



UvA-DARE (Digital Academic Repository)

Interactions between microorganisms and oxic-anoxic transitions

Diao, M.

Publication date

2018

Document Version

Other version

License

Other

[Link to publication](#)

Citation for published version (APA):

Diao, M. (2018). *Interactions between microorganisms and oxic-anoxic transitions*. [Thesis, fully internal, Universiteit van Amsterdam].

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, P.O. Box 19185, 1000 GD Amsterdam, The Netherlands. You will be contacted as soon as possible.



**Seasonal succession of bacteria and archaea
involved in the nitrogen cycle of a
seasonally stratified lake**

Seasonal succession of bacteria and archaea involved in the nitrogen cycle of a seasonally stratified lake

Muhe Diao, Cherel Balkema, María Suárez Muñoz, Jef Huisman, Gerard Muyzer

Department of Freshwater and Marine Ecology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, 1090 GE Amsterdam, The Netherlands

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

This chapter is submitted for publication.

Abstract

Microorganisms involved in the nitrogen cycle ('nitrogen microorganisms') play important roles in the transformation of nitrogen compounds in aquatic ecosystems. Yet, information on the succession of nitrogen microorganisms in seasonally stratified lakes is far from complete. Here, we used functional marker genes to investigate the seasonal succession of nitrogen microorganisms in the water column and sediment of freshwater Lake Vechten over a period of 19 months. Ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB) and anaerobic ammonium-oxidizing (anammox) bacteria were abundantly present in the sediment during the winter period, accompanied by relatively high nitrate concentrations in the water column. The abundance of AOA was one order of magnitude higher than AOB, indicating a dominance of archaea in ammonia oxidation. Nitrogen-fixing bacteria and denitrifying bacteria increased in the water column in spring, when nitrate was gradually depleted and the hypolimnion became anoxic. 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 the anoxic hypolimnion. After the lake was mixed during fall turnover, AOA, AOB and anammox bacteria increased to high abundances again, and ammonium was oxidized to nitrate. In general, nitrogen microorganisms in the water column and sediment displayed a pronounced seasonal succession, which was closely linked with a seasonal shift from nitrate to ammonium as dominant nitrogen source during the transition from oxic to anoxic conditions induced by seasonal stratification of the lake.

Importance

Although microorganisms play vital roles in the nitrogen cycle, an integrative view on the dynamics of nitrogen bacteria and archaea in seasonally stratified lakes is still missing. This study followed the dynamics of nitrogen bacteria and archaea in the water column and sediment of a seasonally stratified lake over a period of 19 months. Ammonia-oxidizing bacteria, archaea and anaerobic ammonium-oxidizing bacteria were abundant in the sediment when the lake was mixed in winter, but diminished when the hypolimnion became anoxic during summer stratification. These changes were accompanied by pronounced seasonality in the nitrate and ammonium concentrations, with high numbers of nitrogen-fixing and denitrifying bacteria in spring when nitrate was gradually depleted. This information will be conducive for a better understanding of how environmental changes (e.g., eutrophication, global warming) may affect the nitrogen cycle in seasonally stratified lakes.

Introduction

Human activities are increasingly disturbing the nitrogen cycle worldwide (Galloway *et al.*, 2004; Erisman *et al.*, 2008; Finlay *et al.*, 2013). Elevated nitrogen discharge contributes to the eutrophication of lakes and coastal waters, leading to blooms of harmful algae and cyanobacteria, expansion of hypoxia, and disruption of aquatic food webs (Huisman *et al.*, 2005; Paerl and Huisman, 2008). Therefore, it is increasingly important to gain a better understanding of the nitrogen cycle in aquatic ecosystems, and how it can be affected by environmental stressors.

Microorganisms play important roles in the transformation of nitrogen compounds in various ecosystems (Falkowski *et al.*, 2008; Nelson *et al.*, 2016). They consume nitrate and ammonium or fix molecular nitrogen to build proteins and nucleic acids, and some microorganisms also gain energy from the transformation of nitrogen compounds. 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) (Turk *et al.*, 2011; Gaby and Buckley, 2012). Ammonia-oxidizing archaea (AOA) and bacteria (AOB) can oxidize ammonia to nitrite, which is catalyzed by the enzyme ammonia monooxygenase (*amoA*) (Könneke *et al.*, 2005; Li *et al.*, 2015). Subsequently, nitrite is oxidized to nitrate by nitrite-oxidizing bacteria. Under anaerobic conditions, anammox bacteria use hydrazine synthase (*hzsA*) to convert ammonium and nitrite to dinitrogen gas (Strous *et al.*, 1999; Harhangi *et al.*, 2012). Increasing evidence shows that anammox bacteria play significant roles in the nitrogen loss from marine and freshwater habitats (Kuypers *et al.*, 2003; Schubert *et al.*, 2006). Denitrifying bacteria (DNB) perform the reduction of nitrate to dinitrogen gas. In this process they use either cytochrome *cd₁* nitrite reductase (*nirS*) or copper-containing nitrite reductase (*nirK*) to catalyze the reduction of nitrite to nitric oxide (Zumft, 1997). Nitrous oxide (N₂O), which is produced in the denitrification process, is a potent greenhouse gas and a dominant ozone-depleting substance (Ravishankara *et al.*, 2009; Harter *et al.*, 2014). Hence, different transformations in the nitrogen cycle tend to be carried out by different microorganisms.

Numerous studies have been performed on the diversity, abundance and distribution of microorganisms involved in the nitrogen cycle, the 'nitrogen microorganisms' (Halm *et al.*, 2009; Nelson *et al.*, 2016; Yang *et al.*, 2017). The

activity and growth of these organisms depends 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.*, 2010; Santos *et al.*, 2014). Therefore, the population dynamics of different microorganisms involved in the nitrogen cycle are likely to be sensitive to changes in environmental conditions, and different nitrogen transformations may be carried out at different times of the year. However, comparative studies that investigate the seasonal succession of different functional groups of nitrogen microorganisms are scarce (Auguet *et al.*, 2011; Lipsewers *et al.*, 2014; Lu *et al.*, 2015).

Here, we studied the seasonal succession of several important functional groups of nitrogen microorganisms in Lake Vechten, a seasonally stratified lake in the center of the Netherlands (Steenbergen and Verdouw, 1982). The main objectives of our study were: (i) to elucidate seasonal changes in the distribution and abundance of different nitrogen microorganisms and the processes they catalyze, and (ii) to identify environmental variables that affect the seasonal succession of these nitrogen microorganisms. For these purposes, samples from different water layers and from the sediment of Lake Vechten were collected monthly over a period of 19 months. Quantitative real-time PCR (qPCR) of different N-cycling marker genes was applied to determine the abundance and distribution of nitrogen-fixing bacteria using *nifH*, ammonia-oxidizing bacteria and archaea using *amoA*, anammox bacteria using *hzsA*, and denitrifiers using *nirS* and *nirK*. These results were correlated with environmental parameters, such as temperature, pH, and concentrations of oxygen, nitrate and ammonium. In addition, amplification products of these genes were cloned and sequenced to validate specificity of the PCR and to determine representative members of these functional groups.

Materials and methods

Study site, sampling and general analyses

Lake Vechten (52°04'N, 5°05'E) is located in the center of The Netherlands, near the city of Utrecht (Steenbergen and Verdouw, 1982). It 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 that particular depth. Water samples were filtered through 0.20 μm nylon membrane filters (Millipore, GNWP) to collect microorganisms. The filters were frozen immediately 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 sediment was determined after drying for 2 days in a 60°C oven.

Subsequent to filtration, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), total dissolved inorganic nitrogen (DIN), sulfate (SO_4^{2-}), phosphorus (PO_4^{3-}) and chloride (Cl^-) were measured with an auto-analyzer (SAN⁺⁺, Skalar, The Netherlands), while dissolved organic carbon (DOC) was measured with a total organic carbon analyzer (TOC-V_{CPH}, Shimadzu, Japan). For sulfide measurements, lake water was filtered through 0.20 μm polyethersulfone membrane filter and fixed with zinc acetate (10% w/v) immediately in the field. Afterwards, sulfide was measured in the laboratory according to the methylene blue spectroscopic method (Trüeper 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 microorganisms were amplified by PCR (**Table 5.1**). 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 denitrifying bacteria, 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 IGK3F and DVVR were used to target the *nifH* gene of nitrogen-fixing bacteria (Gaby and Buckley, 2012). Details on PCR primers and PCR programs can be found in **Supplementary Table S5.1**.

Table 5.1. Target gene, specificity, sequence and annealing temperature of primers used in this study.

Target gene	Specificity	Primers	Primers sequences (5'-> 3')	Ref
<i>amoA</i>	AOA	Arch-amoAF Arch-amoAR	(STAATGGTCTGGCTTAGACG) (GCGGCCATCCATCTGTATGT)	Francis <i>et al.</i> , 2005
<i>amoA</i>	AOB	amoA-1F amoA-2R	(GGGG TTTCTACTGGTGGT) (CCCCTCKGSAAAGCCTTCTTC)	Rotthauwe <i>et al.</i> , 1997
<i>hzsA</i>	Anammox	hzsA_1597F hzsA_1857R	(WTYGGKTATCARTATGTAG) (AAABGGYGAATCATARTGGC)	Harhangi <i>et al.</i> , 2012
<i>nirS</i>	<i>nirS</i>	nirS1F	(CCTAYTGGCCGCCRCART)	Mosier and
	DNB	nirS-q-R	(TCCMAGCCRCCRTCRTGCAG)	Francis, 2010
<i>nirK</i>	<i>nirK</i>	nirK-q-F	(TCATGGTGCTGCCGCGYGA)	Mosier and
	DNB	nirK1040	(GCCTCGATCAGRTRRTGGTT)	Francis, 2010
<i>nifH</i>	NFB	IGK3 DVVR	(GCIWTHTAYGGIAARGGIGGIATHGGIAA) (ATIGCRAAICCICRCAIACIACRTC)	Gaby and Buckley, 2012

AOA, ammonium-oxidizing archaea; AOB, ammonium-oxidizing bacteria; Anammox, anaerobic ammonium-oxidizing bacteria; *nirS* DNB, denitrifying bacteria with the *nirS* gene; *nirK* DNB, denitrifying bacteria with the *nirK* gene; NFB, nitrogen-fixing bacteria.

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. 5 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 in the Supplemental Material.

Phylogenetic analysis

The DNA sequences were first translated into protein sequences and subsequently aligned to published sequences using Cluster Omega with standard parameters. Phylogenetic analysis of the functional genes was performed using MEGA version 7 (Kumar *et al.*, 2016) with the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992). The robustness of the tree topology was tested with bootstrap analysis (1000 replicates).

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/µL). In qPCR, 2 µL template DNA, 5 µL of SYBR Green Supermix (Bio-Rad) and 0.75 µL of each primer (5 µM) were added in a total qPCR reaction volume of 10 µL. 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 1.0×10^8 to 1.0×10^2 gene copy μL^{-1} . 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. 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 S3 in the Supplemental Material.

Redundancy 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 was 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).

Accession numbers. The sequences obtained in this study were deposited in the GenBank database under accession numbers MF993366 - MF993424.

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 (**Figure 5.1A,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 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 (**Figure 5.1B**).

The nitrate concentration in the lake was 2 - 7 μM in early spring of 2013 (**Figure 5.1C**). From May onward, nitrate was depleted throughout the water column and remained $< 1 \mu\text{M}$ until the end of October. After fall turnover, nitrate concentrations increased from 2 μM in December to $\sim 14 \mu\text{M}$ at the end of February 2014. Once the lake became stratified in April 2014, nitrate disappeared from the water column again. There was nearly no ammonium detected in the water column in early spring 2013 (**Figure 5.1D**). Ammonium accumulated in the anoxic hypolimnion when the lake became stratified and reached a maximum of 636 μM in October. After fall turnover, ammonium dispersed throughout the water column with a relatively low concentration of 30 - 60 μM at the end of February. When the water column became stratified in April 2014, ammonium was depleted in the epilimnion and again started to accumulate in the hypolimnion. The concentration of nitrite remained below the detection limit (0.5 μM) during the entire study period.

The sulfate concentration was $\sim 70 \mu\text{M}$ throughout the water column in early spring, but diminished to $< 10 \mu\text{M}$ in the hypolimnion during summer stratification (**Supplementary Figure S5.1A**). Conversely, sulfide was not detectible in the water column during winter and early spring, but accumulated to almost 20 μM in the hypolimnion during summer stratification (**Figure S5.1B**). DOC concentration increased from $\sim 500 \mu\text{M}$ in early spring to $> 1500 \mu\text{M}$ in the hypolimnion during summer stratification (**Figure S5.1C**). Inorganic phosphate was $< 2 \mu\text{M}$ throughout the year (**Figure S5.1D**), and pH varied between 7.5 and 9 in the epilimnion and between 6.2 and 7 in the anoxic sulfidic hypolimnion (**Figure S5.1E**).

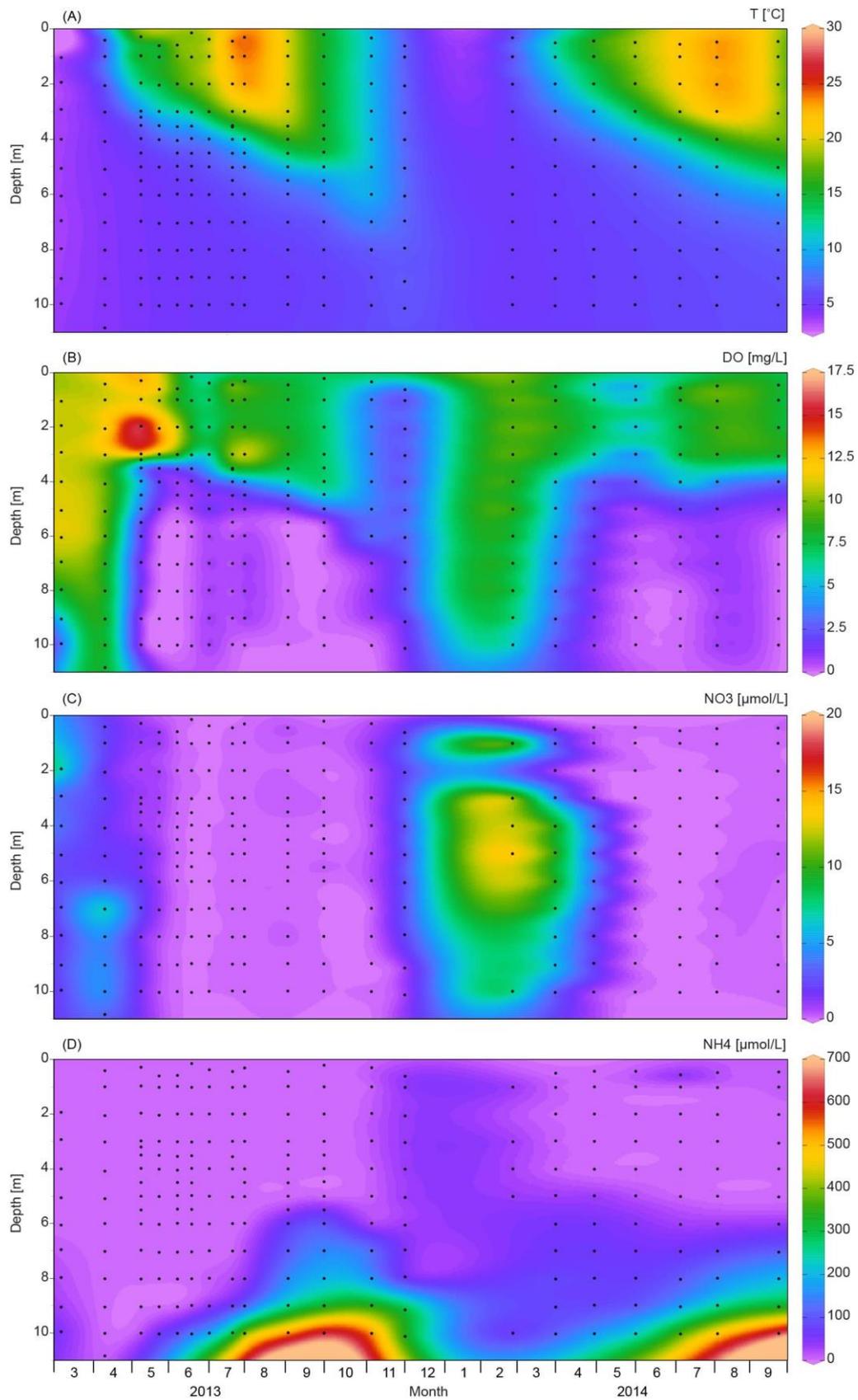


Figure 5.1. Spatio-temporal dynamics of environmental parameters in Lake Vechten over a period of 19 months. (A) Temperature, (B) dissolved oxygen (DO), (C) nitrate, and (D) ammonium.

Abundance and distribution of functional marker genes

We did not find significant amplification of functional marker genes involved in ammonium oxidation (archaeal *amoA*, bacterial *amoA*) and the anammox reaction (*hzsA* genes) in samples from the water column, indicating 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 mainly 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 oxic (**Figure 5.2A,B,C**). The abundance of archaeal *amoA*, bacterial *amoA* and *hzsA* genes in the sediment decreased when the lake stratified and the hypolimnion became anoxic, and reached a minimum between August and October 2013. After fall turnover, when the lake was mixed, the abundance of these three functional marker genes increased again. Compared to the bacterial *amoA* genes, the abundance of archaeal *amoA* genes was one order of magnitude higher.

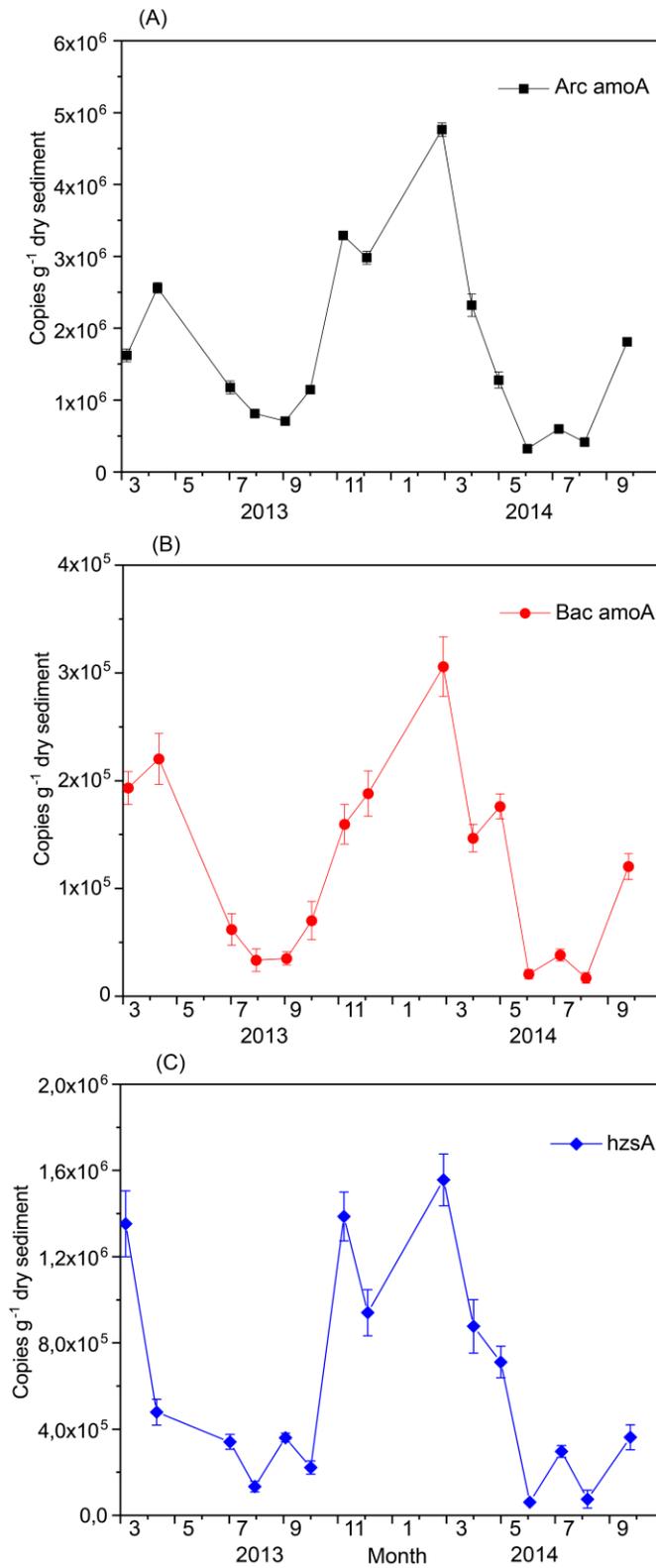


Figure 5.2. Dynamics of (A) archaeal *amoA* genes (ammonia-oxidizing archaea), (B) bacterial *amoA* genes (ammonia-oxidizing bacteria), and (C) *hzsA* genes (anammox bacteria) in the sediment of Lake Vechten.

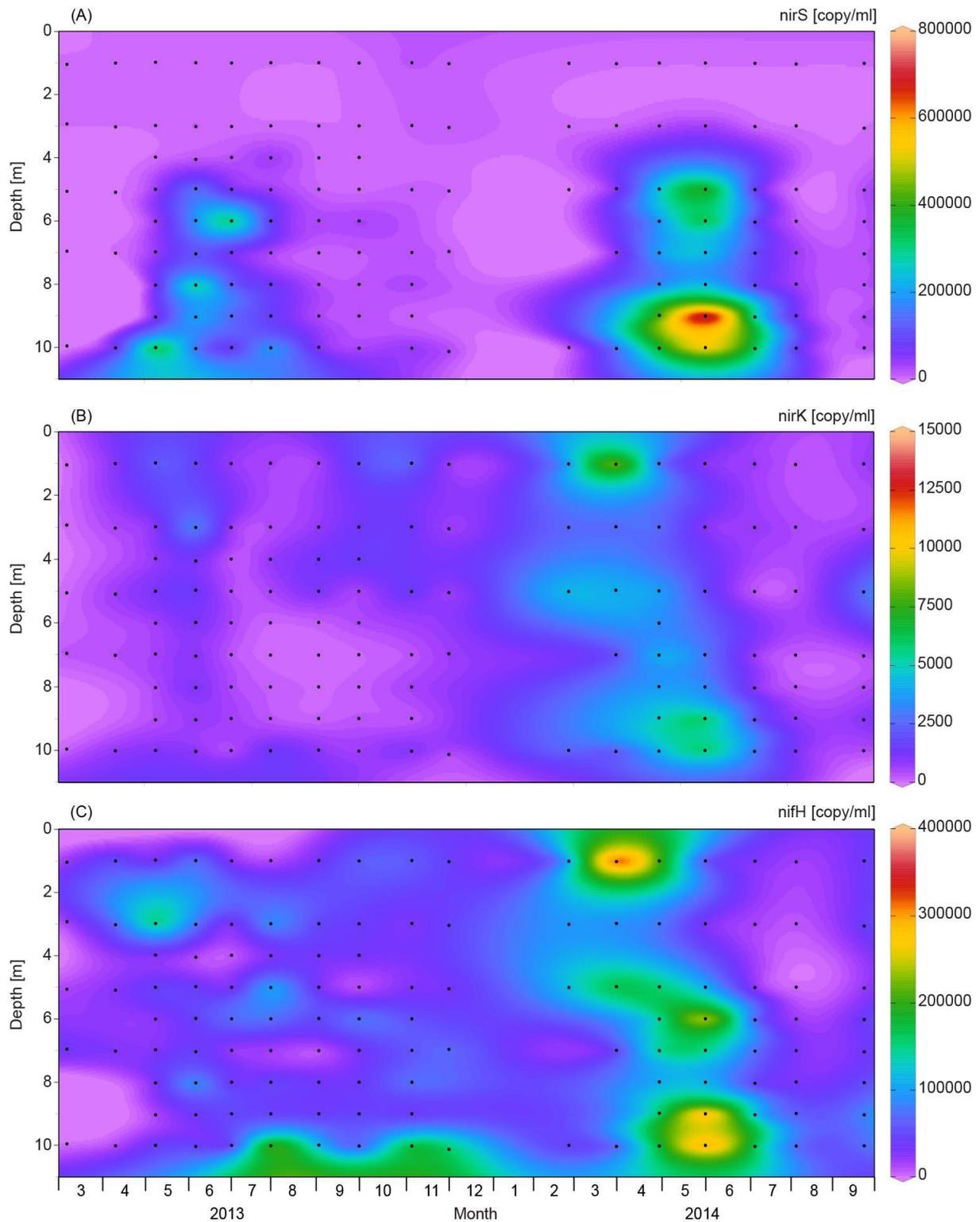


Figure 5.3. Spatio-temporal dynamics of (A) *nirS* genes (denitrifying bacteria), (B) *nirK* genes (denitrifying bacteria), and (C) *nifH* genes (nitrogen-fixing bacteria) in the water column of Lake Vechten.

Denitrification genes (*nirS*, *nirK*) had low abundances in the water column during winter and early spring, but especially *nirS* reached high abundances in the metalimnion and hypolimnion after the onset of lake stratification in late spring and early summer (**Figure 5.3A,B**). Later in summer, before fall turnover, their abundances declined again. In addition, *nirK* had a relatively high concentration at 1 m depth in spring of 2014. In the sediment, abundances of *nirS* and *nirK* genes varied irregularly over the sampling period (**Supplementary Figure S5.2A,B**), without any clear seasonality. The abundance of *nirS* genes exceeded that of *nirK* genes by one order of magnitude during most of the study period.

Copy numbers of the nitrogen-fixation gene (*nifH*) were relatively high in the top layer of the water column in April and May of both years (**Figure 5.3C**). 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. In the sediment, the abundance of *nifH* genes was relatively high during winter and early spring (**Supplementary Figure S5.2C**), and decreased to a minimum during the stratification period.

Sequence analysis of functional marker genes

To confirm the specificity of the PCR and qPCR, and to identify the nitrogen microorganisms, representative amplified fragments of all 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 AOA in Lake Vechten were similar to their counterparts from other freshwater habitats while some were close to *Nitrosopumilus maritimus* (**Supplementary Figure S5.3A**). Nine of the ten AOB sequences clustered with members of the genus *Nitrosomonas*, while one clone was affiliated to the genus *Nitrosospira* (**Supplementary Figure S5.3B**). Sequences of anammox bacteria were very similar to bacterial sequences from a constructed wetland (Coban *et al.*, 2015), which were close to *Brocadia fulgida* (**Supplementary Figure S5.4A**). *NifH* sequences from Lake Vechten were very diverse, including *Deltaproteobacteria*, *Epsilonproteobacteria*, *Cyanobacteria*, *Chlorobi* and *Euryarchaeota* (**Supplementary Figure S5.4B**). *NirS* sequences were similar to sequences from other freshwater environments, and included *Pseudomonas* spp. and *Thauera* spp. (**Supplementary Figure S5.5A**). The *nirK*

sequences were similar to *Ruegeria mobilis* and *Oceanicola nanhaiensis*, belonging to the *Alphaproteobacteria* (**Supplementary Figure S5.5B**).

Redundancy analysis

Redundancy analysis 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, NH_4^+ , NO_3^- , PO_4^{3-} , SO_4^{2-} , sulfide and DOC. Forward selection revealed that 5 of these 10 variables were significant in the redundancy analysis: DO, pH, PO_4^{3-} , SO_4^{2-} and NH_4^+ (**Table 5.2**).

Table 5.2 Significance of the selected explanatory variables in the RDA correlation triplots (see Figure 5.4).

Explanatory Variable	AIC	Pseudo-F	P
DO	124.52	10.58	0.005
PO_4^{3-}	128.26	6.63	0.005
NH_4^+	129.70	5.15	0.005
pH	130.52	4.32	0.005
SO_4^{2-}	133.26	1.55	0.005

The explanatory variables were selected by forward selection based on the pseudo-F statistic, using 9999 permutations to assess their significance. AIC = Akaike information criterion. Total variation explained by the RDA model was 29.4%.

The first and second axis of the RDA plot explained 20.1% and 8.5% of the variation in the qPCR data, respectively (**Figure 5.4**). *NirS* genes were positively correlated with NH_4^+ and negatively correlated with DO. *NirK* and *nifH* genes were positively associated with the PO_4^{3-} and SO_4^{2-} concentration.

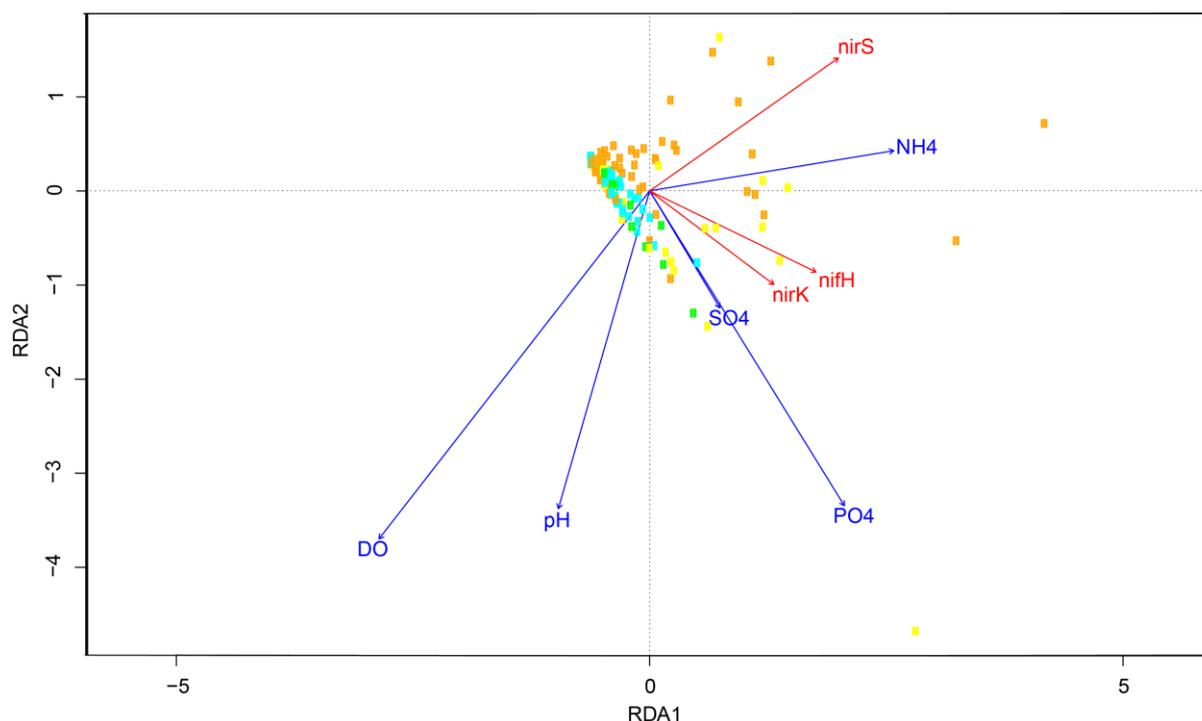


Figure 5.4 Redundancy analysis of the influence of environmental variables in the water column (explanatory variables, blue arrows) on the abundance of *nirS*, *nirK* and *nifH* genes (response variables, red arrows). Symbols represent sampling points (yellow, spring; orange, summer; cyan, fall; green, winter). All explanatory variables in the triplot are significant (see Table 2). Total variation explained by the RDA model was 29.4%.

Discussion

Our results show conspicuous seasonal changes in ammonium and nitrate concentrations, oxygen availability and the abundances of functional marker genes representing different microorganisms involved in the nitrogen cycle. Different nitrogen transformations took place at different depths in the lake and during different times of the year. We will first discuss our observations on the functional groups involved in these nitrogen transformations, and then summarize these results into a comprehensive scheme of the seasonal succession of nitrogen microorganisms in seasonally stratified lakes with anoxic hypolimnia.

Spatio-temporal dynamics of nitrogen bacteria and archaea

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 meet.

However, our findings are only partially in agreement with this expectation. 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. Yet, almost no *amoA* genes were detected in the water column when the oxic-anoxic interface shifted to the metalimnion during summer stratification. Similar results have been reported by other studies, which also found that abundances and activities 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).

The abundance of archaeal *amoA* genes exceeded those from bacteria by one order of magnitude, suggesting a predominant role for archaea in ammonia oxidization. Similar results have been found for freshwater aquaculture ponds (Lu *et al.*, 2015; Lu *et al.*, 2016). Possibly AOA dominated over AOB, because of their higher affinity for ammonia and oxygen (Martens-Habbena *et al.*, 2009; Park *et al.*, 2010; Stahl and de la Torre, 2012). Abundances of AOA and AOB in the sediment of Lake Vechten were high in winter and early spring, but low in summer. A similar seasonal succession of AOA and AOB has been observed in the sediment of an aquaculture pond (Lu *et al.*, 2015), and it was speculated that AOA and AOB also responded similarly to environmental changes in intertidal sediments (Smith *et al.*, 2015).

In line with previous results (Lu *et al.*, 2015), 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). The accumulation of sulfide in the anoxic hypolimnion might be another factor that inhibits the activities of AOA and AOB (Joye and Hollibaugh, 1995; Berg *et al.*, 2015). Because AOA can tolerate periodic exposure to anoxic and sulfidic environments to a certain extent (Berg *et al.*, 2015), the inhibitory effects of sulfide on AOB are likely to be more severe than on AOA (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). Our qPCR result of *hzsA* genes, however, indicates that anammox bacteria were exclusively present in

the sediment of Lake Vechten. 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 winter and increased during summer stratification 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), in coastal sediments (Lipsewers *et al.*, 2014) and in wastewater treatment systems (Gao *et al.*, 2014). Lagostina *et al.* (2015) 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 might be present at different depths in the sediment. However, because we used a box corer to sample the sediment, we could not investigate the vertical distribution of these bacteria in the sediment of Lake Vechten.

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, ammonium oxidation by AOA and AOB may facilitate the activity and abundance of anammox bacteria by providing nitrite and creating anoxic microenvironments in the sediment, which would explain why the dynamics of *hzsA* genes, archaeal and bacterial *amoA* genes followed similar patterns (**Figure 5.2**). For instance, when the abundance of AOA and AOB decreased during summer stratification, *hzsA* genes also dropped to a relatively low abundance. Consistent with these observations, abundances of AOA and anammox bacteria peaked at the same depth in the Black Sea (Kuypers *et al.*, 2003; Coolen *et al.*, 2007), while stable cooperation between AOB and anammox bacteria has been exploited in wastewater bioreactors (Third *et al.*, 2001).

Denitrifying bacteria

In a previous study performed in the late 1980s, mats of sulfur-oxidizing *Beggiatoa* spp. on the sediment were identified to play vital roles in the denitrification process of Lake Vechten (Sweerts *et al.*, 1990). However, although we identified several denitrifying microorganisms, *Beggiatoa* or close relatives were not retrieved from the cloning results of *nirS* and *nirK* genes in our study. The distributions of *nirS*

and *nirK* denitrifying bacteria were quite different in the water column of Lake Vechten. Specifically, *nirS* denitrifying bacteria were restricted to the metalimnion and hypolimnion, whereas *nirK* denitrifying bacteria were also found in the epilimnion and did not show a clear habitat preference. *NirS* and *nirK* denitrifying bacteria also occupied different niches and responded differently to environmental variables in the South China Sea and a variety of other ecosystems (Jones and Hallin *et al.*, 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* denitrifying bacteria reached their highest abundances in the anoxic hypolimnion in late spring and early summer. This seasonal pattern is quite comparable to observations of denitrifying bacteria in other lake studies. In the monomictic Lake Lugano, denitrification rates were highest in the sediment during fully oxic bottom water conditions and shifted to the water column when the bottom water became anoxic during lake stratification (Wenk *et al.*, 2014). In permanently stratified Lake Rassnitzer, anammox bacteria were abundant in the anoxic bottom waters in January and October, while denitrifying bacteria dominated N_2 production in May (Hamersley *et al.*, 2009). In our study in Lake Vechten, denitrifying bacteria were replaced by sulfate-reducing bacteria in the anoxic hypolimnion after nitrate was completely consumed in late summer (Bush *et al.*, 2017). This successional sequence is in agreement with common expectation, because nitrate reduction results in a higher energy yield than sulfate reduction, and hence denitrifying bacteria have a competitive advantage over sulfate-reducing bacteria as long as nitrate is available as 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 the nitrate concentration in the water column was gradually depleted. Nitrogen-fixing bacteria were taxonomically very diverse, including *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and phototrophic sulfur bacteria (e.g. *Chlorobi*), in line with previous studies (Raymond *et*

al., 2004; Turk *et al.*, 2011). 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).

Interestingly, there was pronounced overlap in the spatio-temporal distributions of *nifH*, *nirK* and *nirS* genes in Lake Vechten. In the conventional view of nitrogen cycling, nitrogen fixation and denitrification are temporally and/or spatially segregated. In contrast, we observed co-occurrence of denitrifying and nitrogen-fixing bacteria, which is in line with observations in the meromictic Lake Cadagno (Halm *et al.*, 2009).

Seasonal succession of nitrogen microorganisms

Based on our findings and the preceding discussion, we propose a simple schematic model that depicts the seasonal succession of nitrogen microorganisms in eutrophic and seasonally stratified lakes (**Figure 5.5**). 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 into nitrite and nitrate. In spring, the nitrate concentration in the surface layer is gradually depleted by an increasing biological activity, favoring the growth of nitrogen-fixing bacteria and, at least in Lake Vechten, apparently also *nirK*-containing denitrifying bacteria. Meanwhile, the water column stratifies and the hypolimnion becomes anoxic. The anoxic hypolimnion favors a temporary rise of denitrifying bacteria (both *nirS* and *nirK* strains), which disappear again once the nitrate concentration in the hypolimnion has been fully depleted. Furthermore, the anoxic and sulfidic hypolimnion prevents ammonium oxidation by AOA, AOB and anammox bacteria in the sediment, and their abundances strongly decline during summer stratification while ammonium accumulates in the hypolimnion during summer and early fall. After fall turnover, the entire water column is mixed again, AOA, AOB and anammox bacteria reappear in the sediment and ammonium is oxidized to nitrate (**Figure 5.5**).

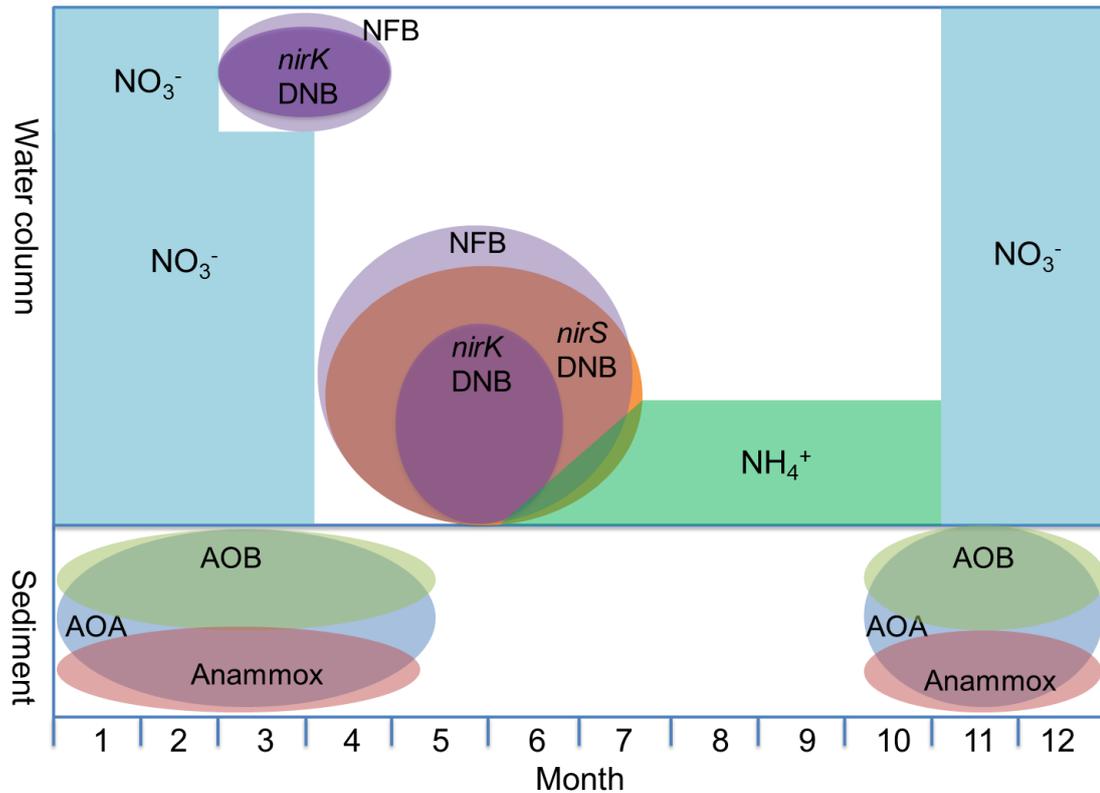


Figure 5.5 Conceptual model of the seasonal succession of nitrogen microorganisms and seasonal transition of inorganic nitrogen (NO_3^- , NH_4^+) in the sediment and water column of Lake Vechten over one year. AOA, ammonium-oxidizing archaea; AOB, ammonium-oxidizing bacteria; Anammox, anaerobic ammonium-oxidizing bacteria; NFB, nitrogen-fixing bacteria; *nirK* DNB, denitrifying bacteria with the *nirK* gene; *nirS* DNB, denitrifying bacteria with the *nirS* gene.

Of course, this simple model is only a first attempt to capture the seasonal succession of different functional groups of microorganisms involved in the nitrogen cycle of seasonally stratified lakes. It may be modified by future studies of other lakes in other environmental settings. Furthermore, it may be refined by more detailed process-based studies at a higher spatio-temporal and taxonomic resolution. Nevertheless, we believe that such simple schematic models will provide a very useful benchmark in our efforts to understand and possibly predict how the nitrogen cycle is likely to be affected by the increasing environmental pressures on our freshwater ecosystems through eutrophication, aquatic pollution and climate change.

Acknowledgments

We thank Pieter Slot and Bas van Beusekom for assistance during the fieldwork, and Leo Hoitinga and Peter Serné for nutrient analyses.

This work was financially supported by the China Scholarship Council (No. 201206220165), the Research Priority Area *Systems Biology* of the University of Amsterdam, and the ERC Advanced Grant PARASOL (No. 322551).

Supplementary Information

Seasonal succession of bacteria and archaea involved in the nitrogen cycle of a seasonally stratified lake

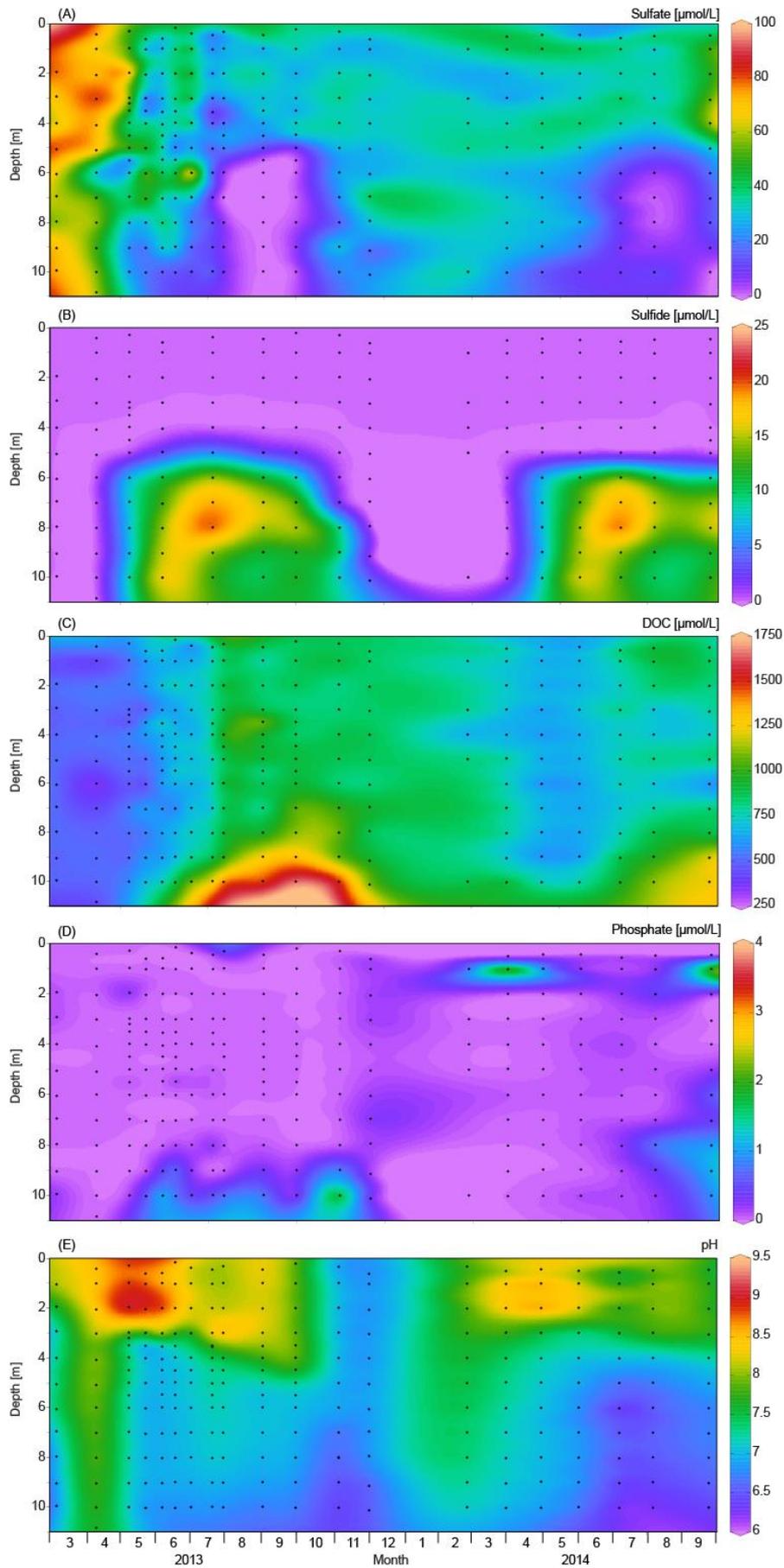


Figure S5.1. Spatio-temporal dynamics of environmental parameters in Lake Vechten over a period of 19 months. (A) Sulfate, (B) Sulfide, (C) DOC, (D) Phosphate, and (E) pH.

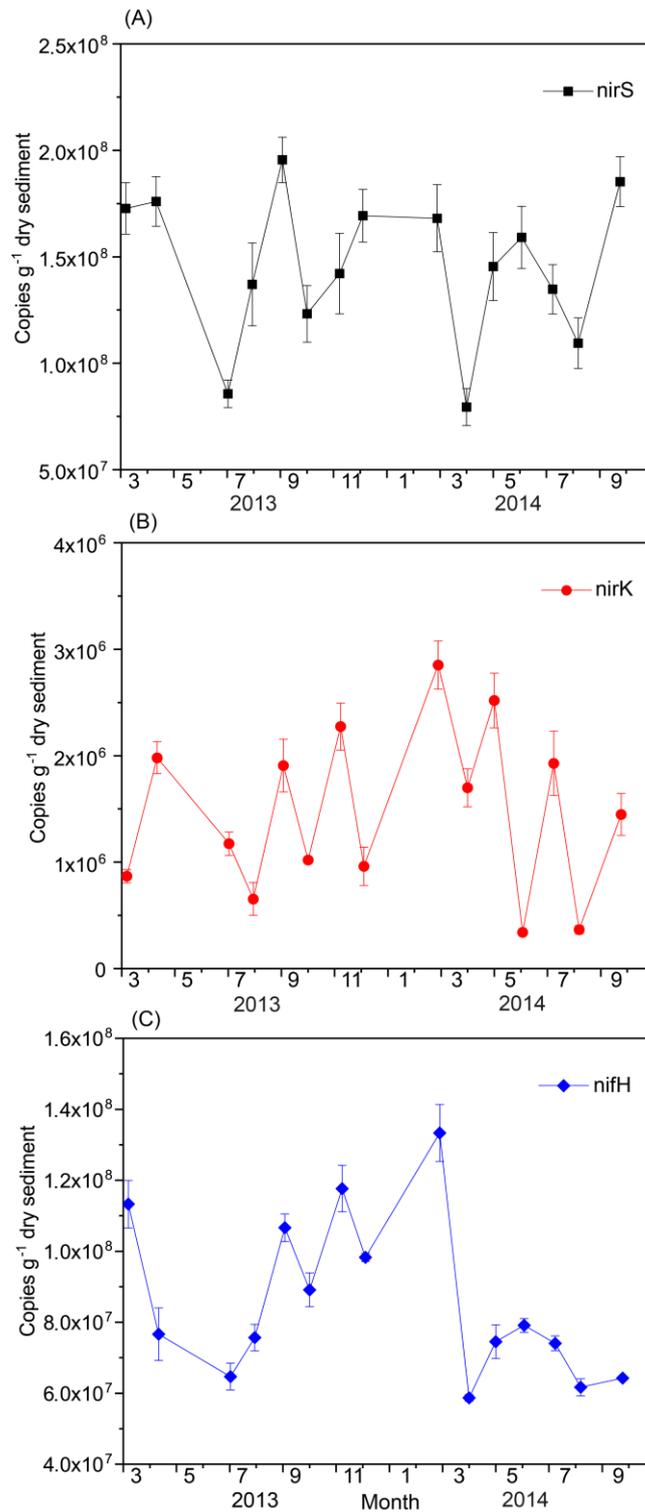


Figure S5.2. Dynamics of (A) *nirS* genes (denitrifying bacteria), (B) *nirK* genes (denitrifying bacteria), and (C) *nifH* genes (nitrogen-fixing bacteria) in the sediment of Lake Vechten.

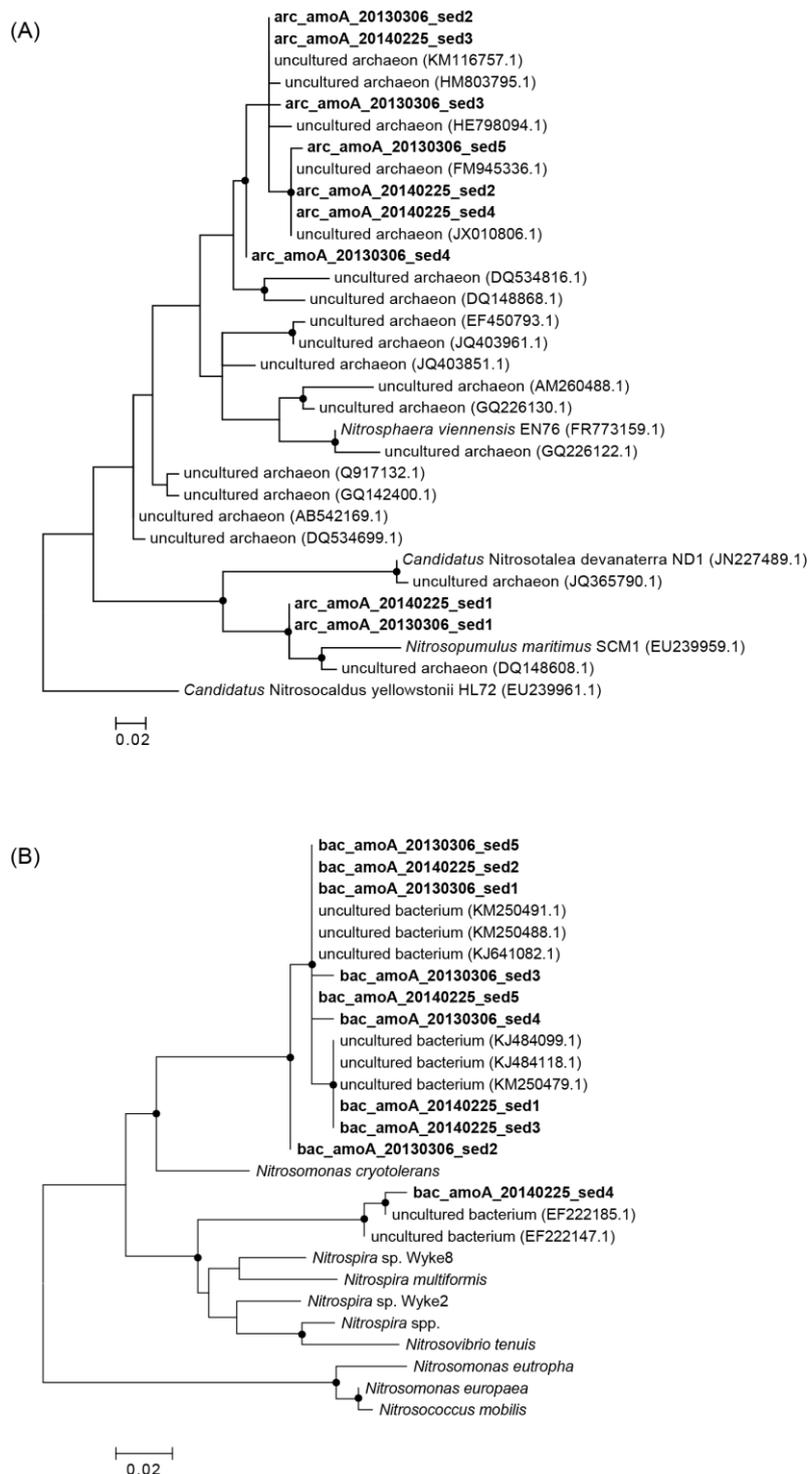


Figure S5.3. Maximum likelihood trees based on protein sequences of (A) archaeal *amoA* genes (ammonia-oxidizing archaea), and (B) bacterial *amoA* genes (ammonia-oxidizing bacteria) of Lake Vechten. Names in boldface are sequences determined in our study. The scale bars indicate the number of amino acid substitutions per site. Black dots on the branches indicate bootstrap values between 90 and 100%.

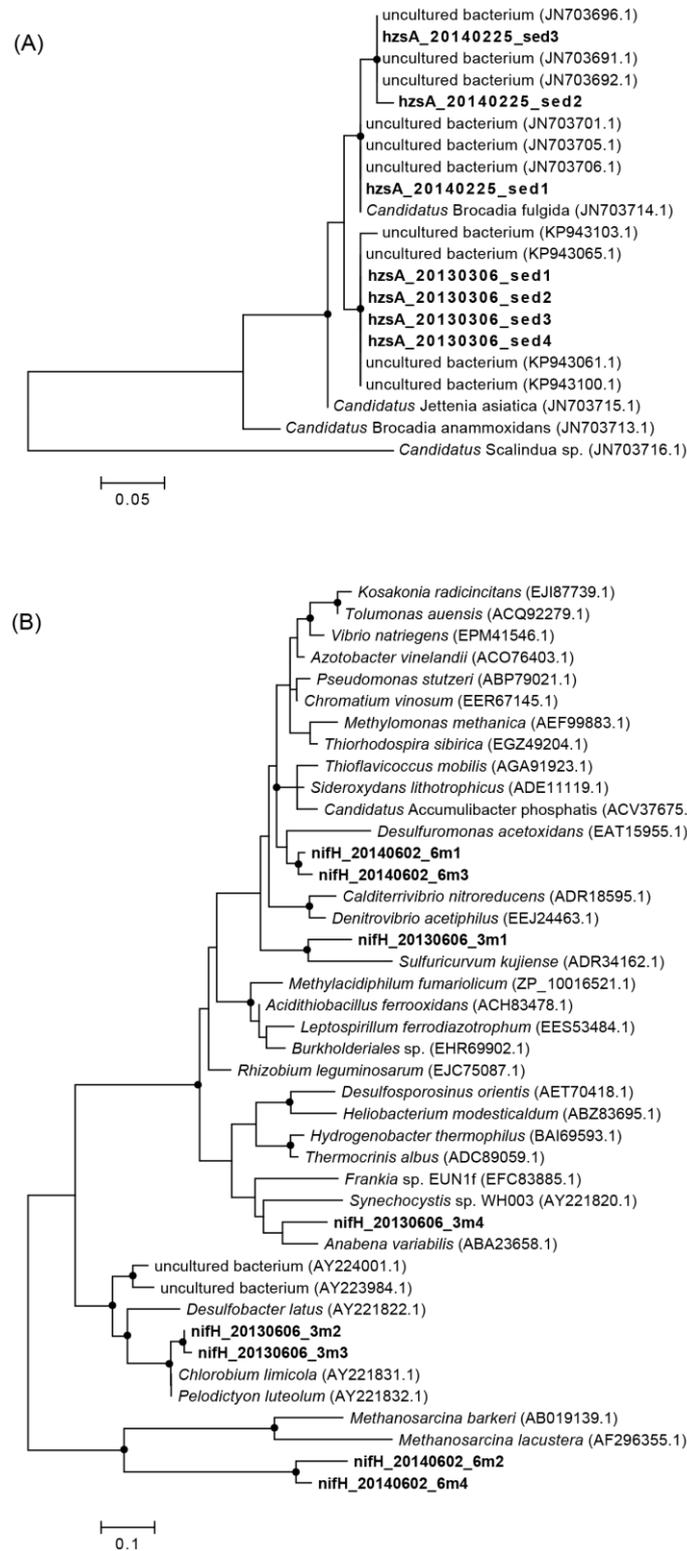


Figure S5.4. Maximum likelihood trees based on protein sequences of (A) *hzsA* genes (anammox bacteria), and (B) *nifH* genes (nitrogen-fixing bacteria) of Lake Vechten. Names in boldface are sequences determined in our study. The scale bars indicate the number of amino acid substitutions per site. Black dots on the branches indicate bootstrap values between 90 and 100%.

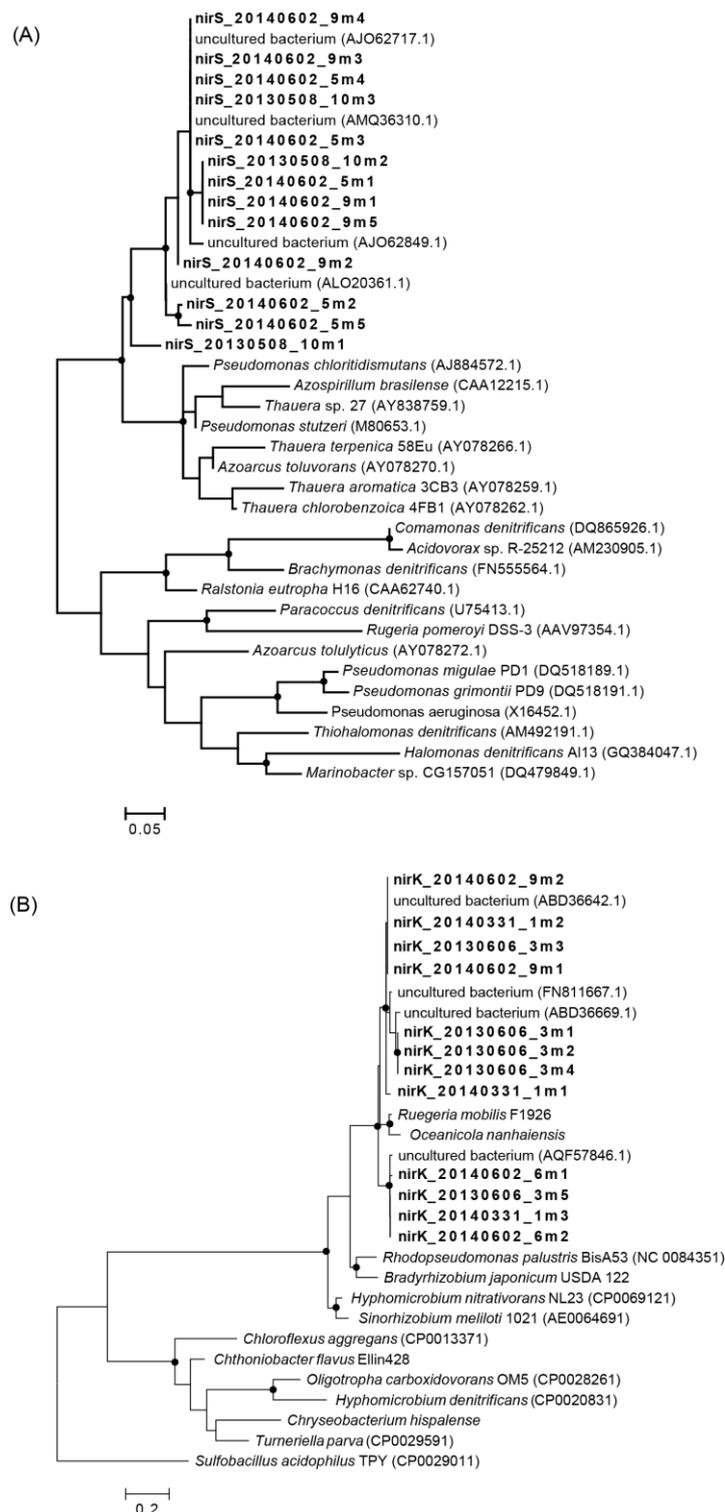


Figure S5.5. Maximum likelihood trees based on protein sequences of (A) *nirS* genes (denitrifying bacteria), and (B) *nirK* genes (denitrifying bacteria) of Lake Vechten. Names in boldface are sequences determined in our study. The scale bars indicate the number of amino acid substitutions per site. Black dots on the branches indicate bootstrap values between 90 and 100%.

Table S5.1. Fragment length and thermal programs of PCR primers used in this study.

Primers	Target gene	Fragment (bp)	PCR program
Arch-amoAF Arch-amoAR	<i>Arc amoA</i>	635	3 min at 95.0°C; 35 cycles of: 45 s at 95.0°C, 45 s at 53.0°C, 45 s at 72.0°C; 5 min at 72.0°C.
amoA-1F amoA-2R	<i>Bac amoA</i>	491	3 min at 95.0°C; 35 cycles of: 40 s at 95.0°C, 40 s at 48.0°C, 40 s at 72.0°C; 5 min at 72.0°C.
hzsA_1597F hzsA_1857R	<i>hzsA</i>	260	3 min at 95.0°C; 35 cycles of: 30 s at 95.0°C, 30 s at 50.0°C, 30 s at 72.0°C; 5 min at 72.0°C.
nirS1F nirS-q-R	<i>nirS</i>	265	3 min at 95.0°C; 35 cycles of: 30 s at 95.0°C, 30 s at 56.0°C, 30 s at 72.0°C; 5 min at 72.0°C.
nirK-q-F nirK1040	<i>nirK</i>	472	3 min at 95.0°C; 35 cycles of: 30 s at 95.0°C, 30 s at 56.0°C, 30 s at 72.0°C; 5 min at 72.0°C.
IGK3 DVVR	<i>nifH</i>	395	3 min at 95.0°C; 35 cycles of: 30 s at 95.0°C, 30 s at 50.0°C, 30 s at 72.0°C; 5 min at 72.0°C.

Table S5.2. List of samples for cloning.

Functional gene	Date	Depth (m)	Fragment (bp)	Cloning ID
<i>Arc amoA</i>	2013-03-06	Sediment	635	arc_amoA_20130306_sed1 -
<i>Arc amoA</i>	2014-02-25	Sediment	635	arc_amoA_20130306_sed5 arc_amoA_20140225_sed1 -
<i>Bac amoA</i>	2013-03-06	Sediment	491	arc_amoA_20140225_sed4 bac_amoA_20130306_sed1 -
<i>Bac amoA</i>	2014-02-25	Sediment	491	bac_amoA_20130306_sed5 bac_amoA_20140225_sed1 -
<i>hzsA</i>	2013-03-06	Sediment	260	bac_amoA_20140225_sed5 hzsA_20130306_sed1 -
<i>hzsA</i>	2014-02-25	Sediment	260	hzsA_20130306_sed4 hzsA_20140225_sed1 -
<i>nifH</i>	2013-06-06	3	395	hzsA_20140225_sed3 nifH_20130606_3m1 -
<i>nifH</i>	2014-06-02	6	395	nifH_20130606_3m4 nifH_20140602_6m1 -
<i>nirS</i>	2013-05-08	10	265	nifH_20140602_6m4 nirS_20130508_10m1 -
<i>nirS</i>	2014-06-02	5	265	nirS_20130508_10m3 nirS_20140602_5m1 -
<i>nirS</i>	2014-06-02	9	265	nirS_20140602_5m5 nirS_20140602_9m1 -
<i>nirK</i>	2013-06-06	3	472	nirS_20140602_9m5 nirK_20130606_3m1 -
<i>nirK</i>	2014-03-31	1	472	nirK_20130606_3m5 nirK_20140331_1m1 -
<i>nirK</i>	2014-06-02	6	472	nirK_20140331_1m3 nirK_20140602_6m1 -
<i>nirK</i>	2014-06-02	9	472	nirK_20140602_6m2 nirK_20140602_9m1 -
				nirK_20140602_9m2

Table S5.3. qPCR programs, efficiency and R² of functional marker genes.

Primers	Target gene	Fragment (bp)	qPCR program	qPCR efficiency	R ²
Arch-amoAF Arch-amoAR	Arc <i>amoA</i>	635	3 min at 95.0°C; 40 cycles of: 45 s at 95.0°C, 45 s at 59.0°C, 45 s at 72.0°C; 5 min at 72.0°C.	95.1-99.2%	0.991-0.994
amoA-1F amoA-2R	Bac <i>amoA</i>	491	3 min at 95.0°C; 40 cycles of: 30 s at 95.0°C, 30 s at 59.0°C, 30 s at 72.0°C; 5 min at 72.0°C.	90.1-92.7%	0.997-0.999
hzsA_1597F hzsA_1857R	<i>hzsA</i>	260	3 min at 95.0°C; 40 cycles of: 30 s at 95.0°C, 30 s at 50.0°C, 30 s at 72.0°C; 5 min at 72.0°C.	83.8-86.4%	0.998-0.999
nirS1F nirS-q-R	<i>nirS</i>	265	3 min at 95.0°C; 40 cycles of: 30 s at 95.0°C, 30 s at 60.0°C, 30 s at 72.0°C; 5 min at 72.0°C.	92.6- 95.5%	0.996-0.998
nirK-q-F nirK1040	<i>nirK</i>	472	3 min at 95.0°C; 40 cycles of: 30 s at 95.0°C, 30 s at 62.5°C, 30 s at 72.0°C; 5 min at 72.0°C.	95.4-99.8%	0.995-0.997
IGK3 DVVR	<i>nifH</i>	395	3 min at 95.0°C; 40 cycles of: 30 s at 95.0°C, 30 s at 55.0°C, 30 s at 72.0°C; 5 min at 72.0°C.	81.9- 83.4%	0.998-0.999