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# Water column anammox and denitrification in a temperate permanently stratified lake (Lake Rassnitzer, Germany)

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## Abstract

We studied microbial $N_2$ production via anammox and denitrification in the anoxic water column of a restored mining pit lake in Germany over an annual cycle. We obtained high-resolution hydrochemical profiles using a continuous pumping sampler. Lake Rassnitzer is permanently stratified at ca. 29 m depth, entraining anoxic water below a saline density gradient. Mixed-layer nitrate concentrations averaged ca. 200 μmol $L^{-1}$, but decreased to zero in the anoxic bottom waters. In contrast, ammonium was $<5$ μmol $L^{-1}$ in the mixed layer but increased in the anoxic waters to ca. 600 μmol $L^{-1}$ near the sediments. In January and October, $^{15}N$ tracer measurements detected anammox activity (maximum 504 nmol $N_2$ $L^{-1}$ $d^{-1}$ in $^{15}NH_4^+$-amended incubations), but no denitrification. In contrast, in May, $N_2$ production was dominated by denitrification (maximum 74 nmol $N_2$ $L^{-1}$ $d^{-1}$). Anammox activity in May was significantly lower than in October, as characterized by anammox rates (maximum 6 vs. 16 nmol $N_2$ $L^{-1}$ $d^{-1}$ in incubations with $^{15}NO_3^-$), as well as relative and absolute anammox bacterial cell abundances (0.56% vs. 0.98% of all bacteria, and $2.7 \times 10^4$ vs. $5.2 \times 10^4$ anammox cells $mL^{-1}$, respectively) (quantified by catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) with anammox bacteria-specific probes). Anammox bacterial diversity was investigated with anammox bacteria-specific 16S rRNA gene clone libraries. The majority of anammox bacterial sequences were related to the widespread *Candidatus* Scalindua sorokinii/brodae cluster. However, we also found sequences related to *Candidatus* S. wagneri and *Candidatus* Brocadia fulgida, which suggests a high anammox bacterial diversity in this lake comparable with estuarine sediments.


***Keywords:*** Anammox; Denitrification; Water column; Meromictic; Lake; CARD-FISH

## Introduction

Annual global anthropogenic fixation of nitrogen (N) into bioavailable forms represents an increase over natural N fixation of >100% [15]. This additional N has contributed to the eutrophication of both freshwater and marine environments, characterized by excess primary production and consequent water column

*Abbreviations:* CARD, catalyzed reporter deposition; CTD, conductivity, Temperature, and depth device; FISH, fluorescence *in situ* hybridization; PCR, polymerase chain reaction.

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anoxia, which imperils aquatic metazoan ecosystems. The microbial transformation of dissolved inorganic N to gaseous forms is a pivotal sink that balances the flux of N into the biosphere via N fixation and that can ameliorate the effects of excess anthropogenic N (reviewed in [34]). Denitrification, the anaerobic heterotrophic reduction of nitrate ($NO_3^-$) to dinitrogen gas ($N_2$), was long thought to be the only significant metabolic pathway of $N_2$ production, but classical heterotrophic denitrification now shares its position with chemolithotrophic denitrification (i.e. with sulfide or iron) [7], as well as the more recently discovered anaerobic ammonium oxidation (anammox) process [43]. Anammox, the anaerobic equimolar oxidation of ammonium ($NH_4^+$) with nitrite ($NO_2^-$) to generate $N_2$, is the chemolithotrophic metabolic pathway of a diverse, but apparently monophyletic clade of planctomycete bacteria [42]. Both denitrification and anammox have been shown to play important roles in coastal sediments, but evidence accumulating since 2003 suggests that anammox may in fact be the dominant N-loss process in suboxic marine water columns [18]. The identities of the metabolic processes responsible for $N_2$ production have significant implications for the N cycle, as well as for carbon (C) and oxygen ($O_2$) budgets, particularly in water columns [28].

Although anammox has been shown to play a significant role in marine systems, less attention has been paid to freshwater and lacustrine (lake) systems. Denitrification is thought to play a significant role in lacustrine N cycling (reviewed in [34]); however, earlier methods could not differentiate between denitrification and anammox. More recently, $N_2$ production via anammox activity has been detected in low-salinity estuarine sediments, where its contribution to total $N_2$ flux ranged up to 22% [11,35,46].

As anthropogenic eutrophication increases nutrient loading to fresh waters, and these nutrients increase the frequency and duration of water column anoxia in lakes, there is an increasing need for an improved understanding of denitrification and anammox [47]. However, little is known about the importance and distribution of anammox in lakes, particularly within the water column. Early work detected the production of labeled $N_2$ from a $^{15}NO_3^-$ tracer in subarctic lake water [14]. Other lake studies using mass balance, isotope fractionation, or acetylene inhibition approaches also suggested $N_2$ production that was identified as water column denitrification [3,29,49]. Recently, anammox activity was detected in the anoxic water column of a deep, permanently stratified tropical lake (Lake Tanganyika), where on one date, anammox was estimated to contribute 13% of the total $N_2$ production, with the balance accounted for by denitrification [40].

Although the importance of anammox in a variety of environments has been demonstrated, little is yet known about the factors that control the spatial and temporal distribution of denitrification *vis-à-vis* anammox. A number of studies have examined the role of organic carbon and $NO_3^-$ availability on anammox in sediments, but have been somewhat inconclusive (reviewed in [13]). The factors controlling the relative contribution of anammox and denitrification to $N_2$ production in water columns are even less well understood. In addition, the significance of denitrification to water column $N_2$ production remains unresolved because the few published instances of significant directly measured water column denitrification (e.g. [12,19,40]) have relied on long *in vitro* incubations of 48 h or more.

As in marine systems, the anoxic conditions necessary to support lacustrine water column denitrification and/or anammox arise when density stratification of the water column prevents atmospheric replenishment of dissolved $O_2$ consumed by respiration. Water column stratification can be maintained by thermal density gradients, which may be ephemeral or seasonal, or permanent, as in deep lakes. Meromixis (permanent stratification) may also be maintained by salinity-related density gradients (haloclines). Deep-sea brine pools result from the hydrogeothermal solution of mineral deposits. In coastal lakes, estuaries, and fjords, haloclines form by the intrusion of seawater. Solution lakes develop haloclines via the dissolution of minerals by groundwater. The resulting halostratification can lead to the formation of very steep oxygen and chemical gradients (chemoclines), which support thin "lenses" of microorganisms adapted to particular chemical microenvironments [10]. The entrainment of particular microbial communities may be enhanced by the attendant steep density gradients (pycnoclines), which trap particles of different densities at specific depths [45]. Special sampling techniques are required to study microbial metabolism in such steep halostablilized chemoclines [10,22].

In the present report, we studied physical and chemical profiles and $N_2$-production mechanisms within the monimolimnion (unmixed bottom waters) of a halostratified meromictic solution lake in northern Germany on three occasions distributed over an annual cycle. Lake Rassnitzer is a restored mining pit lake. Highly saline groundwater seepage had filled the bottom 6 m of the lake before it was filled in 1998 with diverted river water. To study anammox and denitrification in the water column of the lake, we measured hydrochemical properties in profiles at the high vertical resolution appropriate for studying steep chemoclines, and used short incubations with isotopic tracers to measure the metabolic activities of anammox and denitrification. We also looked for molecular markers of anammox bacteria in order to understand their distribution, abundance, and diversity within the lake.

## Materials and methods

### Study site

Lake Rassnitzer (formerly known as Merseburg-Ost 1b) is a restored mining pit lake located near Leipzig, Germany. The physical limnology of the lake and the stability of its halocline have been previously characterized [4,5,16,48]. During four field campaigns on 27 January, 19 May, 14 July, and 18 October 2005, we characterized the hydrochemical structure of the water column. Further, in January, May, and October, we performed incubations of halocline waters with the isotopically labeled tracers $^{15}NH_4^+$ and $^{15}NO_3^-$ to determine the pathways of $N_2$ formation (denitrification and/or anammox). We also collected water samples for detection of anammox bacteria-related 16S ribosomal ribonucleic acid (rRNA) gene sequences and for catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH). All sampling and instrument casts were made from a station over the deepest portion of the lake (36 m).

### Water column profiles

We obtained profiles of salinity, temperature, and oxygen with a conductivity, temperature, and depth (CTD) device (YSI 600XLM, Yellow Springs, Ohio), equipped with a Clark-type pulsed polaragraphic electrode (13 μm high-sensitivity membrane, 6 s response time to a 75% increase in $O_2$ saturation). $O_2$ profiles were measured in upcasts at a vertical velocity of 1 m $min^{-1}$. pH, turbidity, and chlorophyll fluorescence were measured with a second multiparameter CTD device (Idronaut Ocean Seven 316, Brugherio, Italy). Water samples for high-resolution nutrient analyses and $^{15}N$ incubations were obtained by a custom pump-CTD system employing a progressive cavity pump to minimize disruption of *in situ* particulates (Geotech Keck SP, Denver, Colorado). Water samples were collected continuously while the pump was lowered at a constant rate; sample depths were determined from the known pumping rate and were intercalibrated with the CTD data using conductivity measurements. Dye tracer studies demonstrated that "smearing" as the sample was pumped to the surface limited vertical resolution to ±0.15 m (data not shown). In May and October, water samples for nutrient analysis were immediately acidified (pH = 2) with HCl to prevent the formation of Fe oxide particulates upon aeration of anoxic waters; all samples were held at 4 °C during transport to our laboratory (3 h), where they were filtered (0.2 μm) and frozen (−20 °C) for later analysis for $NO_3^-$, $NO_2^-$, $NH_4^+$, and total Fe (detection limits 0.1, 0.1, 0.3, and 0.1 μmol $L^{-1}$, respectively). Nitrate and $NO_2^-$ analysis were by reduction to nitric oxide followed by chemiluminescence detection [6]. Ammonium analysis was by the colorimetric indophenol method [17]. Total Fe was determined by the ferrozine method [17]. Mixed-layer $PO_4^{3-}$ concentrations were determined by the molybdate method [17]. Monimolimnetic sulfide ($S^{2-}$) was determined in May only by the methylene blue method [17]. All sampling casts within and across sampling dates were intercalibrated with reference to the 18.0 psu (practical salinity units) salinity isopleth, which is known to be stable over the annual cycle of our study [4].

### $^{15}N$ incubations and analysis

$^{15}N$-labeling experiments were performed in January, May, and October 2005. Water was collected by pump-CTD from 4 to 6 depths beginning within the oxycline and extending 2–4 m into the monimolimnion. Lake water was collected in quadruply-flushed serum bottles (to eliminate atmospheric contamination), which were then stoppered, sealed, and stored at *in situ* temperatures for transport back to the laboratory (3 h). The $^{15}N$ incubations were begun immediately upon reaching the laboratory. The experimental method was slightly modified from that previously published by Dalsgaard et al. [12]. Briefly, 250 mL of lake water were flushed with He for 15 min to remove background $N_2$ and amended with $^{15}NH_4^+$, $^{15}NH_4^+ + {}^{14}NO_2^-$, or $^{15}NO_3^-$ (Campro Scientific, Berlin, Germany). Each of the substrates ($^{15}NH_4^+$, $^{14}NO_2^-$, and $^{15}NO_3^-$) was added to a final concentration of 8.3 μmol $L^{-1}$ in January and October and to 20.8 μmol $L^{-1}$ in May, with the exception of October, when $^{15}NH_4^+$ was added to a final concentration of 33.3 μmol $L^{-1}$.

Isotopically labeled lake water was transferred into 12-mL Exetainers (Labco, High Wycombe, UK) and incubated up to 24 h in the dark at *in situ* temperatures. The Exetainers were destructively sampled at ca. 0, 8, 16, and 24 h using $HgCl_2$ to stop biological activity. $^{14}N^{15}N$:$^{14}N^{14}N$ and $^{15}N^{15}N$:$^{14}N^{14}N$ ratios of He-equilibrated headspaces were determined by gas chromatography/isotope ratio mass spectrometry (Fisons VG Optima, UK). Estimates of total $N_2$ production ($^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$) from anammox and/or denitrification were derived from the isotope pairing equations [43].

### Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) of anammox bacteria

Bacterioplankton samples were obtained with our custom pump-CTD system (above). Water samples were fixed with particle-free paraformaldehyde solution (final concentration, 1% v/v) for 1 h at room temperature or

overnight at 4 °C. Aliquots of 10–30 mL were vacuum-filtered onto membrane filters (GTTP, 47 mm diam., 0.22 μm pores, Millipore, Schwalbach, Germany) and stored at −20 °C until further processing. The CARD-FISH procedure was as described in Pernthaler et al. [33] and Woebken et al. [50]. The probe AMX-368 [38] as well as a mixture of probes AMX-820 [37], BS-820 [26], and BS-820C [18] were chosen to target the entire group of known anammox bacteria. After CARD-FISH, filter sections were treated with 4,6-diamidino-2-phenylindole (DAPI, a non-specific DNA stain) and subsequently evaluated by microscopy (Carl Zeiss Axioplan, Jena, Germany). We counted a minimum of 2000 DAPI-stained cells per filter section (equivalent to approximately 20 fields of view). The relative abundance of anammox bacteria labeled with the probe mix (above) was determined as a percentage of the DAPI-stained cells. Quantification of the labeled cells was performed manually on 3 replicates per water depth and errors are standard deviations.

## Construction of 16S rRNA gene clone libraries

Clone libraries of Lake Rassnitzer anammox bacteria from January 2005 (30 m depth) were constructed following the protocol for the filter polymerase chain reaction (PCR) [24] using filters prepared as described above. For the PCR reaction, cells on ca. 1 $mm^2$ filter sections were lysed with 3 freeze–thaw cycles (−80 and 42 °C) in sterile PCR-grade water. One planctomycete-specific clone library (primers PLA46F [31] and 1392R [41]) and one anammox bacteria-specific clone library (primers AMX368 and 1392R) were constructed (32 PCR cycles; annealing temperatures 58 and 56 °C, respectively) following standard PCR protocols including a optimized reduction of PCR cycles to minimize PCR bias.

Three clone libraries were also constructed from DNA isolated from an October 2005 Lake Rassnitzer water sample. For DNA extraction, 450 mL of untreated water were filtered as described above. DNA was isolated from the filter from the 29-m depth according to Zhou et al. [53]. For primer pair PLA46F/1392R, four replicates were conducted with 28 PCR cycles and 58 °C annealing temperature. Three replicate PCR reactions each were conducted for primer pairs AMX368F/1392R and PLA46F/AMX820R, with 35 PCR cycles and 56 °C annealing temperature. For the latter two pairs, a preparative gel was necessary since multiple bands were observed on agarose gel. PCR reactions were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), prior to cloning with TOPO TA Cloning kits for sequencing with vector pCR4 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Clones were screened by PCR for inserts of correct size and the PCR products were sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

## Phylogenetic analysis

16S rRNA sequences of sequenced PCR products were edited with the Sequencing Analysis software (Applied Biosystems) and analysed for their closest relatives using the ARB 16S rRNA gene database (version 2004 with continuous updates for anammox bacterial-related sequences) or GenBank using the BLAST program [1]. Plasmids were isolated from clones containing anammox bacterial-related 16S rRNA genes with the Montage Plasmid Miniprep96 Kit (Millipore) and inserts were completely sequenced with vector primers M13F and M13R. Sequences of about 800 and 1050 base pair length, respectively, were assembled from individual reads with the Sequencher v4.5 software (Gene Codes Corporation, Ann Arbor, USA).

The 16S rRNA gene sequences were investigated for the presence of chimeric sequences by using the CHIMERA_CHECK program from the RDP II [9] and imported into the ARB 16S rRNA gene database. Phylogenetic analyses of the 16S rRNA sequences were conducted using distance matrix, maximum parsimony, and maximum likelihood algorithms in ARB [30] with and without 50% variability filters, followed by construction of a consensus tree. Sequence identities were calculated using the similarity function of the distance matrix algorithm in ARB. Anammox bacteria-related sequences are published in GenBank under accession numbers FJ830380–FJ830386.

# Results

In order to understand the role of anammox and denitrification in water column $N_2$ production in Lake Rassnitzer, we developed profiles of hydrochemical properties through the anoxic bottom waters, incubated samples of anoxic water with $^{15}N$-labeled substrates, measuring the production of $^{15}N$-labeled $N_2$, and investigated the abundance and diversity of anammox cells using molecular approaches.

## Hydrochemical structure of the water column

Summer solar heating of Lake Rassnitzer surface water caused seasonal thermal stratification at ca. 10 m depth (Fig. 1A). However, the lake was permanently stratified by dense saline water which formed a steep halocline beginning at ca. 29 m (Figs. 1B and G). Lake water salinity ranged from 3 psu in the mixed upper

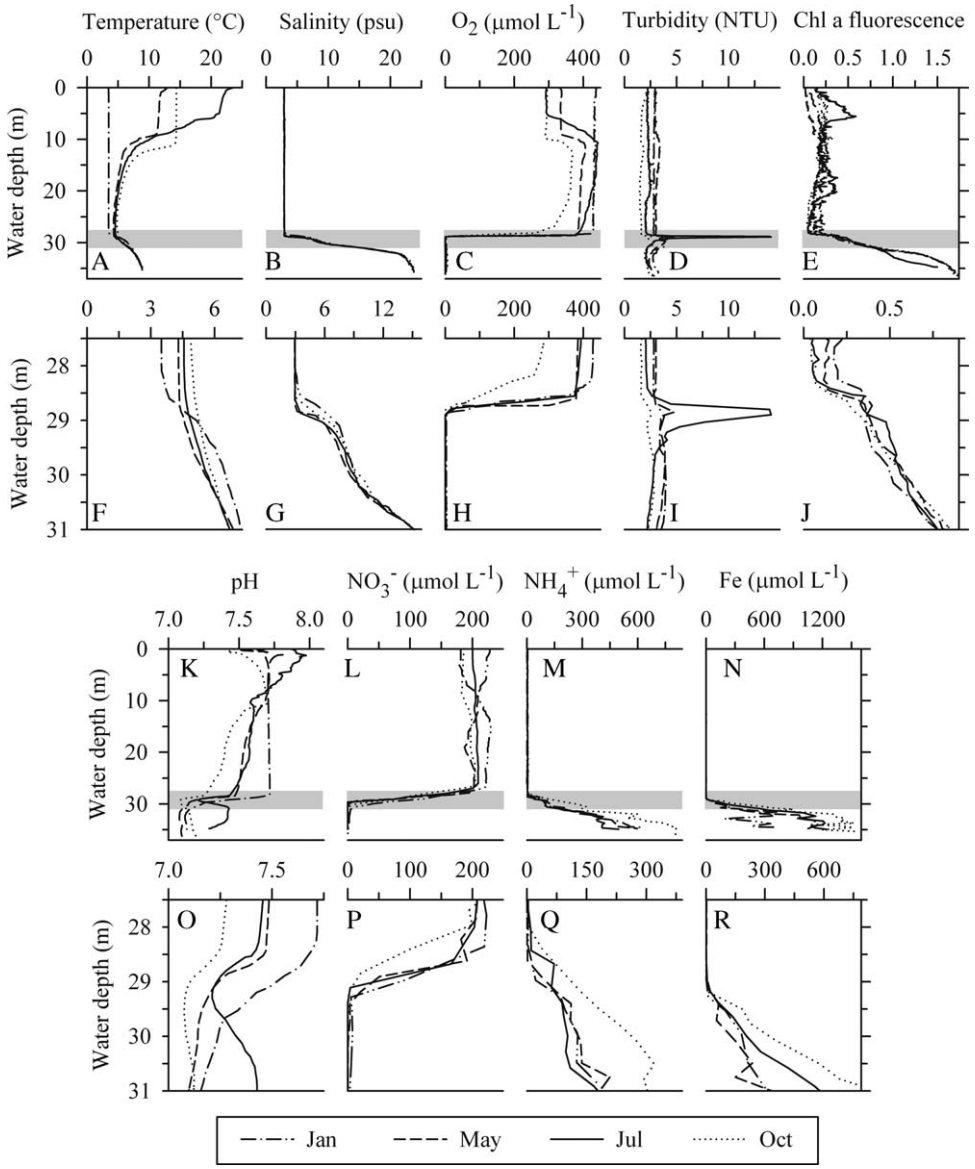


**Fig. 1.** Water column profiles of temperature, salinity, [$O_2$], turbidity, and chl *a* fluorescence (top set of 10 panels: A–J), and pH, [$NO_3^-$], [$NH_4^+$], and total [Fe] (lower set of 8 panels: K–R) on four sampling dates (27 January, 19 May, 14 July, and 18 October) at Lake Rassnitzer. Upper row of panels in each set (panels A–E and K–N) shows the entire water column. The oxycline region (shaded gray) is shown expanded in the lower row of panels (F–J and O–R).

layer, or mixolimnion, to 23 psu at the lake bottom. The resulting abrupt change in water density entrained an unmixed deep layer, or monimolimnion, bounded at its upper reach by a very steep oxycline, where dissolved $O_2$ concentrations fell from near-saturation in the mixolimnion to near-zero over a vertical distance of ca. 30 cm (Figs. 1C and H). Turbidity was constant throughout the mixolimnion, but a lens of high turbidity was found in May and July within the oxycline (Figs. 1D and I). Peaks in chlorophyll fluorescence likely associated with living phytoplankton were observed within the mixolimnion in May and July, but the high monimolimnetic fluorescence likely indicated an accumulation of humic material and/or pheophytin [8] (Figs. 1E and J). The pH of lake waters ranged from ca. 7 to 8, with the highest values found in summer surface waters, and the lowest in monimolimnetic waters (Figs. 1K and O).

High-resolution water sampling with our pump-CTD allowed nutrient profiles to be resolved to $\pm 0.15$ m. Nitrate concentrations were ca. 200 µmol $L^{-1}$ in the mixolimnion, but decreased to near-zero at the top of the monimolimnion (Figs. 1L and P). Nitrite was always below the detection limit of 0.2 µmol $L^{-1}$ (data not

shown). Ammonium concentrations were ca. 600 μmol $L^{-1}$ near the sediments, but declined gradually through the monimolimnion to reach $<5\,\mu mol\,L^{-1}$ within the mixolimnion (Figs. 1M and Q). Total Fe concentrations showed the same pattern, ranging from up to 1500 μmol $L^{-1}$ near the sediments in the anoxic monimolimnion, down to $<2\,\mu mol\,L^{-1}$ in the mixolimnion (Figs. 1N and R). Bottom water concentrations of both $NH_4^+$ and Fe rose over the course of the study year (Figs. 1Q and R). Phosphate within the mixolimnion was always undetectable ($<0.05\,\mu mol\,L^{-1}$; data not shown). Although sulfide ($H_2S$) was detectable by its characteristic odor below 32 m in January (pers. obs.), $H_2S$ was undetectable ($<1\,\mu mol\,L^{-1}$) when it was measured in May (data not shown), and no detectable smell was present in monimolimnetic samples in July or October.

The zones of $O_2$ and $NO_3^-$ consumption began at ca. 28.5 m depth, and averaged ca. 0.5 and 1 m in thickness, respectively, before $O_2$ and $NO_3^-$ became undetectable (Figs. 1H and P). The oxycline grew thinner (<0.3 m) in May and July, as a result of high specific oxygen consumption rates in those months (14.0 and 5.8 μmol $O_2\,L^{-1}\,d^{-1}$, respectively, compared to 1.3 and 0.6 μmol $O_2\,L^{-1}\,d^{-1}$ in January and October). Seasonal changes in specific nitrate consumption rates were less dramatic, but also were highest in the spring and summer (0.26 and 0.39 μmol $N\,L^{-1}\,d^{-1}$ in May and July, compared to 0.21 and 0.20 μmol $N\,L^{-1}\,d^{-1}$ in January and October).

## $^{15}N$-labeling incubations

We anaerobically incubated lake waters from below the oxycline with the isotopic tracers $^{15}NO_3^-$ or $^{15}NH_4^+$ to elucidate the contributions of the two known $N_2$-producing microbial processes – anammox and denitrification – to N cycling in lake water. The production of the two labeled $N_2$ isotopologues – $^{15}N^{15}N$ and $^{14}N^{15}N$ – from waters amended with $^{15}NH_4^+$, $^{15}NH_4^+ + {}^{14}NO_2^-$, or $^{15}NO_3^-$ is shown in Fig. 2. No $^{15}N^{15}N$ production was detected in incubations with $^{15}NH_4^+$ or $^{15}NH_4^+ + {}^{14}NO_2^-$ (data omitted from Fig. 2, panels D to F for clarity).

Two distinct patterns of labeled $N_2$ production were seen. In January and October, $^{14}N^{15}N$ was produced from $^{15}NH_4^+$-amended samples (Figs. 2A and C), but $^{15}N^{15}N$ production was undetectable (Figs. 2D and F), indicating that the added $^{15}NH_4^+$ combined with endogenous $^{14}NO_2^-$ to produce $N_2$ via anammox. There was no delay in the onset of $^{14}N^{15}N$ production, suggesting that anammox cells were metabolically active *in situ*. Amendment with $^{14}NO_2^-$ in addition to $^{15}NH_4^+$ in January and October increased anammox $N_2$ production over $^{15}NH_4^+$ alone at several depths (Figs. 2A and C). The absence of $^{15}N^{15}N$ production

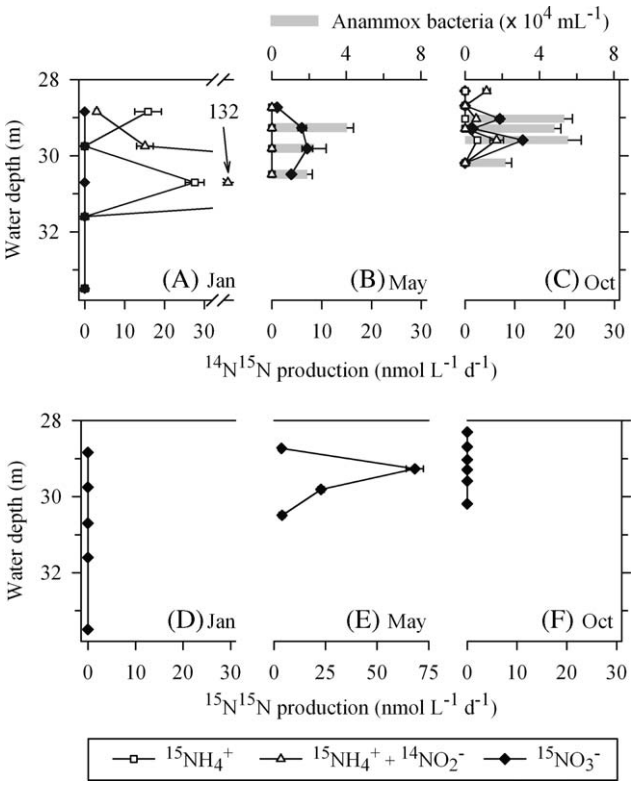


**Fig. 2.** $^{15}N$-labeled $N_2$ production during 24-h incubations of anoxic Lake Rassnitzer water with $^{15}N$-labeled inorganic substrates (see legend) (note different *x*-axis scale in panel E). All rates are *in vitro* $^{15}N$–$N_2$ production, not measures of anammox or denitrification rates. Top row of panels (A–C) shows singly labeled $^{14}N^{15}N$ production. Bottom row of panels (D–F) shows doubly labeled $^{15}N^{15}N$ production. No $^{15}N^{15}N$ production was detected in incubations with $^{15}NH_4^+$ or $^{15}NH_4^+ + {}^{14}NO_2^-$ (data omitted for clarity). In January and October, the production of $^{14}N^{15}N$ alone in incubations with $^{15}NH_4^+$ or $^{15}NO_3^-$ indicates anammox activity, but no denitrification. In May, the production of $^{15}N^{15}N$ in incubations with $^{15}NO_3^-$ indicates denitrification activity. For estimations of rates of anammox and denitrification $N_2$ production derived from the isotope pairing equations [43] (see Table 1). Anammox bacterial abundances were determined by CARD-FISH in May and October. Anammox bacteria were detected, but not enumerated in January.

in $^{15}NO_3^-$-amended incubations – despite the substantial degree of $^{15}N$ labeling of the *in situ* $NO_3^-$ pool ($^{15}N/^{14}N$ ratio $F_N > 0.15$) – shows that denitrification was not a significant contributor to $N_2$ production during these months (Figs. 2D and F). Since denitrification produces $N_2$ from two N atoms that originate from $NO_3^-$, incubations with a high degree of $^{15}N$ labeling of the $NO_3^-$ pool would be expected to produce significant amounts of $^{15}N^{15}N$ if denitrification were active [13,27].

A contrasting pattern was observed in May, when no labeled $N_2$ was produced in $^{15}NH_4^+$-amended

incubations (Figs. 2B and E). However, $^{15}NO_3^-$-amended incubations produced primarily doubly labeled $^{15}N^{15}N$, accompanied by a smaller amount of $^{14}N^{15}N$, consistent with $N_2$ production via denitrification (Figs. 2B and E) (see above). Estimates of the rates of total $N_2$ production ($^{14}N+^{15}N$) by anammox and denitrification were made using the isotope pairing equations [43] (Table 1). Denitrification accounted for all of the labeled $N_2$ flux at the upper two depths, but anammox was estimated to have contributed nearly 20% and 50% of the total $N_2$ flux at the lower two depths, respectively (Table 1). No anammox was detected in incubations with $^{15}NH_4^+$ at these depths, possibly because $^{14}N^{15}N$ production was below detection limit in these incubations where $^{15}NH_4^+$ labeling was low ($F_N<0.13$). Although the oxidized N substrate for anammox proper is $NO_2^-$ rather than $NO_3^-$, anammox $N_2$ production was detected from labeled $^{15}NO_3^-$ in May and October, indicating that, as in marine water columns, anammox likely was coupled to nitrate reduction to nitrite (Figs. 2B and C), e.g. [18,27,28]. In contrast, in January, no labeled $N_2$ was produced in incubations with $^{15}NO_3^-$. Both anammox and denitrification activity began immediately below the oxycline, in a zone where both $NO_3^-$ and $NH_4^+$ were found simultaneously (Figs. 1 and 2). However, activity was also found in deeper layers where $NO_3^-$ was undetectable.

## Abundance and phylogeny of anammox bacteria

We were not able to use standard fluorochrome-labeled fluorescence *in situ* hybridization (FISH) probes because of high sample background fluorescence. Instead, we conducted catalyzed reporter deposition (CARD)-FISH with horseradish peroxidase-labeled probes. Anammox bacteria were undetectable in aerobic mixolimnetic (mixed layer) waters, but were found in a ca. 2-m-thick stratum near the top of the anoxic hypolimnion during all of the three sampling dates for which $N_2$ production was also measured (January, May, and October) (Table 1). Although we were able to visually detect anammox bacteria via CARD-FISH in January at the 29.8- and 30.7-m depths, we were unable to enumerate them because of the high density of iron oxide particulates that formed when the anoxic waters became aerated during filtration. May and October water samples were acidified to prevent the formation of particulates, which permitted CARD-FISH enumeration of anammox bacteria (Figs. 2B and C). Anammox bacteria were detected between ca. 29 and 31 m water depth on both of these dates. The abundance of anammox bacteria relative to total bacterial numbers (DAPI count) within the $N_2$ production zone was significantly lower in May (mean $0.56\pm0.06\%$) than in October (mean $0.98\pm0.03\%$) (one-tailed *t*-test, d.f. = 17, $p=8\times10^{-6}$). Anammox cell numbers in the

**Table 1.** Estimated $N_2$ production (total $^{14}N+^{15}N$) by anammox and denitrification calculated with the isotope pairing equations [43] in incubations of Lake Rassnitzer water with three different combinations of $^{15}N$-labeled inorganic N substrates.

| Date | Depth (m) | $N_2$ production rates (total $^{14}N+^{15}N$) (nmol $N_2$ $L^{-1}$ $d^{-1}$) | | | |
|---|---|---|---|---|---|
| | | Anammox | | | Denitrification |
| | | $^{15}NH_4^+$ | $^{15}NH_4^+ + {}^{14}NO_2^-$ | $^{15}NO_3^-$ | $^{15}NO_{3-}$ |
| **27 Jan 2005** | 28.8 | 50 (10) | 10 (2) | nd | nd |
| | 29.8[a] | nd | 220 (30) | nd | nd |
| | 30.7[a] | 540 (50) | 2600 (600) | nd | nd |
| | 31.6 | nd | nd | nd | nd |
| | 33.5 | nd | nd | nd | nd |
| **19 May 2005** | 28.7 | nd | nd | nd | 4.7 (0.3) |
| | 29.3[b] | nd | nd | nd | 74 (5) |
| | 29.8[b] | nd | nd | 6 (1) | 23 (2) |
| | 30.5[b] | nd | nd | 4 (1) | 4 (2) |
| **18 Oct 2005** | 28.3 | nd | 8 (1) | nd | nd |
| | 28.7[a] | nd | nd | nd | nd |
| | 29.0[b] | nd | 10 (2) | 16 (1) | nd |
| | 29.3[b] | nd | nd | 1.8 (0.2) | nd |
| | 29.6[b] | 14.2 (0.4) | 37 (8) | 13.2 (0.2) | nd |
| | 30.2[b] | nd | nd | nd | nd |

nd indicates non-detection of $N_2$ production. Numbers in parentheses are standard errors (from linear regressions).

[a] Anammox cells detected but could not be quantified.

[b] Anammox cells detected and quantified at this depth with CARD-FISH.

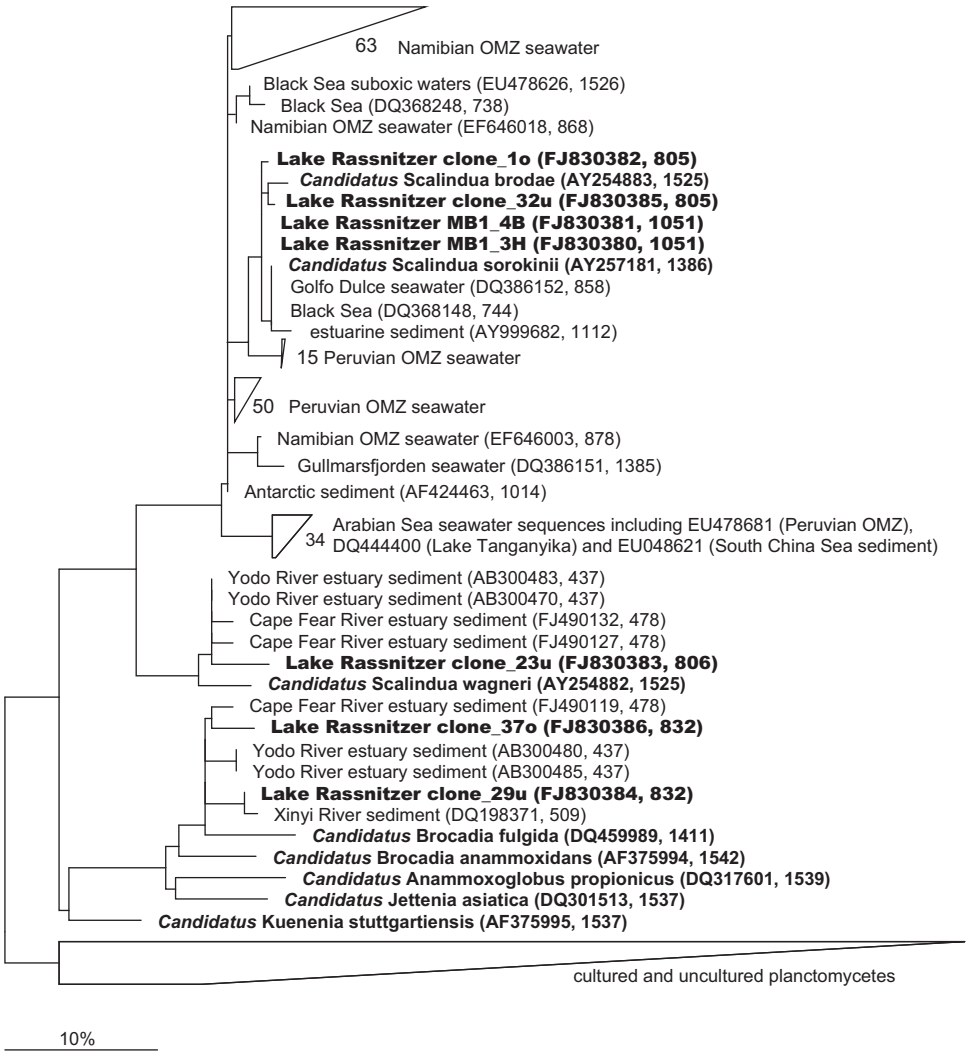


**Fig. 3.** Phylogenetic tree showing the affiliation of representative Lake Rassnitzer anammox bacterial 16S rRNA gene sequences. The consensus tree was built based on maximum likelihood, neighbor joining, and maximum parsimony trees, calculated with and without 50% position variability filters using other cultured and uncultured planctomycete-sequences as an outgroup. The bar represents 10% estimated sequence divergence. Anammox bacterial sequences from Lake Rassnitzer are depicted in bold and all sequence descriptions are followed by their GenBank accession number and sequence length (in base pairs) in parentheses.

$N_2$-production zone were also significantly lower in May (mean $2.7 \pm 0.3 \times 10^4\,mL^{-1}$) than in October (mean $5.2 \pm 0.2 \times 10^4\,mL^{-1}$) (one-tailed *t*-test, d.f. = 17, $p = 2 \times 10^{-6}$) (Figs. 2B and C). Previous work has shown that CARD-FISH may underestimate the abundance of anammox bacteria [50], so these numbers should be considered minima. The significantly lower absolute and relative anammox bacterial cell numbers in May compared to October are consistent with the lower anammox $N_2$ production rate in that month (Table 1). Cell-specific anammox rates in May ranged from 0.4 to $0.5\,fmol\,N\,d^{-1}$, whereas in October, the range was $0.08–0.6\,fmol\,N\,d^{-1}$.

Clone libraries from January and October 2005 constructed with the planctomycete-specific primer Pla46F and the universal primer 1392R did not result in any sequences related to anammox bacteria. The anammox bacteria-specific forward primer Amx-368F with 1392R also failed to produce anammox bacteria-related sequences in October samples. However, the primer combinations Amx368F/1392R and Pla46F/Amx820R retrieved 6 anammox bacteria-related sequences from the January sample and 58 from the October sample, respectively.

The majority (61 of 64) of the anammox bacterial 16S rRNA gene sequences from Lake Rassnitzer formed one cluster (based on 99% sequence identity) and were closely related to *Candidatus* Scalindua brodae [38] (98.8–99.1% sequence identity). These sequences are represented in Fig. 3 by the clones clone_32u, clone_1o, MB1_3H, and MB1_4B. The remaining 3 Lake Rassnitzer sequences (of the 64) clustered

independently. Clone_23u showed 95.1% sequence identity with *Candidatus* S. wagneri [38]. However, it was most closely related to two partial sequences from sediments of the Yodo River Estuary, Japan (AB300483 and AB300470, 98.9% sequence identity) [2] and two sequences from sediments of the Cape Fear River Estuary, North Carolina, USA (FJ490127 and FJ490132, 98.7% sequence identity) [11]. Clone_29u and clone_37o were related to *Candidatus* Brocadia fulgida (94.1% and 93.2% sequence identity, respectively), an anammox bacterium enriched from an anammox wastewater treatment plant in Rotterdam, The Netherlands [23]. The closest relatives of clone_29u were a sequence from freshwater sediments of the Xinyi River (DQ198371, 99.8% sequence identity) [52] and sequences from Yodo River Estuary sediment (AB300485 and AB300480, 99.3% sequence identity) [2]. Clone_37o was most closely related to a sequence from Cape Fear River Estuary sediments (FJ490119, 99.6% sequence identity) [11].

## Discussion

### Seasonal variation of anammox and denitrification in the Lake Rassnitzer water column

Our measurements of $N_2$ production patterns in the anoxic monimolimnion of Lake Rassnitzer showed an unexpected seasonal shift in the dominant process responsible for water column $N_2$ production – from anammox in the fall and winter to denitrification in the spring (Fig. 2, Table 1). The reasons for this shift were not clear. Water temperatures at the depths where anammox and denitrification were measured are quite stable throughout the year ($\pm 1\,^{\circ}C$), as were the concentrations of $NO_3^-$ and $NH_4^+$ (Fig. 1). Although anammox activity was detected in all sampling dates (Fig. 2), anammox $N_2$ production rates estimated from our isotope tracer experiments were lowest in May, the only date when denitrification was detected (Table 1). The increase in anammox rates from May to October (from a maximum of 6–16 nmol $N_2\,L^{-1}\,d^{-1}$) was accompanied by a concomitant and significant increase in relative and absolute anammox cell abundances (from 0.56% to 0.98% of DAPI bacteria, and from $2.7 \times 10^4$ to $5.2 \times 10^4$ anammox cells $mL^{-1}$, respectively). Cell-specific anammox rates remained relatively unchanged (maxima 0.5 and 0.6 fmol N $d^{-1}$), suggesting that the increase in anammox activity in October was due to the growth of anammox bacteria. Thus the early summer appears to be a relatively unfavorable season for anammox bacterial growth.

Evidence from marine sediments suggests that the contribution of anammox to total $N_2$ production in sediments increases as indices of organic loading decrease (e.g. chlorophyll *a*, decreasing water depth, $O_2$ consumption) (reviewed in [13]). This trend has been proposed to result from an increase in competition for $NO_2^-$ by heterotrophic denitrifiers when sufficient reductant is present. Although there is insufficient evidence to suggest that a similar relationship between organic loading and denitrification exists in the water column, we observed a sharp summer increase in $O_2$ consumption within the oxycline from $<1.3\,\mu mol\,O_2\,L^{-1}\,d^{-1}$ in January and October (when anammox alone was detected) to $14.0\,\mu mol\,O_2\,L^{-1}\,d^{-1}$ in May (when denitrification dominated), suggestive of increased organic C oxidation in May. Water column denitrification in May might also have been driven by the availability of reductants other than organic C, such as reduced Fe or sulfide. A similar, but *interannual* shift from denitrification to anammox was observed in the Baltic Sea, where it was attributed to the removal of sulfide substrate for denitrification by an influx of oxygenated water [19]. Although sulfide was not generally available as a reductant for chemoautotrophic denitrification in Lake Rassnitzer, reduced Fe was present in very high concentrations all year (Fig. 1), and chemolithotrophic iron-oxidizing bacteria are known from lake sediments [20]. However, the identity of the reductant for Lake Rassnitzer denitrification as well as the controls on the relative importance of denitrification and anammox remain unknown.

Anammox $N_2$ production rates estimated from our $^{15}N$ incubations using the isotope pairing equations [43] (up to 540 nM $N_2\,d^{-1}$ in incubations with $^{15}NH_4^+$; Table 1) ranged slightly higher than maximum rates reported earlier from marine water columns (17–430 nM $N_2\,d^{-1}$ [12,18,27,44]), as well as from freshwater Lake Tanganyika (240 nM $N_2\,d^{-1}$ [40]). However, most of the anammox rates we determined in Lake Rassnitzer were similar to previously reported rates. CARD-FISH-derived anammox bacterial cell numbers in Lake Rassnitzer also ranged higher ($5.2 \times 10^4\,mL^{-1}$) than previously reported maxima from marine systems ($1.3–2.2 \times 10^4\,mL^{-1}$ [19,26,27]) with the exception of the Peruvian upwelling system, where anammox cell abundances ranged up to $15 \times 10^4\,mL^{-1}$ [18]). Anammox bacteria in Lake Rassnitzer accounted for a maximum of 1.05% of total bacterial cells (DAPI), at the low end of the range reported earlier (maxima 0.75–3.1% [18,19,27]). In contrast, denitrification rates in the present study ($\leq 74$ nM $N_2\,d^{-1}$) were at the lower of the range of denitrification maxima previously reported from marine and freshwater water columns (480–2700 nM $N_2\,d^{-1}$ [12,19,40]).

Anammox activities observed in incubations with $^{15}NH_4^+$ vs. $^{15}NO_3^-$ were not always consistent. Anammox activity was detected in January in incubations with $^{15}NH_4^+$, but not with $^{15}NO_3^-$. Since the $NO_3^-$ pool

in the incubations was highly labeled with $^{15}N$ at 30.7 m where anammox activity was high, we can only conclude that nitrate was not being reduced to nitrite to support anammox in January, unlike in May or October, or as observed in marine water columns (e.g. [18,27,28]). The opposite condition was observed in May, when anammox was observed in incubations with $^{15}NO_3^-$, but not $^{15}NH_4^+$. A possible explanation for the May observation is that the $NH_4^+$ pool in May was weakly labeled ($F_N < 0.13$) relative to the $NO_3^-$ pool ($F_N > 0.89$), so low rates of anammox activity may not have resulted in the production of detectable $^{14}N^{15}N$. These differences emphasize the value of conducting incubations with multiple $^{15}N$-labeled substrates.

## Anammox bacterial diversity

A number of studies have investigated the diversity of anammox bacteria in marine and freshwater sediments and water columns by culture-independent approaches, using PCR with primers targeting either planctomycete or anammox bacteria-related 16S rRNA genes (e.g. [2,11,18,25–27,32,36,39,51]). However, in marine systems, only sequences related to the *Candidatus* S. brodae/sorokinii cluster have so far been recovered.

In contrast, freshwater and brackish systems appear to harbor a greater diversity of anammox bacteria. *Candidatus* Brocadia anammoxidans-related sequences were recovered from sediments of the Xinyi River in China in addition to a *Candidatus* Scalindua-related sequence (after 36 days of enrichment) [52]. A recent study of hot springs sediments and microbial mats in California and Nevada, USA, found anammox bacterial sequences related to two anammox bacterial genera, *Candidatus* Kuenenia and *Candidatus* Brocadia, but not *Candidatus* Scalindua [21]. An even higher anammox bacterial diversity was found in a Japanese estuary, where sequences clustering with *Candidatus* Scalindua, *Candidatus* Brocadia, and *Candidatus* Kuenenia were recovered from sediments of the Yodo River Estuary [2]. A similarly high diversity was found in sediments of the Cape Fear River Estuary, USA [11]. The anammox bacterial sequences recovered from Lake Rassnitzer in the present study also grouped with three distinct clusters: *Candidatus* S. sorokinii/brodae, *Candidatus* S. wagneri, and *Candidatus* B. fulgida (Fig. 3). Three of these sequences (clustering with *Candidatus* B. fulgida as well as *Candidatus* S. wagneri) were closely related to sequences from the Xinyi River, the Yodo River, and Cape Fear River estuaries (Fig. 3).

The reasons for the increased diversity in these freshwater and brackish systems are still unclear. In the Cape Fear River Estuary, an increase in anammox bacterial diversity was observed along a gradient of increasing salinity and salinity variation. The diversity of anammox bacteria in Lake Rassnitzer is similar to that of the most haline and diverse Cape Fear River Estuary site. However, at both sites, the majority of the sequences were related to the *Candidatus* S. sorokinii/brodae cluster. The salinity of the $N_2$ production zone of Lake Rassnitzer ranges from ca. 6 to 13 psu, similar to the maximum salinity observed at the most haline Cape Fear River Estuary site, although the estuary site was highly temporally variable (0.4–9.9 psu) compared to Lake Rassnitzer. Dale and co-workers [11] propose that *Candidatus* Scalindua is the most halotolerant of the anammox genera and that *Candidatus* Kuenenia and *Candidatus* Brocadia are more sensitive to salinity. Our results from Lake Rassnitzer, where most of the sequences we found were related to *Candidatus* Scalindua, support this suggestion. However, we also found a sequence related to *Candidatus* Brocadia at 6.2 psu, indicating that some members of this genera may also be somewhat halotolerant. The only other known lacustrine water column 16S rRNA gene sequence (recovered from the tropical freshwater Lake Tanganyika) was most closely related to *Candidatus* S. sorokinii/brodae, but only a single sequence was recovered from this site [40].

Although anammox bacteria in lakes have been much less studied than in marine systems, our results support the possibility that freshwater and brackish environments such as lakes and estuaries may harbor a greater diversity of anammox bacteria than marine systems. Our observations of an apparent seasonal shift between anammox and denitrification in the water column of Lake Rassnitzer underline the importance of seasonal studies of $N_2$ production processes in lacustrine, as well as marine water columns in order to understand the relative contributions of the two processes and their impacts on suboxic nutrient balances. The continuing impact of anthropogenic nutrient inputs to lakes, and the accompanying increases in the extent of water column anoxia, are likely to increase the role of microbial $N_2$ production in lacustrine N balances. Our study indicates that the microbiology of $N_2$ production in lacustrine water columns may be more complex than previously recognized.

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