonium oxidation consisted of one nitrogen atom from NO_3^- and one from NH_4^+ (van der Graaf *et al.*, 1995; Thamdrup and Dalsgaard, 2002; Dalsgaard *et al.*, 2003). Thus, in incubations with $^{15}\text{NO}_3^-$, denitrification produced $^{28}\text{N}_2$, $^{29}\text{N}_2$ and $^{30}\text{N}_2$ in the ratio $(1 - F)^2:2(1 - F)F:F^2$, where F denotes the mole fraction of $^{15}\text{NO}_3^-$ in NO_3^- , while anaerobic ammonium oxidation produced $^{28}\text{N}_2$ and $^{29}\text{N}_2$ in the ratio $(1 - F):F$. These calculations neglect minor kinetic isotope effects associated with the processes.

Lipid analysis

Particulate organic matter for lipid analyses was collected from specific water depths by filtration of large volumes (up to 500 l) of water through 142-mm-diameter pre-combusted (at 370°C) glass fibre filters (GFF Whatman; nominal pore size $0.7\ \mu\text{m}$) with *in situ* pumps. The filters were extracted for 24 h in a Soxhlet apparatus to obtain the total lipid extracts. Aliquots of the total extracts were saponified after addition of an internal standard and separated into fatty acid and neutral lipid fractions. The fatty acid fraction was methylated ($\text{BF}_3\text{-MeOH}$, Sigma) and analysed by gas chromatography and gas chromatography-mass spectrometry for quantification and identification.

Bacterial abundance and FISH

Water samples were collected throughout the water column to determine total cell abundance and the abundance of anammox cells (only at depths where anammox rates were detected). Microbial cells were fixed by the addition of concentrated formaldehyde solution (5% final concentration) for 15 min at room temperature and thereafter recovered by gentle vacuum filtration (20 and 50 ml for each sample) on to polycarbonate filters with a pore size of $0.2\ \mu\text{m}$ (GTPB, Millipore). Then, the filters were washed with PBS buffer and transferred into sterile PP Petri dishes, sealed, and stored frozen at -20°C for DAPI staining and FISH. The oligonucleotide probes Amx-0820-a-A-22 and BS-820-a-A22 (MWG, Germany) specific for anammox bacteria were used for hybridization and a 'nonsense' probe was used to minimize autofluorescence and unspecific binding. The protocols of Schmid and colleagues (2000; 2005) were used for the hybridization procedure.

DNA extraction, polymerase chain reaction, DNA sequencing and phylogenetic analyses

Prokaryotic DNA was extracted from subsampled portions of the same glass fibre filters as for lipid analyses, at 100 and 110 m water depth. Protocols for DNA extraction and polymerase chain reaction (PCR) followed that by Schmid and colleagues (2003). *Planctomycetales*-specific oligonucleotide

Pla46 (Neef *et al.*, 1998) and anammox-specific oligonucleotide Amx-368 (Schmid *et al.*, 2003) were used as forward primers in separate PCR reactions with the universal 1392R (Lane, 1991) as the reverse primer. Clone libraries were constructed using TOPO TA Cloning kit with vector pCR4.0 (Invitrogen). One hundred clones were screened from each of the four clone libraries with M13F and M13R primers. DNA sequencing was performed with ABI BigDye and ABI Prism 3100 Genetic Analyser (Applied Biosystems). The phylogeny of these sequences were analysed with the ARB software package (Ludwig *et al.*, 2004), along with closely related sequences (over 100 bp long) retrieved from the GenBank through BLAST searches (Altschul *et al.*, 1990). The 16S rDNA sequences obtained in this study have been submitted to the GenBank under Accession No. DQ444362 to DQ444455.

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