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# Succession of bacteria and archaea involved in the nitrogen cycle of a seasonally stratified lake

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## Abstract

Human-driven changes affect nutrient inputs, oxygen solubility, and the hydrodynamics of lakes, which affect biogeochemical cycles mediated by microbial communities. However, information on the succession of microbes involved in nitrogen cycling in seasonally stratified lakes is still incomplete. Here, we investigated the succession of nitrogen-transforming microorganisms in Lake Vechten over a period of 19 months, combining 16S rRNA gene amplicon sequencing and quantification of functional genes. Ammonia-oxidizing archaea (AOA) and bacteria (AOB) and anammox bacteria were abundant in the sediment during winter, accompanied by nitrate in the water column. Nitrogen-fixing bacteria and denitrifying bacteria emerged in the water column in spring when nitrate was gradually depleted. Denitrifying bacteria containing *nirS* genes were exclusively present in the anoxic hypolimnion. During summer stratification, abundances of AOA, AOB, and anammox bacteria decreased sharply in the sediment, and ammonium accumulated in hypolimnion. After lake mixing during fall turnover, abundances of AOA, AOB, and anammox bacteria increased and ammonium was oxidized to nitrate. Hence, nitrogen-transforming microorganisms in Lake Vechten displayed a pronounced seasonal succession, which was strongly determined by the seasonal stratification pattern. These results imply that changes in stratification and vertical mixing induced by global warming are likely to alter the nitrogen cycle of seasonally stratified lakes.

**Keywords:** ammonia-oxidizing bacteria, ammonia-oxidizing archaea, anaerobic ammonium oxidation, denitrification, nitrogen fixation, stratified lake

## Introduction

Anthropogenic activities increasingly disturb the nitrogen cycle worldwide (Finlay et al. 2013, Hutchins and Capone 2022). Elevated nitrogen discharge contributes to the eutrophication of lakes and coastal waters, leading to blooms of harmful algae and cyanobacteria and disruption of aquatic food webs (Huisman et al. 2018, van der Lee et al. 2021). Furthermore, global warming increases the strength and duration of thermal stratification in seasonally stratified lakes (North et al. 2014, Jenny et al. 2016), which may reduce oxygen availability in the hypolimnion and consequently alter the dynamics of the microbial community therein (Diao et al. 2017). For example, the dynamics of bacteria involved in sulfur cycling closely interact with oxic–anoxic transitions in seasonally stratified lakes (Bush et al. 2017, Diao et al. 2018). However, the succession of bacteria and archaea involved in nitrogen cycling in seasonally stratified lakes, and how their succession might be affected by environmental stressors such as oxic–anoxic transitions, is still unclear.

It is well known that microorganisms play important roles in transformations of nitrogen compounds in ecosystems (Kuypers et al. 2018). Biological nitrogen fixation reduces atmospheric dinitrogen to ammonia for assimilation, which is performed by nitrogen-fixing bacteria (NFB) containing nitrogenase, encoded by the *nif* genes (Gaby and Buckley 2012). Ammonia-oxidizing ar-

chaea (AOA) and bacteria (AOB) can oxidize ammonia to nitrite, which is catalyzed by the enzyme ammonia monooxygenase (Li et al. 2015). Subsequently, nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). Under anoxic conditions, anammox bacteria use hydrazine synthase (HZS) to convert ammonium and nitrite to dinitrogen gas (Strous et al. 1999). Denitrifying bacteria (DNB) reduce nitrate to dinitrogen gas. In this process, they use either cytochrome *cd<sub>1</sub>* nitrite reductase (NirS) or copper-containing nitrite reductase (NirK) to catalyze the reduction of nitrite to nitric oxide (Zumft 1997). It is imperative to note that there are complex interactions between the different microbially mediated transformations in the nitrogen cycle (Kuypers et al. 2018). For instance, nitrous oxide (N<sub>2</sub>O), which is a potent greenhouse gas and a dominant ozone-depleting substance, can be produced in both the nitrification and denitrification processes (Ravishankara et al. 2009). Thus, understanding the dynamics and functions of the various microorganisms involved in nitrogen cycling is essential to predict its response to environmental disturbances.

Numerous studies have been performed on the diversity, abundance, and distribution of bacteria and archaea involved in nitrogen cycling (Halm et al. 2009, Yang et al. 2017). The activity and growth of these organisms depend on redox status, availability of the different nitrogen species, and a myriad of other factors including temperature and pH (Erguder et al. 2009, Dang et al.

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2010). Therefore, it was hypothesized that environmental changes induced by eutrophication and global warming might lead to large perturbations to nitrogen cycling and the population dynamics of the associated nitrogen-transforming microorganisms (Junium et al. 2018). Despite an increasing number of studies on the dynamics of nitrogen-transforming microorganisms in lakes (Krausfeldt et al. 2017, Zhang et al. 2022), understanding of the seasonal succession of different functional groups remains elusive. Furthermore, most previous studies on AOA have been performed in marine and estuarine environments (Könneke et al. 2005, Li et al. 2015), whereas much less information is available on the AOA of freshwater environments (Klotz et al. 2022, Ren and Wang 2022). These knowledge gaps make it difficult to accurately predict the response of the nitrogen cycle to environmental change.

Here, we studied the seasonal succession of several important nitrogen-transforming microorganisms in Lake Vechten using a systems approach, which integrated field sampling of the microbial community, physicochemical analysis, and the quantification of genes involved in nitrogen transformations. Specifically, samples from different depths in the water column and from the sediment of Lake Vechten were collected monthly over a period of 19 months. Environmental parameters (temperature, pH, concentrations of oxygen, nitrate, ammonium, sulfate, and sulfide) were measured in the field or in the lab. 16S rRNA gene amplicon sequencing was employed to take a census of the diversity and dynamics of different functional groups of nitrogen-transforming bacteria. Quantitative real-time PCR (qPCR) of different nitrogen cycling marker genes was applied to determine the abundance and distribution of ammonia-oxidizing bacteria and archaea (*amoA* genes), anammox bacteria (*hzsA* gene), NFB (*nifH* gene), and denitrifiers (*nirS* and *nirK* genes). The main objectives of our study were (i) to elucidate seasonal changes in the distribution and abundance of nitrogen-transforming microorganisms and their corresponding processes, and (ii) to identify environmental variables that affect the seasonal succession of nitrogen-transforming microorganisms.

## Material and methods

### Study site, sampling, and general analyses

Lake Vechten (52°04'N, 5°05'E) is located in the center of The Netherlands, a few kilometers southeast of Utrecht, which is a large city with >350 000 people. The lake is located next to a golf court, squeezed between two highways and two major railways. The lake was formed by sand excavation to build the highway A12 in 1941 (Steenbergen and Verdouw 1982). There are no waste dumpsites and no ditches or streams connecting the lake with the city or the agricultural area east of the city. The lake is largely fed by groundwater and rainwater, including surface runoff from the golf court and the adjacent highway. The lake consists of two basins with a total surface area of 4.7 ha, and has a maximum depth of 11.9 m.

Vertical profiles of temperature, dissolved oxygen (DO), chlorophyll *a*, photosynthetically active radiation (PAR), specific conductivity, and pH of the lake water were measured *in situ* using a multiprobe Hydrolab DataSonde 4a (Hydrolab Corporation, Austin, TX, USA). From every meter depth in the Western Basin, water samples were collected monthly or biweekly from March 2013 to September 2014. Water was pumped via a hose connected to the Hydrolab Datasonde to make sure the water samples matched the conditions measured by the Hydrolab Datasonde at each particular depth. Water samples were filtered through 0.20 µm nylon

membrane filters (Millipore, GNWP) to collect microorganisms. The filters were immediately frozen and stored at -20°C until further analysis. Sediment samples (0–10 cm) were collected monthly with a box-corer from the same location, transported to the laboratory in a dry shipper and stored at -20°C until further analysis. Dry weight of the sediment was determined after drying for 2 days at 60°C.

Subsequent to filtration, ammonium (NH<sub>4</sub><sup>+</sup>), nitrite (NO<sub>2</sub><sup>-</sup>), nitrate (NO<sub>3</sub><sup>-</sup>), dissolved inorganic nitrogen (DIN), sulfate (SO<sub>4</sub><sup>2-</sup>), phosphate (PO<sub>4</sub><sup>3-</sup>), and chloride (Cl<sup>-</sup>) were measured with an auto-analyzer (SAN<sup>++</sup>, Skalar, The Netherlands), while dissolved organic carbon (DOC) was measured with a total organic carbon analyzer (TOC-V<sub>CPH</sub>, Shimadzu, Japan). For sulfide (S<sup>2-</sup>) measurements, lake water was filtered through 0.20 µm polyethersulfone membrane filter and fixed with zinc acetate (10% w/v) immediately in the field. Afterward, sulfide was measured in the laboratory according to the methylene blue spectroscopic method (Trüper and Schlegel 1964). The data were visualized with Ocean Data View version 4.7.8 (Schlitzer 2002).

### DNA extraction

DNA was extracted from the biomass-containing filters using the PowerSoil DNA Isolation Kit according to the manufacturer's instructions (Mo Bio, Laboratories Inc., USA). The concentration of extracted DNA was quantified with the Qubit dsDNA BR Assay Kit (Invitrogen, USA).

### PCR and cloning

Six functional marker genes that are commonly used to detect and characterize nitrogen-transforming microorganisms were amplified by PCR (Table S1 in the Supplementary Information). The archaeal *amoA* gene was amplified with primers Arch-amoAF and Arch-amoAR (Francis et al. 2005), while the bacterial *amoA* gene was amplified with primers amoA-1F and amoA-2R (Rotthauwe et al. 1997). The *hzsA* gene of anammox bacteria was targeted with primers hzsA 1597F and hzsA 1857R (Harhangi et al. 2012). For DNB, primers nirS1F and nirS-q-R were used to amplify the *nirS* gene, while the *nirK* gene was amplified with primers nirK-q-F and nirK1040 (Mosier and Francis 2010). Primers IKG3F and DVVR were used to target the *nifH* gene of NFB (Gaby and Buckley 2012). Details on PCR primers and PCR programs can be found in Table S1.

PCR products were checked by electrophoresis in 1.5% (w/v) agarose gels. Products with the expected fragment length were purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, USA). Subsequently, purified PCR products were used for cloning with TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) according to the manufacturer's instructions. Cloning of PCR products was conducted in triplicate. Transformants were selected on LB plates containing 50 µg/mL ampicillin. Five colonies from each plate were transferred into 10 mL liquid LB medium (50 µg/mL ampicillin) and cultivated at 37°C in a shaking incubator (Edmund Bühler, Germany). Plasmid DNA from 2 mL liquid culture was isolated with the QIAprep Spin Miniprep Kit (Qiagen, Germany). The DNA of plasmid inserts was sequenced by the company Baseclear (Leiden, The Netherlands). The samples selected for cloning are listed in Table S2.

### Phylogenetic analysis

The DNA sequences were first translated into protein sequences and subsequently aligned to published sequences using Clustal Omega with standard parameters. Phylogenetic analysis of the

functional genes was performed using MEGA version 7 with the Maximum Likelihood method based on the Jones–Taylor–Thornton (JT) matrix-based model (Jones et al. 1992, Kumar et al. 2016). The robustness of the tree topology was tested with bootstrap analysis (1000 replicates).

### Amplicon sequencing and operational taxonomic unit assignments

To compare the bacterial diversity among samples from different depth and different seasons, the PCR-amplified 16S rRNA genes of 189 water samples and 11 sediment samples were profiled by denaturing gradient gel electrophoresis (DGGE). Based on the DGGE results and physicochemical parameters of Lake Vechten, 51 samples from the water column and 4 samples from the sediment were selected for 16S rRNA gene amplicon sequencing (Table S3). Sequencing was performed on an Illumina MiSeq system by Research and Testing Laboratory (Lubbock, Texas, USA). The details of amplicon sequencing and operational taxonomic unit assignments were described in a previous study (Diao et al. 2017).

### qPCR

qPCR assays of functional genes were run in 96-well white qPCR plates (Bio-Rad, Hercules, CA, USA) with adhesive seals in a Real-Time PCR Detection System (Bio-Rad). DNA of all samples was diluted to the same concentration (5 ng/ $\mu$ L). In qPCR, 2  $\mu$ L template DNA, 5  $\mu$ L of SYBR Green Supermix (Bio-Rad), and 0.75  $\mu$ L of each primer (5  $\mu$ M) were added in a total qPCR reaction volume of 10  $\mu$ L. qPCRs consisted of 40 cycles, with each cycle consisting of denaturation, annealing, elongation, and melting curve detection. Standard curves were made with plasmid DNA containing the respective gene insert. The concentration of plasmid DNA was measured by the Qubit dsDNA BR Assay Kit (Invitrogen, USA). A seven-serial dilution series was prepared from  $1.0 \times 10^8$  to  $1.0 \times 10^2$  gene copy/ $\mu$ L. All samples and standards reactions were performed in triplicate and an average value was calculated. Melting curve analysis was performed at the end of each qPCR run. The qPCR results were visualized with Ocean Data View version 4.7.8 (Schlitzer 2002). Furthermore, agarose gel electrophoresis was performed to verify the fragment sizes of qPCR products. The primers, thermal programs, efficiencies, and  $R^2$ , and other information of the qPCR assays are listed in Table S4.

In total, 15 environmental parameters in 244 samples, 6 functional genes in 140 samples, and the 16S rRNA gene sequences in 55 samples were analyzed to elucidate microbial nitrogen cycling in Lake Vechten.

### Statistical analysis

Possible relationships between the seasonal succession of functional marker genes and environmental variables in the water column were investigated using redundancy analysis (RDA) (Zuur et al. 2009). The analysis was performed using the software package R (version 3.0.3) supplemented by the “vegan” package (Oksanen et al. 2013). All environmental parameters, except pH, were  $\log(x + 1)$ -transformed and used as explanatory variables. The abundances of functional marker genes were response variables in the RDA model. First, the number of explanatory variables were reduced by eliminating variables with a high collinearity through calculation of the variance inflation factors (VIF) using the R function VIF in the “car” package (Fox and Weisberg 2011). Explanatory variables were analyzed step-wise until only those with a VIF < 10 remained. Subsequently, RDA was applied using forward selection with the Ordstep function in the R package “vegan” to select

only those explanatory variables that contributed significantly to the RDA model, while removing nonsignificant terms (Oksanen et al. 2013). Significance was determined using a permutation test with a multivariate pseudo-F statistical test and 9999 permutations (Zuur et al. 2009).

To visualize the diversity of nitrogen-transforming bacteria based on the amplicon sequencing data, the relative abundances of well-known nitrogen-transforming bacteria were z-score transformed and then visualized using the R-package pheatmap 1.0.8 (Kolde 2015).

### Accession numbers

The sequences of functional genes obtained in this study were deposited in the GenBank database under accession numbers MF993366–MF993424. The 16S rRNA gene sequences have been deposited as dataset SAMN06314865–SAMN06314918 in the Sequence Read Archive (SRA, NCBI).

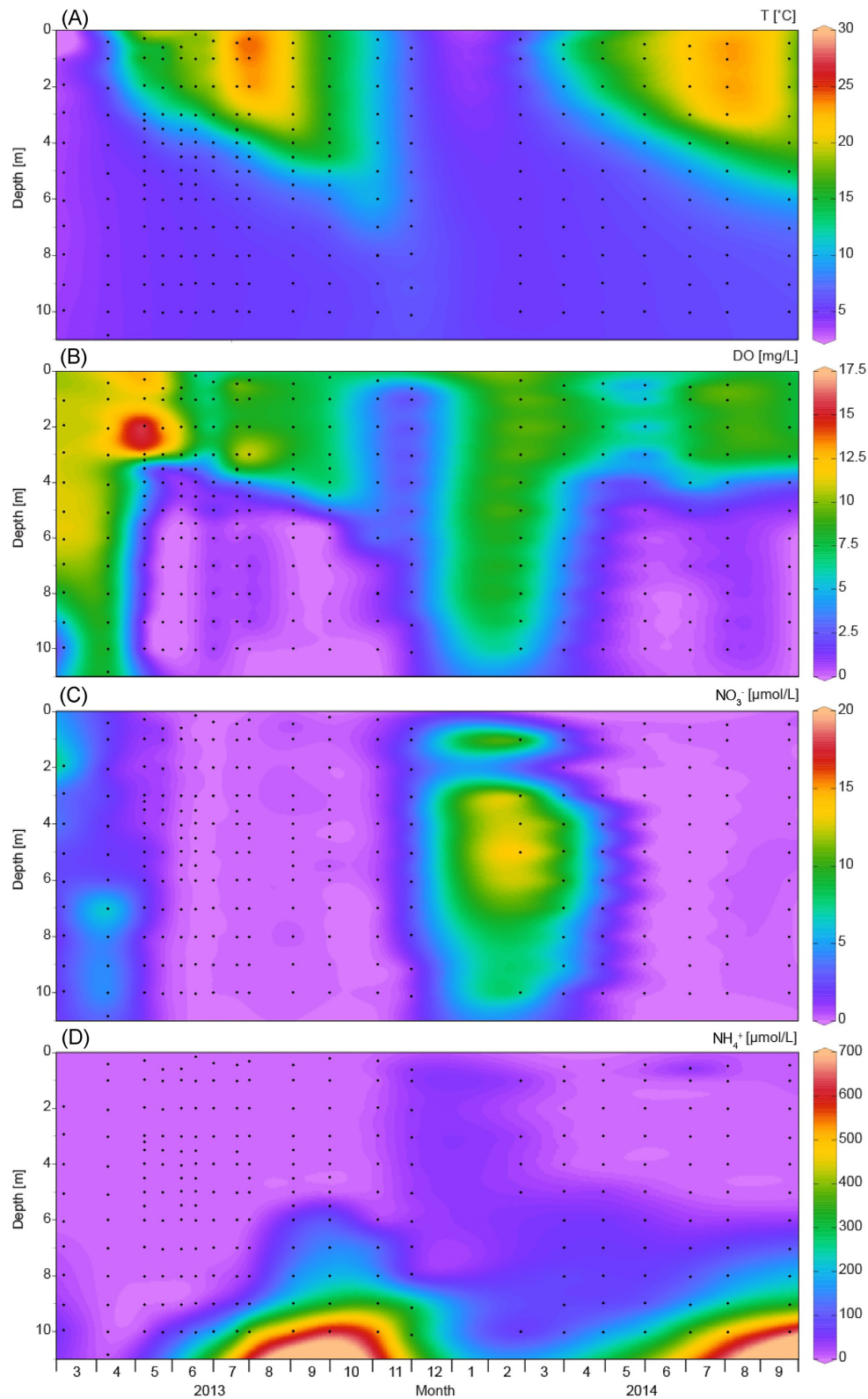
## Results

### Environmental conditions

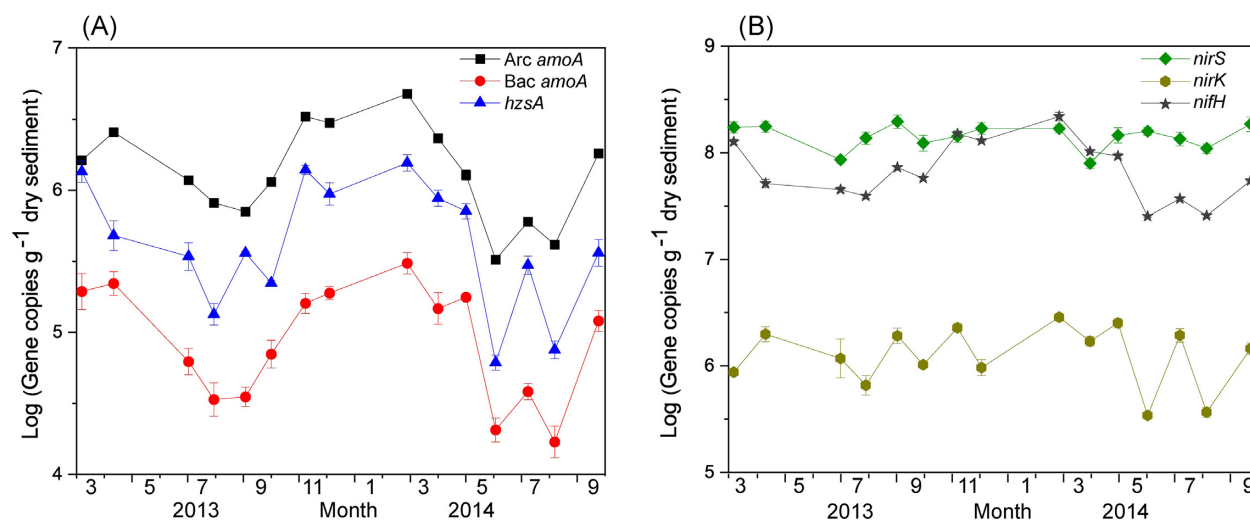
The temperature in Lake Vechten was approximately uniform over depth in early spring when deep mixing resulted in oxic conditions throughout the water column (Fig. 1A and B). From April onwards, the temperature in the surface layer increased, creating a typical stratified lake consisting of an epilimnion, metalimnion, and hypolimnion. During summer stratification, the epilimnion remained oxic, whereas the hypolimnion turned anoxic. The stratification persisted until late fall when mixing of the water column resulted in a uniform temperature profile of ca. 7°C. It is noteworthy that the oxygen concentration in the surface layer decreased during fall turnover, creating hypoxia throughout the water column in November (Fig. 1B).

The nitrate concentration in the lake was 2–7  $\mu$ M in the early spring of 2013 (Fig. 1C). From May onward, nitrate was depleted throughout the water column and remained <1  $\mu$ M until the end of October. After the fall turnover, nitrate concentrations increased from 2  $\mu$ M in December to ~14  $\mu$ M at the end of February 2014. Once the lake became stratified in April 2014, nitrate in the water column was again depleted. There was nearly no ammonium detected in the water column in early spring 2013 (Fig. 1D). Ammonium concentrations in the epilimnion remained <2  $\mu$ M when the lake became stratified in summer, whereas ammonium accumulated in the anoxic hypolimnion during the summer stratification and reached a maximum of 636  $\mu$ M in October. After fall turnover, ammonium dispersed throughout the water column with a concentration of 30–60  $\mu$ M at the end of February. When the water column became stratified in April 2014, ammonium was again depleted in the epilimnion and started to accumulate in the hypolimnion. The concentration of nitrite remained below the detection limit (0.5  $\mu$ M) during the entire study period.

The sulfate concentration was ~70  $\mu$ M throughout the water column in early spring but diminished to <10  $\mu$ M in the hypolimnion during summer stratification (Fig. S1A). Conversely, sulfide was not detectable in the water column during winter and early spring but accumulated to almost 20  $\mu$ M in the hypolimnion during summer stratification (Fig. S1B). DOC concentration increased from ~500  $\mu$ M in early spring to >1500  $\mu$ M in the hypolimnion during summer stratification (Fig. S1C). Inorganic phosphate was <2  $\mu$ M throughout the year (Fig. S1D), and pH varied between 7.5 and 9 in the epilimnion and between 6.2 and 7 in the anoxic, sulfidic hypolimnion (Fig. S1E).



**Figure 1.** Spatio-temporal dynamics of environmental parameters in Lake Vechten over a period of 19 months. (A) Temperature, (B) dissolved oxygen (DO), (C) nitrate, and (D) ammonium. Environmental parameters were measured for 244 water samples indicated by black dots.



**Figure 2.** Dynamics of functional genes in the sediment of Lake Vechten. **(A)** Archaeal *amoA* genes (AOA), bacterial *amoA* genes (AOB), *hzsA* genes (anammox bacteria), **(B)** *nirS* genes (DNB), *nirK* genes (DNB), and *nifH* genes (NFB). Values show the mean  $\pm$  standard deviation (SD) of three technical replicates (qPCR measurements) per sample.

### Abundance and distribution of functional marker genes

We did not observe significant amplification of functional marker genes involved in ammonia oxidation (archaeal *amoA* genes, bacterial *amoA* genes) and the anammox reaction (*hzsA* genes) in samples from the water column. This indicates that these genes and the corresponding microorganisms were rare or absent in the water column. Instead, qPCR showed that archaeal *amoA*, bacterial *amoA*, and *hzsA* genes were present in the sediment. The abundance of these three functional marker genes changed synchronically during the seasons, with a relatively high abundance during winter and early spring, when the water column was oxidic (Fig. 2A). The abundance of archaeal *amoA*, bacterial *amoA*, and *hzsA* genes in the sediment decreased when the lake became stratified and the hypolimnion turned into anoxic, and then reached a minimum between August and October 2013. After the fall turnover, when the lake was mixed, the abundance of these three functional marker genes increased again. The abundance of archaeal *amoA* genes was one order of magnitude higher than the bacterial *amoA* genes.

In the sediment, the abundances of *nirS* and *nirK* genes varied irregularly during the entire sampling period without any clear seasonality (Fig. 2B). The abundance of *nirS* genes exceeded that of *nirK* genes by approximately two orders of magnitude during the majority of the sampling period. In the sediment, the abundance of *nifH* genes was relatively high during winter and early spring (Fig. 2B) and decreased to a minimum during the stratification period.

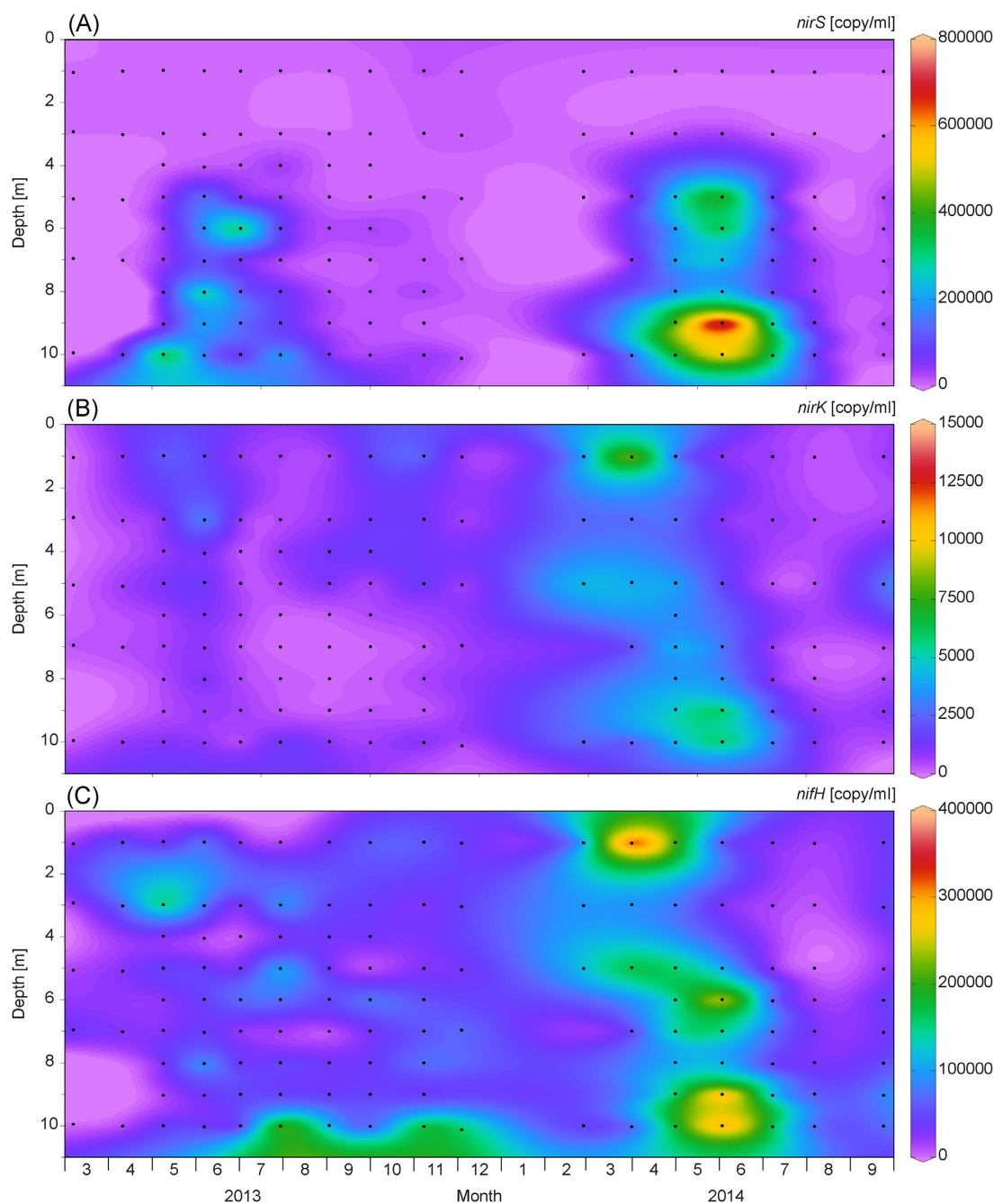
Denitrification genes (*nirS*, *nirK*) had low abundances in the water column during winter and early spring, but *nirS* genes reached relatively high abundances in the metalimnion and hypolimnion after the onset of lake stratification in late spring and early summer (Fig. 3A and B). Later in summer, before the fall turnover, their abundances declined. In addition, *nirK* genes had a relatively high concentration at a depth of 1 m in the spring of 2014. Copy numbers of the nitrogen fixation (*nifH*) gene were relatively high in the top layer of the water column in April and May of both years (Fig. 3C). The *nifH* gene was also abundant in the bottom layer (10 m) from July to December of 2013, whereas it peaked in the meta- and hypolimnion during late spring of 2014.

### Sequence analysis of functional marker genes

To confirm the specificity of PCR and qPCR, and to identify the nitrogen-transforming bacteria and archaea, amplified fragments of the functional marker genes were cloned and sequenced. Subsequently, the sequences of clones were analyzed, and phylogenetic trees were calculated. Results of clone libraries showed that most AOA in the sediments of Lake Vechten were similar to uncultured AOA from freshwater and soil habitats, while two AOA sequences clustered with *Nitrosopumilus maritimus*, *Nitrosoarchaeum koreensis*, and *Candidatus Nitrosotenuis cloaca* (Fig. S2A). Nine of the ten AOB sequences clustered with freshwater members of the genus *Nitrosomonas*, while one clone was affiliated to the genus *Nitrospira* (Fig. S2B). Sequences of *hzsA* genes were very similar to anammox bacteria from a constructed wetland (Coban et al. 2015), which were closely related to *Brocadia fulgida* (Fig. S3A). *NifH* genes from Lake Vechten were very diverse, including *Deltaproteobacteria*, *Epsilonproteobacteria*, *Cyanobacteria*, *Chlorobi*, and *Euryarchaeota* (Fig. S3B). *NirS* genes were similar to sequences from other freshwater environments, including *Pseudomonas* spp. and *Azospirillum* (Fig. S4A). *NirK* genes were similar to *Ruegeria mobilis* and *Oceanicola nanhaiensis*, belonging to the *Alphaproteobacteria* (Fig. S4B).

### 16S rRNA gene amplicon sequencing

The composition of the bacterial community was investigated by 16S rRNA gene amplicon sequencing and the relative abundances of the presumed nitrogen-transforming organisms in the top water layer (1–6 m), bottom water layer (6–10 m), and sediments are summarized in Fig. 4. The AOB were represented by *Nitrosomonas* and *Nitrospira*, while *Nitrospina* and *Nitrospira* can oxidize nitrite into nitrate. Additionally, *Nitrospira* can perform complete nitrification (van Kessel et al. 2015), although their identity as complete ammonia oxidizers (comammox bacteria) cannot be verified from amplicon sequencing results alone. Interestingly, these bacterial groups were mainly present in the sediment. Anammox bacteria, such as *Brocadia* or other affiliated genera, were not discovered by the amplicon sequencing approach. *Acidithiobacillus*, *Anabaena*, *Burkholderia*, *Chlorobium*, *Clostridium*, *Desulfuromonas*, *Methylobacterium*, *Methylomonas*, *Rhizobium*, and *Rhodobacter* were the potential NFB. *Azospirillum*, *Bacillus*, *Bradyrhizobium*, and *Pseudomonas*



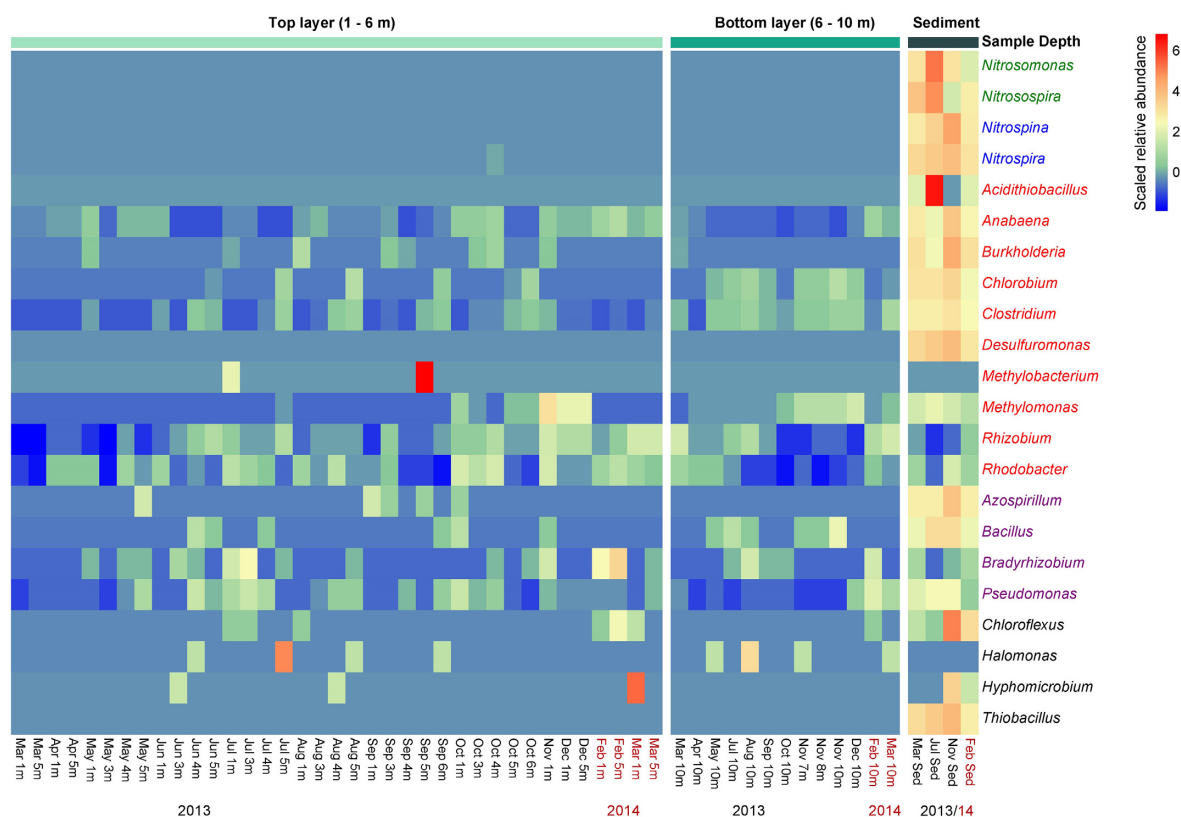
**Figure 3.** Spatio-temporal dynamics of (A) *nirS* genes (DNB), (B) *nirK* genes (DNB), and (C) *nifH* genes (NFB) in the water column of Lake Veichten. Functional genes were quantified for 125 water samples indicated by black dots.

can perform both nitrogen fixation and denitrification, while *Chloroflexus*, *Halomonas*, *Hyphomicrobium*, and *Thiobacillus* were the potential DNB. Contrary to the functional marker genes, the 16S rRNA gene sequencing data did not show pronounced seasonal patterns at the aggregated level of the functional groups. The 16S rRNA gene data do reveal distinct seasonal variation for specific taxa within the functional groups, however. For example, relative abundances of the potential nitrogen-fixing cyanobacterium *Anabaena* and purple nonsulfur bacterium *Rhodobacter* were high in the bottom layer when the lake was mixed during winter and early spring, whereas relative abundances of the green sulfur bacterium *Chlorobium* and firmicute *Closterium* were high when the epilimnion was anoxic during the summer stratification (Fig. 4).

### Redundancy analysis

RDA was applied to correlate the abundances of *nirS*, *nirK*, and *nifH* genes in the water column with associated environmental variables. In total, 10 explanatory variables had a VIF <10 including temperature, DO, PAR, pH,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{S}^{2-}$ , and DOC. Forward selection revealed that 5 of these 10 variables were significant in the RDA: DO,  $\text{PO}_4^{3-}$ ,  $\text{NH}_4^+$ , pH, and  $\text{SO}_4^{2-}$  (Table S5).

The first and second axis of the RDA plot explained 20.1% and 8.5% of the variation in the qPCR data, respectively (Fig. 5). *NirS* genes were positively correlated with  $\text{NH}_4^+$  and negatively correlated with DO. *NirK* and *nifH* genes were positively associated with the  $\text{PO}_4^{3-}$  and  $\text{SO}_4^{2-}$  concentration.



**Figure 4.** Heatmap of z-score transformed relative abundances of putative nitrogen-transforming bacterial genera in the water column and sediments of Lake Vechten. The colors of bacterial genus names indicate different functional groups: green, AOB; blue, NOB; red, NFB; purple, bacterial genera performing both nitrogen fixation and denitrification; and black, DNB. Only genera representing  $>0.02\%$  of the total bacterial community are shown in the figure.

## Discussion

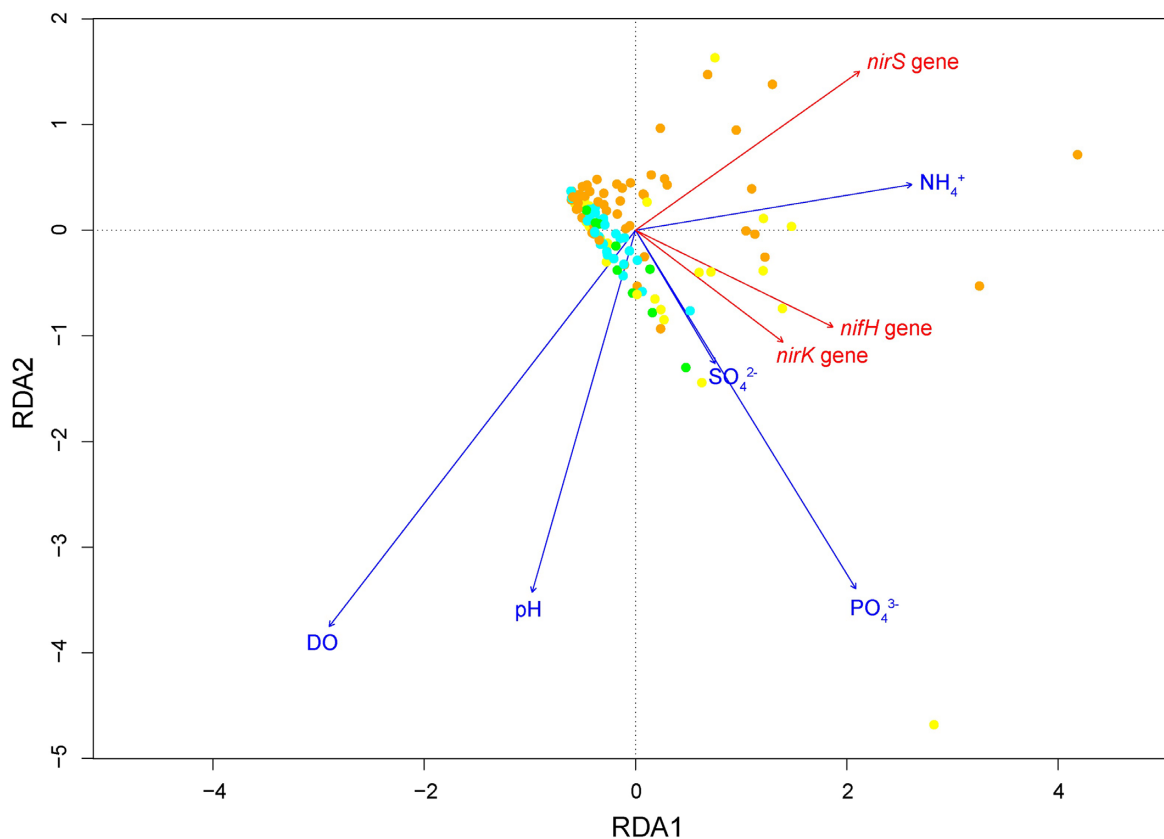
Our results show marked seasonal changes in ammonium and nitrate concentrations as well as oxygen availability in association with the seasonal stratification of the lake, in line with previous records from Lake Vechten in the 1980s (Steenbergen and Verdouw 1982, Verdouw and Dekkers 1982). This seasonal variation in environmental conditions was accompanied by pronounced seasonal patterns in microbial community composition and the abundances of functional marker genes of bacteria and archaea involved in the nitrogen cycle. The dynamics of functional marker genes indicate that different nitrogen transformations took place at different depths in the lake, and during different times of the year. We will first discuss our observations of the functional groups involved in these nitrogen transformations, and then summarize these results into a conceptual scheme of the seasonal succession of nitrogen-transforming microorganisms in seasonally stratified lakes with anoxic hypolimnia.

## Diversity of nitrogen-transforming microorganisms

Our analysis combined investigation of the diversity of nitrogen-transforming microorganisms by 16S rRNA gene amplicon sequencing with cloning of specific functional genes involved in nitrogen transformations. AOA in Lake Vechten likely belonged to *Nitrososphaeria*, and some of them were closely related to the genus *Nitrosopumilus*, which is in line with other observations in freshwater lakes (Herber et al. 2020, Ren and Wang 2022). Ammonia-oxidizing *Nitrosomonas* and *Nitrosospira* and nitrite-

oxidizing *Nitrospina* and *Nitrospira* have been reported in other freshwater lakes (Yang et al. 2016, Alfreider et al. 2018, Mori et al. 2019). We did not detect any nitrifiers belonging to the Gammaproteobacteria in Lake Vechten, and hence there is likely no mixotrophic activity combining nitrification and denitrification at the oxygen transition zone as recently described for an oil sands pit lake (Mori et al. 2019). It was reported that *Candidatus Brocadia* and *Kuenenia* were the dominant anammox bacteria in Dianchi Lake and Erhai Lake (Yang et al. 2017), while we only detected *Candidatus Brocadia* in Lake Vechten. NFB in Lake Vechten were taxonomically diverse, ranging from phototrophic *Cyanobacteria* and *Chlorobi* to chemotrophic *Firmicutes*, *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*, in line with previous studies (Dixon and Daniel 2004, Raymond et al. 2004). In a previous study performed in the late 1980s, mats of sulfur-oxidizing *Beggiatoa* spp. on the sediment were identified to play a vital role in the denitrification process of Lake Vechten (Sweerts et al. 1990). Although we identified several denitrifying microorganisms, *Beggiatoa* or close relatives were not retrieved from amplicon sequencing or the cloning results of *nirS* and *nirK* genes in our study.

Previously, nitrogen-transforming microorganisms were classified into distinct groups based on their taxonomic identity and the presumed functional roles of these taxonomic groups in nitrogen cycling. However, the emerging genomic information of these microorganisms has revealed their versatile metabolic pathways. For instance, it was discovered that NOB *Nitrospira* also have *amoA* genes, fundamentally expanding our understanding of the nitrification process (van Kessel et al. 2015). DNB can switch from canonical denitrification to dissimilatory nitrate reduction to am-



**Figure 5.** RDA of the influence of environmental variables in the water column (explanatory variables, blue arrows) on the abundance of *nirS*, *nirK*, and *nifH* genes (response variables, red arrows). Symbols represent sampling points (yellow, spring; orange, summer; cyan, fall; green, winter). All explanatory variables in the triplot are significant (see Table S5). Total variation explained by the RDA model was 29.4%.

monium in nitrate-limited and sulfidic environments (van den Berg et al. 2015, Jones et al. 2017). Furthermore, horizontal gene transfer enables different taxonomic groups to contain the same functional genes (Brochier-Armanet et al. 2008, McKay et al. 2019), making it more difficult to infer the functional roles of microorganisms solely from their phylogenetic classification (Kuypers et al. 2018). Therefore, in addition to classic taxonomic data, refined information on functional genes and cellular metabolism is a prerequisite to determine the functional roles of nitrogen-transforming microorganisms.

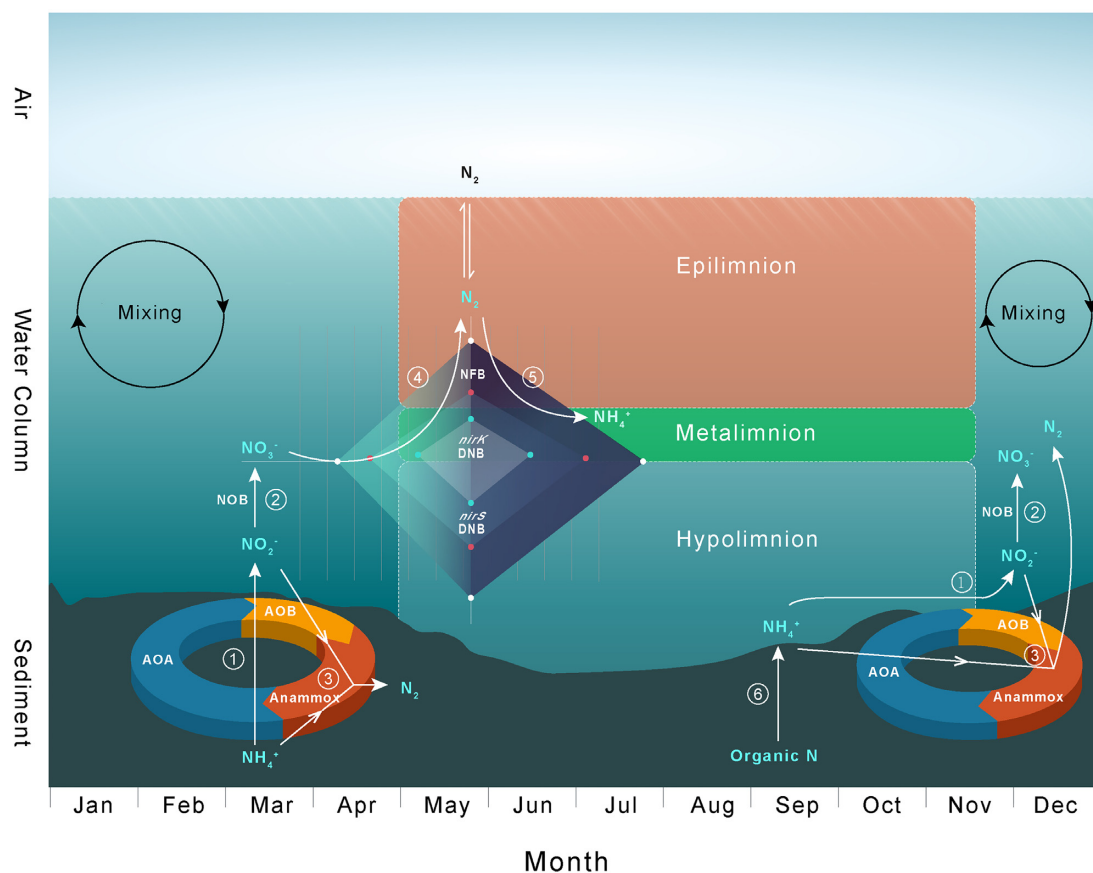
### Spatio-temporal dynamics of nitrogen-transforming microorganisms

#### AOA and AOB

AOA and AOB have been detected in different water layers in freshwater lakes (Auguet et al. 2012, Yang et al. 2016). Theoretically, one might expect ammonia oxidizers to thrive at the oxic-anoxic interface where ammonium and oxygen coexist. However, our findings are only partially in agreement with this expectation. The functional marker genes indicate that AOA and AOB were abundant in the sediment of Lake Vechten during winter and early spring, when deep mixing of the lake provided oxygen to the bottom water layers. Consequently, ammonia was oxidized to nitrate at the sediment–water interface, and nitrate was spread over the water column by vertical mixing. Yet, almost no *amoA* genes were detected in the water column when the oxic–anoxic interface shifted to the metalimnion during summer stratification. The prevalence of AOB in the sediment was also confirmed by the 16S rRNA gene sequencing data. Similar results have been

reported by other studies, which also found that abundances of ammonia oxidizers were very low in the water column (Hastings et al. 1998, Pauer and Auer 2000), but high in the sediments (Wu et al. 2013, Bollmann et al. 2014). When the lake was stratified in summer and early autumn, ammonium and nitrate concentrations in the epilimnion remained low, probably because inorganic nitrogen was rapidly consumed by primary producers (Verdouw and Dekkers 1982, Diao et al. 2017). However, ammonium accumulated in the deeper hypolimnion during summer, which can be attributed to anaerobic mineralization of dead organic matter while the absence of oxygen and nitrate prevented oxidation of the released ammonium.

Similar to observations from freshwater aquaculture ponds (Lu et al. 2016), the abundance of archaeal *amoA* genes exceeded those from bacteria by one order of magnitude. There are several potential factors, including pH, oxygen, ammonium, and sulfide that might have led to higher abundance of AOA than AOB (Stahl and de la Torre 2012). In line with previous results (Bollmann et al. 2014), oxygen appears to be a key limiting factor for ammonia oxidizers in the sediments of Lake Vechten, as AOA and AOB declined when the hypolimnion became anoxic during summer stratification. In general, stratification of eutrophic lakes often leads to a decrease of DO in bottom waters, which limits the diffusion of oxygen into sediments (Neubacher et al. 2011). Hydrogen sulfide with a concentration >0.002 mg/L is generally considered toxic for most freshwater organisms and an  $H_2S$  concentration >0.01 mg/L is hazardous to marine organisms (Beauchamp et al. 1984, Wang and Chapman 1999). The highest  $H_2S$  concentration in the hypolimnion of Lake Vechten was 20  $\mu M$  (0.68 mg/L) during the sum-



**Figure 6.** Conceptual model of the seasonal succession of nitrogen-transforming microorganisms and seasonal transition of inorganic nitrogen ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ ) in the sediment and the water column of Lake Vechten over one year. AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; Anammox, anaerobic ammonium oxidizing bacteria; NFB, nitrogen-fixing bacteria; *nirK* DNB, denitrifying bacteria with the *nirK* gene; and *nirS* DNB, denitrifying bacteria with the *nirS* gene. (1) aerobic ammonium oxidation; (2) aerobic nitrite oxidation; (3) anaerobic ammonium oxidation; (4) denitrification; (5) nitrogen fixation; and (6) mineralization.

mer stratification. Therefore,  $\text{H}_2\text{S}$  in the hypolimnion would be toxic to most organisms in Lake Vechten, which—in addition to the lack of oxygen—might be another factor that inhibits the activities of AOA and AOB during the summer (Joye and Hollibaugh 1995, Berg et al. 2015). As AOA can tolerate periodic exposure to anoxic and sulfidic environments (Berg et al. 2015), the inhibitory effects of sulfide on AOB are likely to be more severe (Erguder et al. 2009).

### Anammox bacteria

Anammox bacteria have been detected in both the water column and sediment of freshwater lakes (Schubert et al. 2006, Yang et al. 2017). The 16S rRNA gene amplicon sequencing approach did not find any known anammox bacteria in the water column of Lake Vechten, which may be due to their low abundance or primer mismatches. However, the qPCR result of *hzsA* genes showed anammox bacteria were present in the sediment. In Dianchi Lake and Erhai Lake, the abundance of anammox bacteria was higher in summer than in spring (Yang et al. 2017). Similarly, anammox rates were undetectable during the mixing period in the monomictic Lake Lugano (Wenk et al. 2014). Contrary to these results, we observed a relatively high abundance of *hzsA* genes in winter and early spring but a low abundance during summer stratification in Lake Vechten.

Coexistence of AOA, AOB, and anammox bacteria has also been observed in other freshwater lakes (Yang et al. 2016, Yang et al. 2017). It was reported that AOA and AOB were present at different depths in the sediment (Lagostina et al. 2015). Since AOA and AOB need oxygen to oxidize ammonia, while anammox bacteria favor anoxic conditions, they may inhabit different depths in the sediment. However, we could not elucidate the fine-scale vertical distributions of these bacteria in the sediment of Lake Vechten as we used a box corer to collect sediment samples. Nitrite produced by AOA and AOB appears to be a key regulator of anammox bacteria abundance (Third et al. 2001, Dang et al. 2010). Thus, the ammonia oxidation performed by AOA and AOB can facilitate the activity and growth of anammox bacteria by providing nitrite, which offers an explanation of why *hzsA* genes, archaeal, and bacterial *amoA* genes followed similar dynamic patterns. Consistent with these observations, abundances of AOA and anammox bacteria peaked at the same depth in the Black Sea (Coolen et al. 2007), and stable coexistence of AOB and anammox bacteria has been observed in wastewater bioreactors (Third et al. 2001).

### Denitrifying bacteria

Distributions of *nirS* and *nirK* DNB in the water column of Lake Vechten were quite different. Specifically, *nirS* DNB were restricted to the metalimnion and hypolimnion, whereas *nirK* DNB were also found in the epilimnion and did not show a clear habitat preference. *NirS* and *nirK* DNB also occupied different niches and re-

sponded differently to environmental variables in the South China Sea and a variety of other ecosystems (Jones and Hallin 2010, Li et al. 2013). *nirS* genes were far more abundant than *nirK* genes in both the water column and sediment of Lake Vechten, indicating a predominant role for *nirS* genes in the denitrification process. Similarly, it was reported that *nirS* genes were consistently more abundant and more diverse than *nirK* genes in San Francisco Bay and in marine sediment (Li et al. 2013, Lee and Francis 2017).

Our results show that *nirS* DNB reached their highest abundances in the anoxic hypolimnion in late spring and early summer, which led to the depletion of nitrate. This seasonal pattern is comparable to observations of DNB in other lake studies. In the monomictic Lake Lugano, denitrification proceeded in the sediment during fully oxic bottom water conditions and shifted to the water column when bottom water became anoxic after lake stratification (Lee and Francis 2017). In the permanently stratified Lake Rassnitzer, DNB dominated  $N_2$  production in May (Hammersley et al. 2009). In our Lake Vechten study, DNB were replaced by sulfate-reducing bacteria in the anoxic hypolimnion only after nitrate was completely consumed in late summer (Diao et al. 2018). This successional sequence was in line with expectation because nitrate reduction results in a higher energy yield than sulfate reduction and, therefore, DNB have a competitive advantage over sulfate-reducing bacteria as long as nitrate is available as a terminal electron acceptor.

### Nitrogen-fixing bacteria

*NifH* genes were present in both the water column and sediment of Lake Vechten. Although there was no distinct seasonality of *nifH* genes, their abundance increased markedly in spring when nitrate in the water column was gradually depleted. Consequently, NFB may have provided an important source of inorganic nitrogen for the lake ecosystem, especially during spring, although we have not quantified nitrogen fixation rates in our study. The RDA results show that the abundance of *nifH* genes was positively correlated with phosphate, which is consistent with observations that the growth of nitrogen-fixing cyanobacteria is stimulated by high phosphate levels (Lehtimäki et al. 1997, Sañudo-Wilhelmy et al. 2001).

From the 16S rRNA gene sequencing results, we observed some bacterial groups that can potentially perform both denitrification and nitrogen fixation. In the conventional view of nitrogen cycling, nitrogen fixation and denitrification are temporally and/or spatially segregated. However, we observed pronounced overlap in the spatio-temporal distributions of *nifH*, *nirK*, and *nirS* genes in Lake Vechten. Similarly, co-occurrence of DNB and NFB was also reported for the meromictic Lake Cadagno (Halm et al. 2009). A recent metagenomics study discovered the coexistence of functional genes related to nitrogen fixation and denitrification in *Methylobacter* (Biderre-Petit et al. 2019), indicating that some microorganisms can perform both nitrogen fixation and denitrification.

Overall, this study focused on the seasonal changes in diversity and abundances of nitrogen-transforming microorganisms. However, some microbial groups were not discussed in detail since we could not confirm their presence or functional roles from our analysis. For instance, comammox bacteria might play a significant role in the nitrification process (Kits et al. 2017), and further research will be required to assess their population dynamics in lakes. To further elucidate the ecological roles of the different nitrogen-transforming microorganisms in stratified lakes, future studies should also investigate microbial activities, as microbial

groups with low abundances can maintain high activities (Sterngren et al. 2015, Hausmann et al. 2019).

### Seasonal succession of nitrogen-transforming microorganisms

Based on our findings and the preceding discussion, we propose a simple conceptual model that depicts the seasonal succession of several groups of nitrogen-transforming microorganisms in eutrophic and seasonally stratified lakes (Fig. 6). In winter and early spring, the entire water column is mixed, providing oxygen-rich water to the sediment-water interface. The sediment is inhabited by a consortium of AOA, AOB, and anammox bacteria. AOA and AOB oxidize ammonium to nitrite, and then NOB oxidize nitrite to nitrate. In spring, ammonium and nitrate in the surface layer is gradually depleted by primary producers, which stimulates the growth of NFB. At approximately the same time in spring, the water column stratifies and the hypolimnion becomes anoxic. The degradation of organic carbon compounds in the anoxic hypolimnion stimulates a temporary rise of DNB (both *nirS* and *nirK* groups), which disappear once nitrate in the hypolimnion has been fully depleted. Furthermore, the anoxic and sulfidic hypolimnion prevents ammonia oxidation by AOA, AOB, and anammox bacteria in the sediment, and their abundances strongly decline during summer stratification. Meanwhile, ammonium accumulates in the hypolimnion during the summer and early fall because of ammonification, as previously observed (Biderre-Petit et al. 2019), while the lack of oxygen and nitrate prevents ammonium oxidation. After the fall turnover, the entire water column is mixed again, thereby supplying oxygen to the deeper water layers, AOA and AOB reappear in the sediment, and ammonium is oxidized to nitrate (Fig. 6). At the same time, anammox bacteria also increase in the sediment and may transform ammonium and nitrite in the anammox reaction.

This simple conceptual model is a first step to capture the succession of different functional groups of microorganisms involved in nitrogen cycling of seasonally stratified lakes. It may be further refined by more detailed process-based studies at a higher functional, spatio-temporal, and taxonomic resolution. Nevertheless, this simple conceptual model clearly shows that the succession of nitrogen-transforming microorganisms is strongly regulated by the seasonal stratification of lakes and the concomitant depletion of oxygen in the hypolimnion. Global warming can expand the duration and strength of the summer stratification in seasonally stratified lakes (North et al. 2014, Jenny et al. 2016, Woolway et al. 2020). Furthermore, especially in lakes in human-dominated landscapes, intensified fertilizer usage may increase eutrophication, which exacerbates oxygen depletion in the hypolimnion of stratified waters (Bush et al. 2017). Together, these changes are likely to shift the nitrogen transformations toward an earlier depletion of dissolved inorganic nitrogen (DIN) in the epilimnion in spring and prolonged accumulation of ammonium in the anoxic hypolimnion during summer and fall. Moreover, a shorter duration of the winter period and higher winter temperatures are likely to diminish the frequency and extent of vertical mixing in seasonally stratified lakes (Woolway and Merchant 2019, Woolway et al. 2020), which will suppress the supply of oxygen to deeper water layers (Bush et al. 2017, Jane et al. 2021) and thereby limit the microbially driven oxidation of ammonium and other reduced compounds during fall and winter. Hence, this simple conceptual model provides a useful benchmark in efforts to understand and predict how the nitrogen cycle and its associated microorganisms are affected by increasing environmental pressures on freshwater ecosystems through eutrophication and global warming.

## Author contributions

M.D., J.H., and G.M. designed the study. M.D., C.B., and M.S.M. performed the fieldwork and lab experiments. M.D., J.H., and G.M. analyzed the data and wrote the manuscript.

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## Supplementary data

Supplementary data are available at [FEMSLE](https://femsle.onlinelibrary.wiley.com/doi/10.1093/femsle/fnad013/7043454) online.

**Conflicts of interest statement.** The authors declare that they have no competing interest.

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