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Seasonal dynamics of anaerobic oxidation of ammonium and denitrification in a dimictic lake during the stratified spring–summer period

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Abstract

In aquatic ecosystems, nitrogen (N) loading is mitigated in redox transition zones principally through the processes of denitrification and anaerobic oxidation of ammonium (anammox). Here, we investigate the N cycling processes in the water column of a seasonal stratified lake influenced by benthic processes in Southern Germany (Fohnsee) during the development of the vertical redox stratification between April and September. Concentration profiles and stable isotope compositions of NO_3^- and NH_4^+ together with numerical modeling and quantification of the hydrazine synthase gene (*hzsB*) and nitrite reductase (*nirK* and *nirS*) genes were used to identify the predominant nitrogen-transformation processes at lake Fohnsee throughout the spring and summer periods. Water chemistry data, quantitative polymerase chain reaction analysis and increases of $\delta^{15}N$ and $\delta^{18}O$ values of nitrate from 7.0‰ to 41.0‰ and 2.0‰ to 28.0‰, respectively, showed that nitrate reduction to nitrite and NO occurs in an upward moving zone of the water column from June to September following the displacement of the oxycline caused by thermal stratification. We also observed an increase in $\delta^{15}N$ of ammonium from 15‰ to 28‰ in the anoxic water column. Modeling results suggest that this shift in $\delta^{15}N\text{-}NH_4^+$ is predominantly controlled by mixing between ammonium stemming from the oxic water column with $\delta^{15}N$ values of 25‰ and ammonium that is likely formed in the lake sediments by oxidation of organic matter with $\delta^{15}N$ values of 11‰. Observed gene abundances (*hzsB*, *nirK*, and *nirS*) in lake water samples collected in June and July indicated the co-occurrence of nitrate reduction and low rates of anammox, while the presence of sulfide in August and September may have inhibited the activity of anammox bacteria near the sulfate-reduction zone at the lake bottom. This study revealed temporal and spatial (e.g., depth dependent) variations in the dominant N-transformation processes in the investigated lake.

Human modification of the nitrogen (N) cycle on our continents throughout the last few decades has been profound (Gruber and Galloway 2008). While the addition of various forms of reactive N to the environment has been predominantly targeted at enhancing food production via fertilization, some reactive N eventually ends up in the environment and is frequently polluting groundwater, rivers, and lakes (Rockström et al. 2009). Anthropogenic and geogenic N loading of natural ecosystems is mostly mitigated by microbially driven redox reactions taking place in oxygen-deficient waters (Harrison et al. 2009). Three anaerobic pathways are known to be involved in the reduction of NO_3^- and NO_2^- in aquatic ecosystems: canonical denitrification, dissimilatory reduction of nitrate to ammonium (DNRA), and anaerobic ammonium oxidation (anammox) (Strous et al. 1999; Kuypers et al. 2003). Since its discovery in nature in 2002 (Thamdrup and Dalsgaard 2002), field and laboratory studies have consistently shown that anammox plays a significant role in the N cycle of marine ecosystems removing up to 50% of the N load from these environments (Dalsgaard et al. 2003; Engström et al. 2005; Hamersley et al. 2007). Recent research also suggested that the anammox process could be ubiquitous in freshwater ecosystems (Schubert et al. 2006; Wenk et al. 2013; Zhu et al. 2013; Crowe et al. 2017; Gao et al. 2018;

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Callbeck et al. 2021). However, the N loss attributed to anammox in freshwater lakes appears to vary significantly depending on environmental conditions (Schubert et al. 2006; Wenk et al. 2013; Zhao et al. 2015). Crowe et al. (2017) showed that anammox was responsible for up to 50% of N_2 released in Lake Superior (North America), whereas results obtained from Lake Kivu (Africa) suggest that anammox account for only ~ 1% of N loss (Roland et al. 2018). These contrasting observations regarding the importance of anammox in N cycling in freshwater lakes is likely linked to the trophic state of the ecosystem dependent on the input of organic matter (Dalsgaard et al. 2012; Babbin et al. 2014; Callbeck et al. 2021), the occurrence of sulfidic redox zones due to the efflux of nitrite and ammonium produced from benthic processes (Callbeck et al. 2021) and the presence of other reductants besides organic carbon such a hydrogen sulfide (Robert Hamersley et al. 2009).

In temperate latitudes, redox conditions in lakes can change markedly during the year due to thermal stratification (Dake and Harleman 1969). During the late spring and summer months, the water layers near the lake bottom shift from oxic to anoxic conditions, facilitating redox processes in order of their energy favorability (Clark 2015). In the absence of O_2, bacterial communities using NO_3^- and NO_2^- may dominate (i.e., denitrifying and anammox bacteria), followed by microorganisms using Mn(IV), Fe(III), SO_4^{2-}, and CO_2 or acetate (Fenchel et al. 1999) as electron acceptors. Sulfate-reducing conditions near the lake bottom could be achieved during later summer producing H_2S while NO_2^- and NH_4^+ are present in the water column due to denitrification and organic nitrogen remineralization in the lake sediments, respectively.

The isotopic composition of NO_3^- and NH_4^+ is often a helpful tool to identify N sources (Kendall 1998; Wild et al. 2018) and to reveal microbial redox reactions converting N compounds in freshwater ecosystems (Granger et al. 2008; Wenk et al. 2014). Microorganisms preferentially metabolize lighter isotopes, converting ^{14}N at a higher rate than ^{15}N containing compounds, leaving the remaining nitrogen pool enriched in ^{15}N. Generally, changes in the $\delta^{15}N$ value of forms of inorganic nitrogen (e.g., NO_3^-, NH_4^+) can be an excellent indication for microbially driven processes. However, biogeochemical N transformations inferred from measurement of the isotopic composition of NO_3^- and NH_4^+ may reflect more complex patterns than previously thought. Particularly, trends in nitrogen isotope ratios of ammonium are often difficult to interpret when nitrification, anammox, and indirectly denitrification or DNRA affect $\delta^{15}N$ of ammonium (Brunner et al. 2013; Granger and Wankel 2016). Therefore, successful interpretation of N and O isotope ratio data requires a detailed understanding of all transport and transformation pathways influencing the $\delta^{15}N$ and $\delta^{18}O$ values of N containing compounds. Changes of the stable isotope signature of a compound in natural ecosystems can also occur due to diffusive transport (Lehmann et al. 2003; de Visscher et al. 2004) and/or mixing between different N sources (Brandes et al. 1998) within the water column. Thus, modified reaction–diffusion transport models differentiating isotopes of a single compound (e.g., ^{14}N and ^{15}N) have been used as a tool to improve the interpretation of stable isotope data in N cycling studies (Lehmann et al. 2003; Norði and Thamdrup 2014; Wenk et al. 2014). The combination of water chemistry data and molecular microbiological techniques is another elegant approach to assess the occurrence of microbial driven redox processes (Michael Beman et al. 2012; Wenk et al. 2013; Deutzmann et al. 2014; Zigah et al. 2015) and to interpret the associated stable isotope patterns.

Initial investigations based on a sampling campaign at the seasonally stratified lake Fohnsee in southern Germany revealed an isotopic enrichment of ^{13}C of methane in the anoxic water column that overlapped with increasing $\delta^{15}N$ of nitrate, ammonium, and nitrite and $\delta^{18}O$ of nitrate. Together with the detection of microbial communities that consist of bacteria known to be involved in denitrification with anaerobic oxidation of methane and anammox the occurrence of both processes was suggested (Einsiedl et al. 2020). Since this initial study was conducted only for a single sampling event at a time when bacterial sulfate reduction in the water column was not prevalent, the relevance of the detected processes as a nitrogen sink throughout the entire spring and summer stratification of the lake remained unclear.

Hence, the objective of this study was to couple chemical, isotopic, modeling, and genomic techniques to investigate the pathways of nitrogen-transformation processes throughout the entire spring and summer stratification at lake Fohnsee in southern Germany. We present a combined approach of ammonium and nitrate isotope compositions ($\delta^{15}N$-NH_4^+ and $\delta^{15}N$, $\delta^{18}O$-NO_3^-) and water chemistry data collected during spring and summer stratification over a period of 6 months, together with numerical modeling and quantitative polymerase chain reaction (qPCR) analysis for the functional genes for anammox (*hszB*) and denitrification (*nirK*, *nirS*), to reveal the co-existence of canonical denitrification and anaerobic ammonium oxidation (anammox) within the water column at lake Fohnsee.

Methods

Study site

The Osterseen is a chain of hydrologically connected lakes that are located at the southern end of Lake Starnberg in southern Germany. Lake Fohnsee is the second biggest of the Osterseen lakes with a surface area of approximately 211,900 m^2 and a water depth of up to 23 m. Seasonal stratification occurs during late spring and summer at lake Fohnsee with an oxic zone in the upper part of the lake water column (epilimnion) and a very stable anoxic redox zone (hypolimnion) between May and October with high resistance to vertical mixing (Braig et al. 2010). Due to its mixing regime, lake Fohnsee can be classified as dimictic with overturn events taking place in March and at the end of October. The spring

turnover is driven by the melting of lake ice due to increasing temperatures, while the fall turnover of the water column occurs when the thermocline vanishes as a result of decreasing temperatures (Peña Sanchez et al. 2022).

Water column sampling

Six sampling campaigns were conducted at Fohnsee from April to September 2019. We obtained depth-resolved water samples from the water column of the lake throughout the 6 months of progressively evolving lake stratification, and establishment of increasingly reducing conditions. The dissolved oxygen (DO) content and temperature of the sampled lake water were measured with a Multi 350i multi-parameter probe (WTW) prior to water sampling with a depth resolution of up to 1 m. Samples for laboratory-based analysis of major cations and anions, methane concentrations, stable isotope ratios of nitrate ($\delta^{15}N$, $\delta^{18}O$), ammonium ($\delta^{15}N$), and methane ($\delta^{13}C$) and for molecular-biological investigation were taken and pre-handled as described in detail in Supplementary Information A1.

Determination of chemical properties

Concentrations of NO_3^-, NO_2^-, and NH_4^+ of the obtained water samples were determined using ion chromatography with a ICS 1100 device (Thermo Fischer Scientific) including a Dionex IonPac AS9-HC analytical column (4 × 250 mm) for anions and a Dionex IonPac CS 12A analytical column (4 × 250 mm) for cations. Ion concentrations were measured in triplicates, and the values presented in this study correspond to the mean value of these measurements. Detection limit and standard deviation (σ) of the data presented in this study were as follows: for NO_3^-, 0.010 and 0.0012 mmol L^{-1}; for NO_2^-, 0.006 and 4.2×10^{-5} mmol L^{-1}; for NH_4^+, 0.008 and 5.5×10^{-4} mmol L^{-1}. Methane concentrations were measured using a static equilibration headspace method as described by Kampbell et al. (2006); detailed description of the method can be found in the Supplementary Information A2.

Stable isotope calculations

Isotope results are expressed in per mil notation relative to the international standards V-PDB, AIR, and V-SMOW for carbon, nitrogen, and oxygen isotope ratios, respectively. The nitrogen and oxygen isotope ratios of NO_3^- dissolved in water were determined by converting nitrate to N_2O using the denitrifier method as described by Sigman et al. (2001). The produced N_2O gas was measured using an HP 6890 gas chromatograph with a PreCon® device coupled to a Finnigan Mat Delta + XL mass spectrometer. Precision and accuracy as σ ($n = 10$) were 0.5‰ for $\delta^{15}N$ and 1‰ for $\delta^{18}O$, respectively.

The nitrogen isotopic composition of dissolved NH_4^+ was determined using the "diffusion-method" as described by Sebilo et al. (2004) (*see* Supplementary Information A).

The carbon isotopic composition of methane was determined from headspace gas resulting from the head-space equilibration using cavity ring-down spectroscopy (G2201-i Isotopic Analyzer for CO_2/CH_4, Picarro Inc). A detailed description of the method can be found in Supplementary Information A.

For a first approximation of the N and O isotope enrichment factors ε of nitrate, and the N isotope enrichment factor for NH_4^+, apparent stable isotope enrichment factors were calculated using a closed-system model.

$$\ln\frac{R_t}{R_0} = \varepsilon \times \ln f \tag{1}$$

where ε is the stable isotope enrichment factor associated with the microbial consumption of the reactant, f is the unreacted portion of the substrate (NH_4^+ or NO_3^-) described by the relation C_i/C_0, and R_0 and R_i are the isotopic ratios of the substrate before alteration (index 0) and at a given time t, respectively.

Diffusion model for concentrations and stable isotopes of NH_4^+

Ammonium is frequently released from anoxic sediments as a result of the mineralization of organic matter (Morin and Morse 1999; Beutel 2006; Wang et al. 2008). Fohnsee water is assumed to be stagnant in the hypolimnion (no advective mixing) so that ammonium is transported within the water column only by diffusion. Thus, vertical transport of NH_4^+ from the lake sediment–water interface along the anoxic water column can be described by the following equation:

$$\frac{\partial C}{\partial t} = K_z \frac{\partial^2 C}{\partial z^2} + r \tag{2}$$

where t is time (day), z is the water depth (m), C is the concentration of ammonium (mmol L^{-1}), K_z is the turbulent diffusive transport coefficient (m^2 d^{-1}), and r is the reaction rate of ammonium (mmol d^{-1}). K_z was calculated independently for each month using density profiles that were derived from the temperature profiles measured at Fohnsee from May to September as described by Wenk et al. (2013) and Blees et al. (2014). Calculation of the turbulent diffusion coefficient used in our model is described in detail in Supporting Information B1.

The model described by Eq. 2 can be modified to consider both the isotopic species $^{14}NH_4^+$ and $^{15}NH_4^+$ separately in order to assess the extent of stable isotope fractionation due to diffusion. The final $^{15}N/^{14}N$ isotope ratio of NH_4^+ can be calculated incorporating both resulting partial differential equations for ^{15}N and ^{14}N into the general formula for stable isotope notation, using N_2 as reference material as follows:

$$\delta N^{15}NH_4^+ = \left[\frac{\left(\frac{\partial C^{15}N}{\partial t} \right)}{\left(\frac{\partial C^{14}N}{\partial t} \right)} \Big/ \left(\frac{C^{15}N}{C^{14}N} \right)_{N_2} - 1 \right] \times 1000 \tag{3}$$

Model development, boundary, and initial conditions are described in detail in Supplementary Information B2. The model considers a source of ammonium in the anoxic zone released from the lake sediments with a constant isotopic signature for $\delta^{15}N\text{-}NH_4^+$ of 11‰ as it was measured near the lake sediment, and the initial $\delta^{15}N\text{-}NH_4^+$ of 28‰, which corresponds to the average signature of the ammonium in the oxic water column at the beginning of June, and thus, it considers mixing with the residual enriched NH_4^+ in the water column from the previous months.

DNA extraction and PCR amplification

The filters obtained after filtering the lake water from the different layers, which were stored at –20°C, were cut for DNA extraction on a sterile petri dish into 2 mm pieces before adding it to a lysing matrix screw cap tube. The protocol was based on phenol/chloroform/isoamyl alcohol mixture as described in Lueders et al. (2004) and Töwe et al. (2011) using PEG8000 instead of PEG6000. The concentration of the DNA was measured fluorometrically and the quality was determined by spectrophotometry. Some samples had to be excluded due to low quality or low DNA concentration (June: 20 m depth, August: 8 m depth, September: 10 and 6 m depth).

qPCR was used to measure the overall bacterial abundance based on the copy numbers obtained for the 16*S* rRNA gene according to Gschwendtner et al. (2011), the abundances of the hydrazine synthase β-subunit (*hzsB*) to determine the abundance of anammox bacteria (Wang et al. 2012) as well as marker genes for nitrite reducers (*nirK* and *nirS*; Töwe et al. 2010). Primers, standards, and PCR conditions were used as described in the original publications. qPCRs were performed on a 7300 Real-Time PCR System (Applied Biosystems) with the Power Sybr Green Master Mix (Applied Biosystems). A dilution series confirmed that there was no inhibition of the PCR for the undiluted samples (data not shown). The R^2 of all standard curves was above 0.99, and the efficiencies of the amplification were as follows: 99.21% 16*S* rRNA gene, 75.55% *hzsB*, 94.33% *nirK*, and 117.51% *nirS*.

Results

Water column stratification and its influence on the redox zonation

Previous field investigations using stable water isotope compositions have shown that Fohnsee is almost exclusively fed by groundwater, which has a strong effect on its mixing type (Braig et al. 2010). Renewal of DO in the water column is therefore accomplished due to wind-induced mixing and diffusion. However, marked increases in the temperatures of the near-surface layers during spring result in a sharp density gradient between the warm epilimnion and the cold hypolimnion, which acts as a barrier against wind-mixing between April and the end of September. As a result, oxygen replenishment from the atmosphere depends almost exclusively on diffusion of DO across the thermocline (Fig. 1).

In addition, a vertical downward flux of organic matter due to spring algal blooms further increases the DO demand in the hypolimnion and may facilitate elevated rates of benthic bacterial sulfate reduction causing a release of sulfide to the

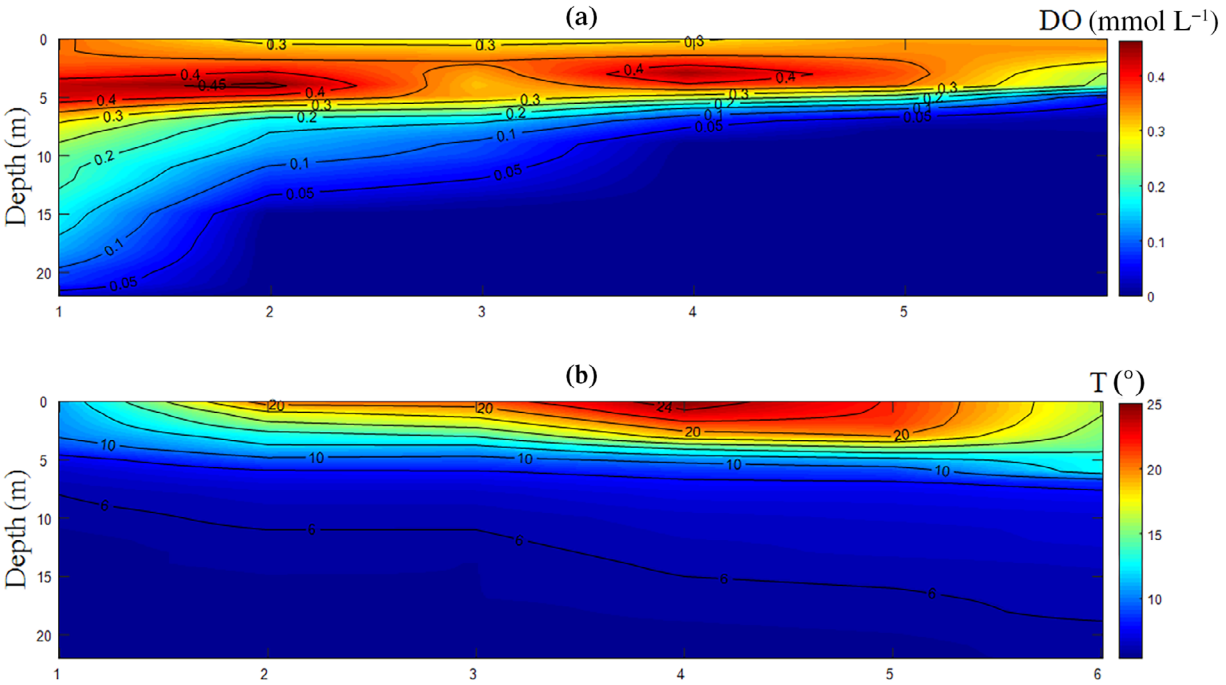

Fig. 1. Temperature and DO development from April to September in the water column of Fohnsee.

overlying water column in late summer (Callbeck et al. 2021). The DO consumption rate in deeper layers of the lake is higher than the rate at which oxygen is renewed in the hypolimnion by diffusion, thus, significant depletion of DO occurs during the summer period. In May, strictly anoxic conditions (DO < 0.3 μmol L^{-1}) were observed below 19 m depth with a constant water temperature of 5.5°C (Fig. 1). Subsequently, the strictly anoxic zone expanded upward in the water column increasing toward a depth of 9.5 m in September. DO concentrations at the lake surface were constantly around 0.3 mmol L^{-1} with maximum concentrations of 0.42 mmol L^{-1} found immediately below the thermocline indicating that the epilimnion remained oxic throughout the observation period.

Depth-profiles of concentrations of NO_3^- and SO_4^{2-}, and N and O isotope ratios of NO_3^-

At the beginning of the stratification period in April, constant concentrations of SO_4^{2-} and NO_3^- of ~ 0.1 mmol L^{-1} were observed throughout the entire water column, with the exception of the anoxic zone between 21 and 23 m depth, where nitrate concentrations decreased to around 0.06 mmol L^{-1} (Fig. 2a). $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ did not show any significant changes throughout the water column with average values of 7.0‰ and 2.6‰, respectively (Fig. 2a).

At the beginning of June, nitrate concentrations significantly decreased from 0.1 mmol L^{-1} at 17 m depth to 0.02 mmol L^{-1} at 23 m depth, while in the same zone $\delta^{15}N$ and $\delta^{18}O$ values increased from 7.2‰ to 15.4‰ and from 1.7‰ to 10.0‰, respectively (Fig. 2b). At the end of June, NO_3^- concentrations at 23 m depth declined to 0.01 mmol L^{-1}, while at the same time $\delta^{15}N$ and $\delta^{18}O$ values increased to 34.0‰ and 12.3‰ (22 m), respectively (Fig. 2c). Sulfate concentrations during these months remained relatively constant.

In July, anoxic conditions were observed below 12 m depth and nitrate concentrations decreased from 0.1 mmol L^{-1} at 12 m depth to concentrations below the detection limit at 21 m depth. The $\delta^{15}N$ value of NO_3^- at 21 m was 25.5‰ and the $\delta^{18}O$ value of NO_3^- was 13.8‰ (Fig. 2d). Measurement of the isotopic composition of nitrate below this depth was not possible due to the low NO_3^- concentrations. In July, sulfate concentrations remained constant around 0.1 mmol L^{-1} in the water column from the lake water surface to 21 m depth.

At the depths from 21 to 23 m where nitrate was completely reduced and a decrease of SO_4^{2-} concentrations from 0.1 to 0.071 mmol L^{-1} was observed (Fig. 2d).

During the following 2 months of August and September, NO_3^- concentrations continuously decreased in the hypolimnion while both $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ of the remaining nitrate progressively increased (Fig. 2e,f). During the final sampling campaign in September, a nitrate free zone was observed below 19 m depth. This depth coincided with the maximum $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ values of 41.0‰ and 27.4‰, respectively (Fig. 2f). In the nitrate-free zone from 19 to 23 m depth, sulfate concentrations decreased significantly from 0.09 to 0.05 mmol L^{-1} at the lake bottom and H_2S was detected with a maximum concentration of 0.02 mmol L^{-1} observed at 23 m depth (Fig. 2f). This reveals that toward the end of the observation period, a zone with bacterial (dissimilatory) sulfate reduction had been established in the lowest section of the water column, representing a different redox zonation pattern compared to spring.

At the beginning of the lake stratification period in June, the nitrate-reduction zone was located close to the lake bottom between a water depth of 18 and 22 m. Following the upward movement of the oxycline indicated by the dashed line in Fig. 2 in subsequent months, the zone where nitrate was reduced moved upward reaching a depth between 13 and 20 m in July. At the end of the sampling period in September, the NO_3^--reduction zone was located between 14 and 19 m depth.

Concentrations and stable isotope composition of ammonium

At the beginning of the observation period in April, ammonium concentrations were highest near the lake bottom with 0.038 mmol L^{-1} accompanied by a $\delta^{15}N\text{-}NH_4^+$ value near 14‰. NH_4^+ concentrations decreased toward the lake surface to 0.012 mmol L^{-1} at 8 m, while $\delta^{15}N\text{-}NH_4^+$ increased to 18‰ at a water depth of 20 m (Fig. 3a). Ammonium was below detection from 0 to 8 m depth.

Figure 3b,c reveals that ammonium concentrations in June decreased from 0.063 mmol L^{-1} at the lake bottom (23 m) to 0.035 mmol L^{-1} at the oxycline (18 m). Simultaneously, $\delta^{15}N\text{-}NH_4^+$ increased from 14.7‰ at the lake bottom to 27.5‰ below the oxycline (18 m). $\delta^{15}N\text{-}NH_4^+$ values indicated that NH_4^+ in the oxic zone was markedly enriched in ^{15}N with an average $\delta^{15}N\text{-}NH_4^+$ value of 37.3‰ (Fig. 3b) compared to ammonium found in the anoxic zone. From the end of June to September 2019, maximum ammonium concentrations at the lake bottom of around 0.085 mmol L^{-1} were observed, which decreased toward the oxycline to concentrations below the detection limit (Fig. 3c–f). The decrease in ammonium concentrations from the lake bottom toward the oxic zone was accompanied by an increase in $\delta^{15}N\text{-}NH_4^+$ values of up to 12‰.

The increase in $\delta^{15}N\text{-}NH_4^+$ that was observed in the anoxic zone of the water column was most pronounced at the end of June (13.6‰ at 23 m to 25.1‰ at 17 m depth) and least pronounced in September (10.2‰ at 23 m to 15.1‰ at 10 m depth). In August, $\delta^{15}N\text{-}NH_4^+$ values remained constant throughout the hypolimnion with an average of 15.6‰, except for the water depth from 23 to 20 m depth where $\delta^{15}N$ of NH_4^+ varied by 3‰ (Fig. 3e). From June to September, the NH_4^+ concentration in the oxic zone was consistently below or very close to the detection limit and therefore measurement of $\delta^{15}N\text{-}NH_4^+$ values was not possible.

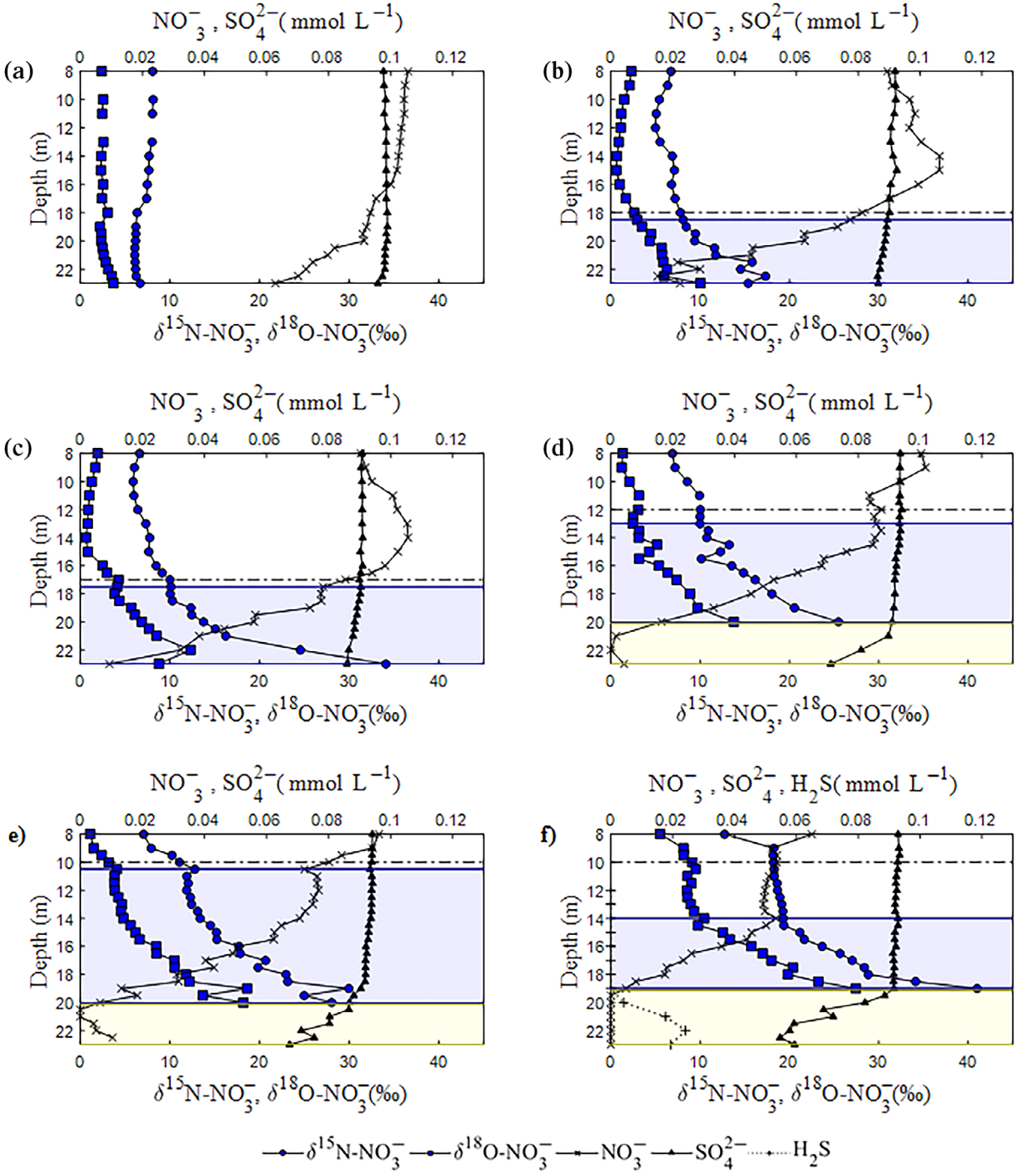

Fig. 2. Depth profiles of nitrate (crosses) and sulfate (triangles) concentrations, and $\delta^{15}N$ (filled circles) and $\delta^{18}O$ values (filled squares) of nitrate for April (**a**), 05 June (**b**), 18 June (**c**), July (**d**), August (**e**), and September (**f**) 2019 at Fohnsee. Horizontal dashed lines represent the depth below which DO was below detection (< 0.3 μmol L^{-1}) and blue boxes highlight the potential denitrification zone below the oxycline based on isotope and chemical profiles. Light yellow boxes highlight the potential bacterial sulfate-reduction zone.

qPCR and microbial abundance for anammox and denitrifying bacteria

The number of bacterial 16*S* rRNA gene copies indicated that bacterial abundance in the water column remained relatively constant throughout the water column in depth and time, independent of changes in redox conditions. The copy numbers ranged in the magnitude of 10^7 copies mL^{-1}, except for some samples from 6, 10, and 14 m depth, where the copy numbers were slightly reduced.

Both nitrite reductase genes, *nirK* and *nirS*, were identified in the water column (Fig. 4). In the oxic zones, bacteria harboring the *nirS* gene was less abundant than their counterpart

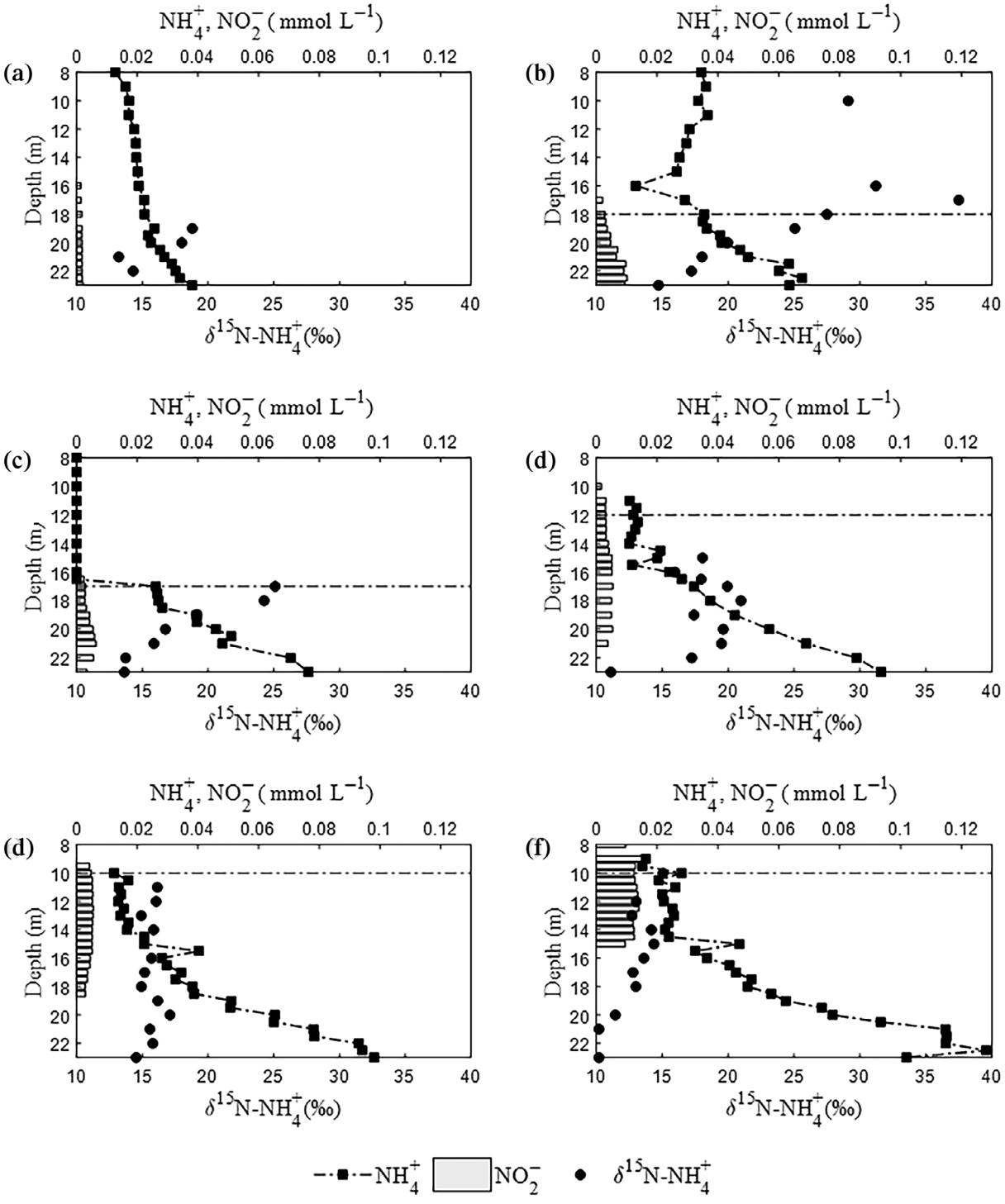

Fig. 3. Depth profiles of ammonium (squares) and nitrite concentrations (bars), and δ^{15}N values of ammonium (circles) for April (**a**), beginning of June (**b**), end of June (**c**), July (**d**), August (**e**), and September (**f**) 2019 at Lake Fohnsee. The horizontal dashed line represents the depth below which DO was below detection (< 0.3 μmol L^{-1}).

carrying the *nirK* gene (Fig. 4a). In the anoxic zones, both groups contributed equally to the overall abundance of nitrite reducers, which was mostly related to a significant increase of bacteria harboring the *nirS* gene. In the anoxic zone, the abundance for *nirK* and *nirS* genes was for both genes in the range of 10^5 copies mL^{-1}. Overall, the dynamics of nitrite reducers

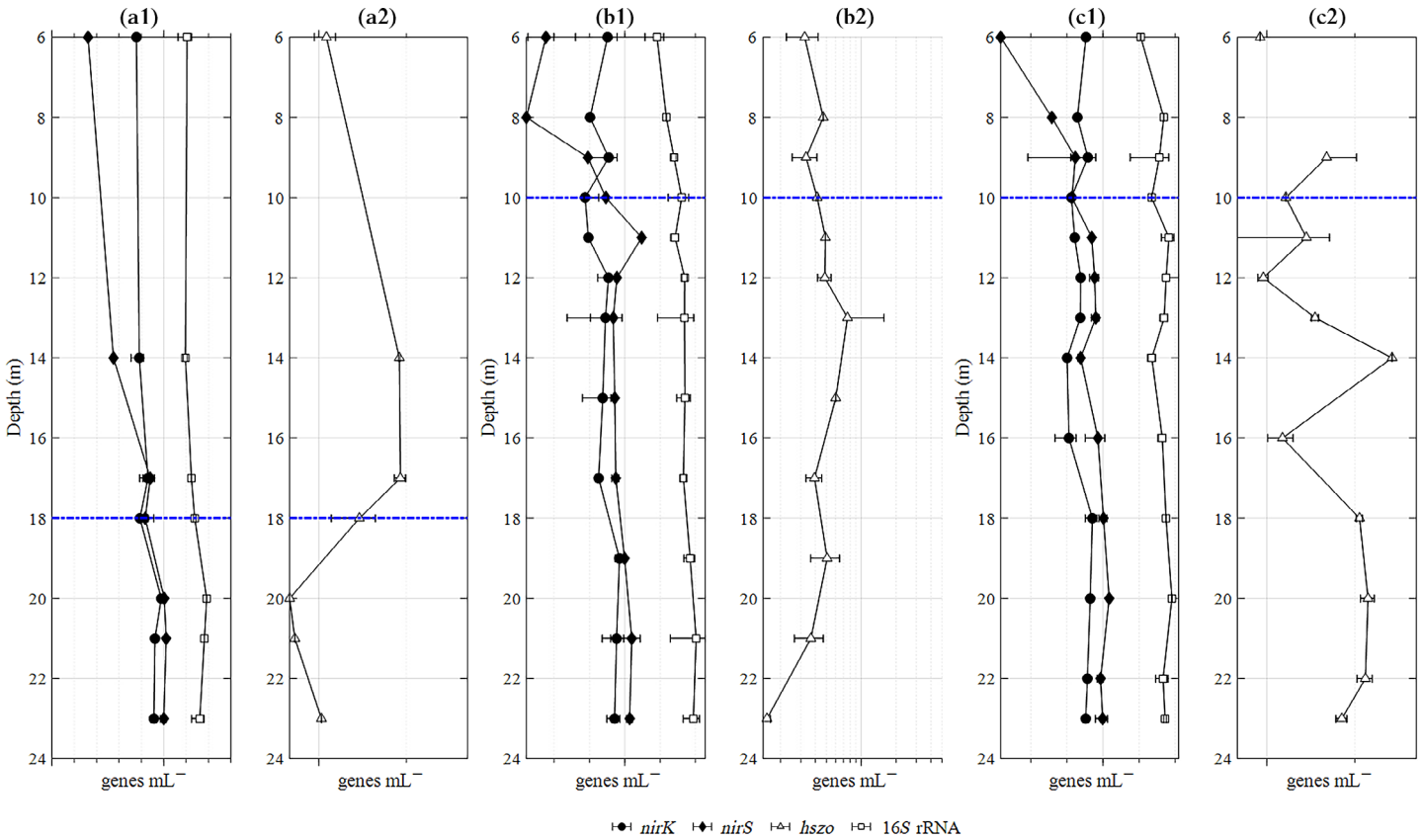

Fig. 4. Depth profile of number of gene copies *hszB*, *nirK*, *nirS*, and 16*S* rRNA for (**a**) June, (**b**) August, and (**c**) September, the dotted blue line shows the depth at where anoxic conditions were observed (DO < 0.3 μmol L^{-1}).

followed the dynamics of the oxycline over the season, resulting in an increase in the upper water layers from April to August. In August, a significant increase of nitrite reducers was observed in 10 m depth, whereas in April this increase was only detectable in 18 m. We did not observe any significant temporal or spatial changes in the number *hzsB* genes, that is, changes between the anoxic and oxic water column. Furthermore, the number of *hzsB* gene copies was considerably lower than *nirK* copies. In most of the analyzed samples, we observed only around 10^1 copies mL^{-1} or less of *hzsB* genes with exception of a sample taken in 14 m depth in September, and 14 and 17 m in June, where the magnitude of number of gene copies was close to 10^2 copies mL^{-1}. Overall, anammox communities were in average 10^4 times less abundant than denitrifying communities.

Discussion

Nitrogen-transformation processes revealed by shifts in $\delta^{15}N$ and $\delta^{18}O$ of nitrate

During predominantly oxic conditions in April, concentrations and $\delta^{15}N$ and $\delta^{18}O$ values (~ 7‰ and 5‰, respectively) of nitrate were relatively constant throughout the entire water depth at Fohnsee as a result of mixing following the spring overturn event. Nitrate reduction during this month occurs predominantly via by benthic denitrification due to elevated O_2 concentrations in the water column. In subsequent months, the depletion of oxygen in the water column observed during the stratification of the lake from June to September resulted in conditions that are thermodynamically favorable for the microbial reduction of nitrate. In the absence of molecular oxygen (DO < 0.3 μmol L^{-1}), concentrations of nitrate decreased from the oxycline toward the bottom of the lake throughout June to September, while $\delta^{15}N$ values of nitrate NO_3^- increased from 7.0‰ to 42.2‰ and $\delta^{18}O$ increased from 5‰ to 28‰ (Fig. 2).

During nitrate reduction, molecules containing the lighter isotopes (^{14}N and ^{16}O) are generally consumed by microorganisms at higher rates than those containing the heavier isotopes (^{15}N and ^{18}O), leaving the remaining nitrate in water enriched in ^{15}N and ^{18}O (Blackmer and Bremner 1977; Böttcher et al. 1990). In addition, nitrite concentrations peaked (up to 0.017 mmol) in the anoxic zone near the lake bottom where the highest $\delta^{15}N$ values of nitrate were observed, suggesting that nitrate is reduced to nitrite (Ge et al. 2012; Wang and Li 2015; Chen et al. 2017). Therefore, the observed chemical and isotopic data in the anoxic zone near the lake bottom suggest that some nitrate is reduced to nitrite in lake water

column. This interpretation is supported by two previous studies (Einsiedl et al. 2020; Peña Sanchez et al. 2022) at Fohnsee that demonstrated using isotopic fingerprinting of NO_3^-, NO_2^-, and methane combined with modeling that denitrification occurred within the water column coupled with methane oxidation as electron donor.

The data patterns showed that the nitrate-reduction zone was located near the lake bottom at the beginning of the stratification period and moved upward following the seasonal vertical displacement of the oxycline.

Additional insights on nitrogen transformations processes can be derived from a dual isotope plot of $\delta^{18}O$ vs. $\delta^{15}N$ of nitrate. Figure 5 reveals that increasing $\delta^{15}N$ and $\delta^{18}O$ values with decreasing nitrate concentrations plot on straight lines with slopes between 0.66 and 0.96 in all 5 months with an anoxic water column which is indicative of denitrification (Böttcher et al. 1990; Aravena and Robertson 1998; Wunderlich et al. 2012). However, it is known that isotope exchange processes between water oxygen and nitrite oxygen (Buchwald and Casciotti 2010; Wunderlich et al. 2012) and inverse isotope effects of N during anammox (Brunner et al. 2013) can occur and it cannot be exclude that the isotopic composition of nitrate is additionally affected by other N transformations, such as nitrate reduction to nitrite coupled to anammox and DNRA. Nonetheless, the presented chemical and isotopic data strongly suggest that nitrate reduction to nitrite and subsequently to NO and N_2 is a key N-removal process at lake Fohnsee once reducing conditions are established starting at the beginning of June, similar to many other studies that have identified denitrification as the dominant N-removal process (i.e., Bulow et al. 2010).

Nitrogen-transformation processes revealed by shifts in $\delta^{15}N$ of ammonium

Ammonium is frequently formed as a result of microbial degradation of nitrogen-containing organic matter in lake sediments (Robertson and Groffman 2007). The observed elevated ammonium concentrations within the oxic water column of Fohnsee in April (Fig. 3a) are likely the result of entrainment of the benthic ammonium into the lake water during the lake turnover in spring and the limited aerobic ammonium oxidation as a result of the low temperatures in the water column. Also, the dilution of the ammonium oxidizing organisms delaying nitrification in the water column for weeks to months has been reported as a cause of limited rates of nitrification in stratified lakes (Haas et al. 2021). Several field studies have shown that nitrification rates drop with the decrease in water temperature (Quinlan 1986; Shammas 1986; Holloway and Lyberatos 1990; Saad and Conrad 1993; Grundmann et al. 1995), where growth of nitrifying bacteria is strongly inhibited at temperatures < 10°C. Temperatures in the water column of lake Fohnsee throughout April range between 5.4°C at the bottom and 10.8°C at the lake surface; thus, nitrification might have been limited during this month, contributing to ammonium accumulation in April. During the sampling campaigns from June to September, the highest ammonium concentrations of ~ 0.09 mmol L^{-1} were always observed near the lake bottom close to the sediment–water interface, while ammonium concentrations decreased toward the oxycline to < 0.02 mmol L^{-1}. This suggests that ammonium is released from the lake sediments at Fohnsee with $\delta^{15}N$ values between 11‰ and 14‰.

Ammonium diffusing from the lake sediment into the water column can be consumed by nitrification, ammonium assimilation, and/or by anammox bacteria with NO_2^- as electron acceptor (Schubert et al. 2006; Roland et al. 2018). Microorganisms performing aerobic and anaerobic oxidation of ammonium preferentially metabolize ^{14}N resulting in an enrichment of ^{15}N in the remaining ammonium as concentrations decrease (Granger et al. 2008; Karsh et al. 2012; Kritee et al. 2012). Thus, the observed trend of $\delta^{15}N\text{-}NH_4^+$ increasing from 5‰ to 15‰ in the anaerobic water column from the lake bottom to the oxycline between June and September (Fig. 3b,c,f) is consistent with microbially driven anaerobic ammonium oxidation. However, some nitrification might also occur at very low rates in anoxic waters due to O_2 production by phototrophic organisms, a process that could also contribute to the observed ^{15}N enrichment in ammonium (Callbeck et al. 2021). In the oxic zone, decomposition of N-containing organic matter and ammonification produce NH_4^+ that is usually rapidly converted to nitrate due to nitrification (Casciotti et al. 2011). Since both processes preferentially metabolize ^{14}N to ammonium and subsequently to nitrate (Mariotti et al. 1981), the remaining ammonium and nitrate become progressively enriched in ^{15}N as observed in our study (Fig. 3a, b). Nitrogen isotope enrichment factors for nitrification in aquatic systems have been reported in the range of 17–19‰ (Lehmann et al. 2004). Such marked N isotope enrichment is very similar to the $_{NH_4}\square_{\times}$ observed for aerobic conditions in our study for samples obtained in April (*see* Supplementary Information D).

Ammonium in the water column of Fohnsee can also be produced by decomposition of plankton in the lake or can stem from vertical fluxes of suspended inorganic particles coated with N sinking through the water column (Wakeham and Lee 1993; Smemo and Yavitt 2007). N coated on organic carbon or inorganic particles may also lead to the observed high shift of $\delta^{15}N$ values of ammonium (15–37‰) at Fohnsee. The NH_4^+ remaining in the oxic zone with elevated $\delta^{15}N$ will mix with newly formed NH_4^+ released from the sediments in the anoxic zone below the oxycline with much lower $\delta^{15}N$ values resulting in a mixing trend that could also be interpreted as an isotope enrichment of ^{15}N in remaining NH_4 from the bottom to the top of the anoxic lake water column that could be interpreted as anaerobic ammonium oxidation. Thus, although hydrochemical and stabile isotope profiles suggest that oxidation of ammonium under anaerobic conditions is taking place, the data are ambiguous regarding possible

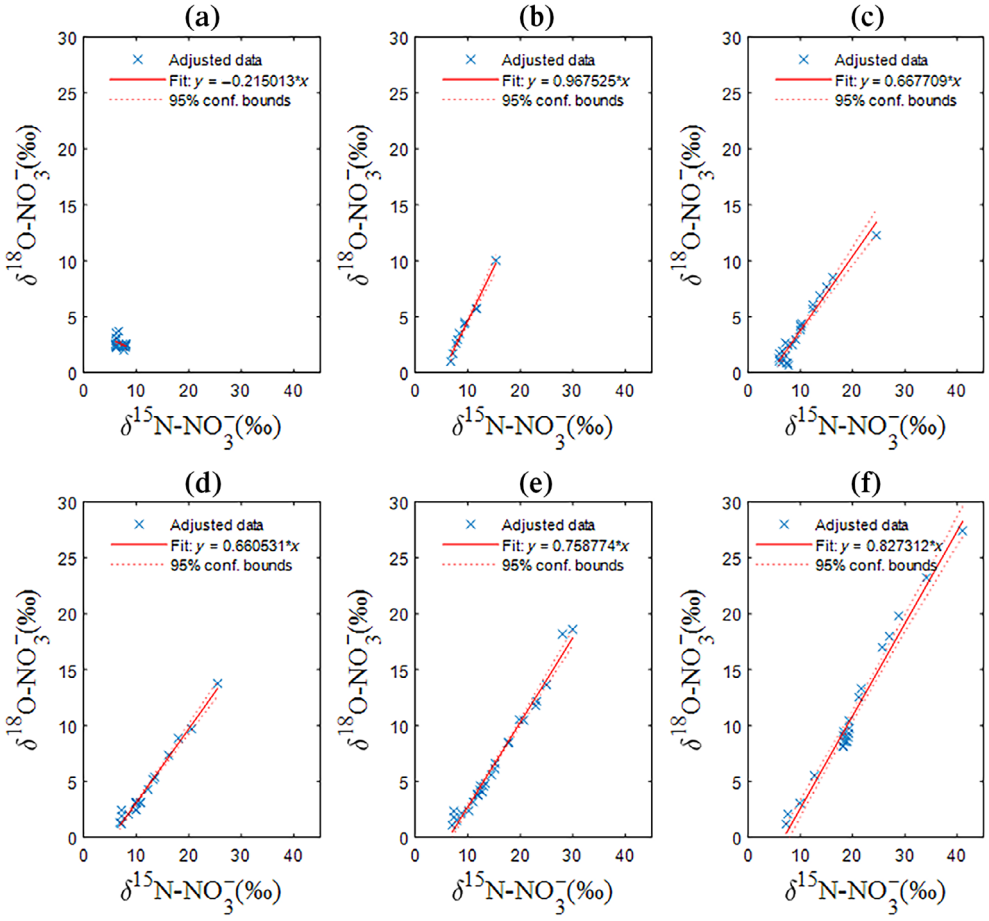

Fig. 5. Linear trajectories of $\delta^{15}N$ vs. $\delta^{18}O$ of nitrate for (**a**) April, (**b**) beginning of June, (**c**) end of June, (**d**) July, (**e**) August, and (**f**) September. Adjusted data refers to the data points included in the fit and the 95% confidence bounds.

processes that could also lead to the observed trends in $\delta^{15}N$-NH_4^+ throughout the water column. For example, the increase in $\delta^{15}N$-NH_4 might be also the result of mixing between the ammonium formed by organic matter decomposition in the lake sediments of the anoxic hypolimnion with a $\delta^{15}N$-NH_4^+ value of ~ 11‰, and the ^{15}N enriched ammonium remaining from ammonification and nitrification in the oxic zone with a $\delta^{15}N$-NH_4^+ value of up to 37‰. To test if this mixing hypothesis and only physical transport could theoretically describe the observed trends in $\delta^{15}N$ of ammonium and the concentration depth profiles in the water column respectively, a numerical model was developed for $\delta^{15}N$-NH_4 values and ammonium concentrations.

Modeling of diffusion-controlled transport of ammonium and $\delta^{15}N$ values of NH_4^+

Ammonium concentrations simulated across the water column of Fohnsee using the diffusion model (Eq. 3) with the initial ammonium concentration and calculated K_z values showed a very good fit between the measured and observed NH_4^+ concentration depth profiles. The numerical simulations based on upward diffusion of sediment-derived ammonium explain more than 90% of the observations made in the field during the months of June, July, August, and September (Fig. 6).

Diffusion-reaction models have been used in other studies to explain concentrations of N-containing dissolved species throughout the water column (Lehmann et al. 2003) and to calculate reaction rates by inverse modeling using the consumption rate (R) or the decay constant (k) as fitting parameters (Wenk et al. 2014). Our results revealed, however, that the observed decrease in the ammonium concentrations from the sediment–water interface in the hypolimnion to the oxycline at Fohnsee can be mostly explained by diffusive transport alone ($R^2 > 0.9$), requiring only minor contributions from ammonium consumption processes such as anammox.

Additional simulations performed with ammonium consumptions rates observed for anammox in other studies in lake water columns (Lake Tanganyika: 240 nmol N_2 L^{-1} d^{-1} [Schubert et al. 2006], Lake Rassnitzer: 504 nmol N_2 L^{-1} d^{-1} [Robert Hamersley et al. 2009]) and water columns in marine

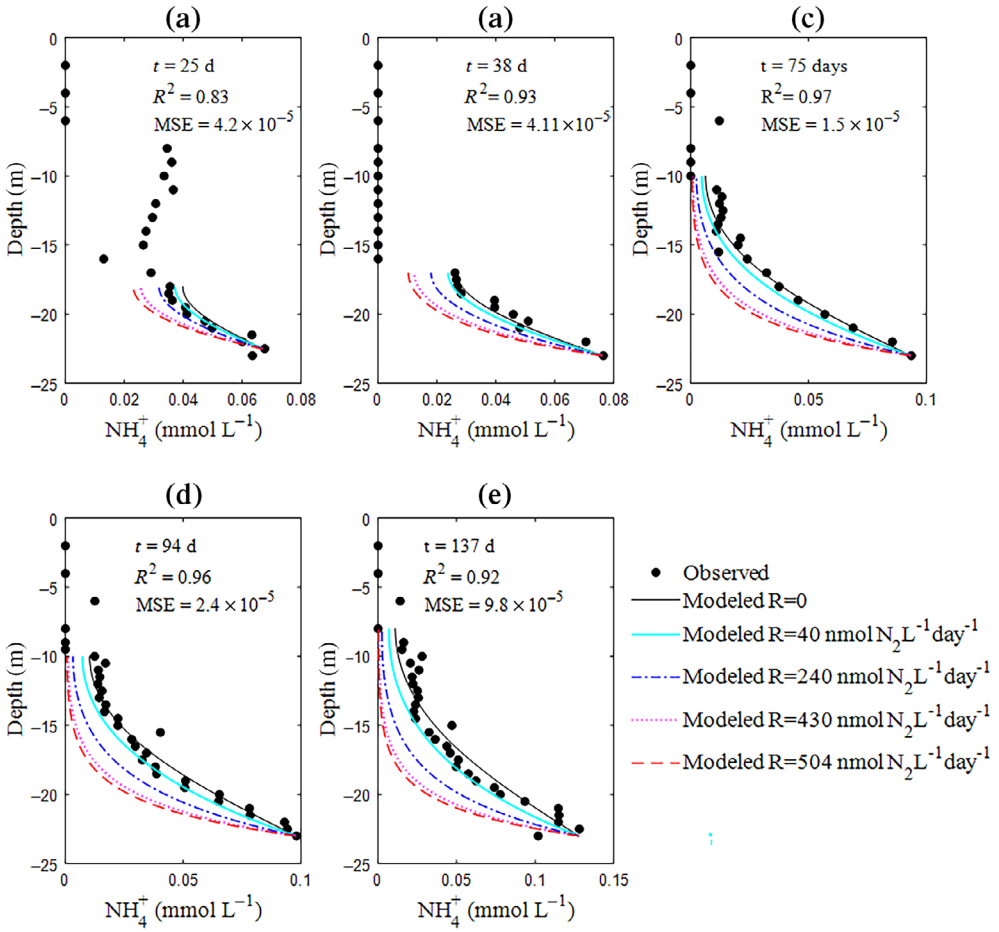

Fig. 6. Simulated and measured ammonium concentrations vs. depth for (**a**) June first sampling, (**b**) June second sampling, (**c**) July, (**d**) August, and (**e**) September. Statistics presented in the figure correspond to fit for this study.

environments (430 nmol N_2 L^{-1} d^{-1}; Dalsgaard et al. 2003; Kuypers et al. 2005; Thamdrup et al. 2006), resulted in concentration profiles which did not match our observed data (Fig. 6). In contrast, simulations performed with anammox rates similar to the ones observed for Lake Lugano (~ 40 nmol N_2 L^{-1} d^{-1}; Wenk et al. 2014) resulted in plausible NH_4^+ concentration profiles matching the observed data, but with a lower fit than simulations performed without anammox ($R^2 > 0.9$ for anammox rates = 0 nmol N_2 L^{-1} d^{-1}, in comparison with $R^2 > 0.8$ for simulations with anammox rates of ~ 40 nmol N_2 L^{-1} d^{-1}). The exception was the month of June (*see* Fig. 6a,b) where the model fit was slightly better or similar for simulations with low anammox rates (R^2 = 0.83 and 0.93 for rates = 0 nmol N_2 L^{-1} d^{-1}, and R^2 = 0.89 and 0.93 for simulations with anammox rates of ~ 40 nmol N_2 L^{-1} d^{-1}). Thus, although NH_4^+ concentrations in the anoxic part of the water column are predominantly caused by diffusion, modeling results suggest that anaerobic oxidation of NH_4^+ likely also occurs especially in June at rates of ~ 40 nmol N_2 L^{-1} d^{-1}. This interpretation is consistent with a previous isotope study and microbiological investigations at Fohnsee that revealed the occurrence of anammox within the water column in the summer period (Einsiedl et al. 2020).

Effect of mixing processes and movement of the oxycline on δ^{15}N-NH_4^+ values

Interpretation of stable isotope data requires a thorough understanding of all biotic and hydrodynamic (mixing, advection, and diffusion) processes influencing the δ^{15}N-NH_4^+ pool in the water column. We have expanded the diffusion-model shown in Eq. 2 to account for both isotope species of ammonium, ^{14}N and ^{15}N. The simulated scenario considers the δ^{15}N-NH_4^+ in the oxic water column in April, a constant NH_4^+ influx from the sediment, and the upward movement of the oxycline toward the lake surface throughout the spring and summer observation period. The model was able to simulate the δ^{15}N-NH_4^+ values measured in the water column with a very good fit with the measured data for June and September ($R^2 > 0.8$) and a satisfactory fit for August ($R^2 > 0.6$; Fig. 7).

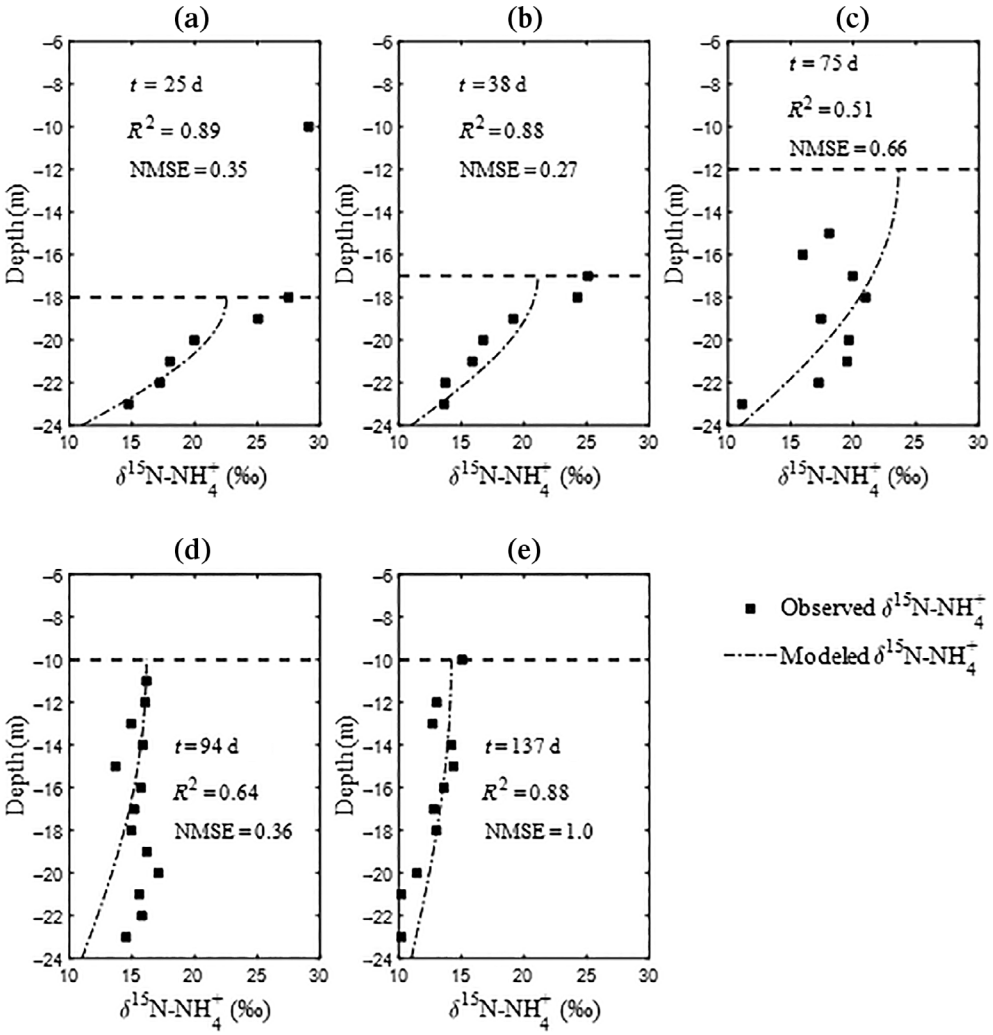

Fig. 7. Simulated and measured $\delta^{15}N$ of ammonium vs. depth for (**a**) June first sampling, (**b**) June second sampling, (**c**) July, (**d**) August, and (**e**) September. Normalized mean square error (NMSE).

At the beginning of the stratification period in April, the oxycline was located close to the lake bottom (Fig. 3a). Ammonium diffusing from the lake sediment into the overlying oxic water column in April was likely mostly oxidized by nitrifying bacteria to dissolved nitrate. Nitrifying bacteria consume ^{14}N at a higher rate than ^{15}N with a kinetic N isotope effect between 14.2‰ and 38.2‰ (Mariotti et al. 1981; Casciotti et al. 2003), leaving the remaining NH_4^+ enriched in ^{15}N resulting in progressively increasing $\delta^{15}N\text{-}NH_4^+$ values. This is consistent with the observed high $\delta^{15}N\text{-}NH_4^+$ values of $\sim +20$‰ at 20 m depth (Fig. 3a) resulting from aerobic oxidation of ammonium to nitrate in the water column under oxic conditions in April.

At the beginning of June, anoxic conditions were observed below 18 m depth (Fig. 7a). $\delta^{15}N\text{-}NH_4^+$ values near the lake water sediment interface were ~ 15‰ and trended to higher values of up to 27‰ below the oxycline. A strong increase of $\delta^{15}N\text{-}NH_4^+$ of ammonium was also observed at the end of June in the anoxic water column from 14‰ near the lake bottom toward 26‰ at the oxycline (Fig. 7b). Our model reveals that the N isotope shift of ammonium observed in the anaerobic water column appears to be result of mixing between the previously ^{15}N enriched NH_4^+ in the oxic water column resulting from nitrification when oxic conditions prevailed, and the newly formed NH_4^+ in the anoxic sediments ($\delta^{15}N\text{-}NH_4^+$ of ~ 11‰) induced by the upward displacement of the oxycline.

As the oxycline moved upward during the observation period throughout the summer, nitrification became progressively limited in the upper 10 m depth of the water column and the remaining NH_4^+ enriched in ^{15}N became a less dominant end member for mixing. Consequently, influx of ammonium with $\delta^{15}N\text{-}NH_4^+$ values near 11‰ from the anoxic lake sediments into the overlying water column resulted in a shift to less positive $\delta^{15}N\text{-}NH_4^+$ values during August and September (Fig. 7d,e).

Since ammonium from different sources with distinct $\delta^{15}N$ values appears to mix within the water column of Fohnsee, the model results suggest that the trends of increasing $\delta^{15}N$-NH_4^+ values with decreasing ammonium concentrations observed in the anoxic water column can be predominantly explained by mixing and diffusion, although anaerobic oxidation of NH_4^+ can also play a minor role.

It is interesting to note that the $\delta^{15}N$-NH_4^+ values predicted by our model for the anoxic water column between 23 and 20 m in August are lower than the $\delta^{15}N$-NH_4^+ values that were observed in the water column in July and August (Fig. 7c,d). In these months, we observed a significant decrease of sulfate concentrations in lower part of the hypolimnion (Fig. 2d,e) and the occurrence of H_2S providing evidence that bacterial sulfate reduction has occurred in the deepest section of the water column after nitrate was completely consumed. The H_2S produced by bacterial sulfate reduction near the sediment–water interface at Fohnsee may serve as an electron donor for chemolithotrophic denitrification (Brunet and Garcia-Gil 1996) in the water column layers above the sulfate reducing zone. Therefore, it is possible that ammonium could be formed as product during the oxidation of H_2S using nitrate as electron acceptor during DNRA. During this process, NO_3^- is reduced to NO_2^-, and subsequently converted to NH_4^+ (Tiedje 1988). The formed NO_2^-, and thus, the subsequently produced NH_4^+ are both enriched in ^{14}N in comparison with the source of NO_3^-. However, if the initial NO_3^- is highly enriched in ^{15}N as observed in the water column of Fohnsee in August ($\delta^{15}N$-NO_3^- 20‰; Fig. 2e), the newly formed NH_4^+ resulting from DNRA may have higher $\delta^{15}N$ values than the NH_4^+ released from the sediments (11‰). Thus, the $\delta^{15}N$-NH_4^+ that is formed by DNRA might have the potential to shift the $\delta^{15}N$ of ammonium to values between 11 and 17‰, as observed near the lake bottom in August (Fig. 7d).

Denitrifying and anammox bacteria abundances in the water column

Gene abundances of *nirK* and *nirS* of up to 1×10^5 copies mL^{-1} in the denitrification zone strongly suggested the presence of denitrifying bacteria in the water column of Fohnsee. *Nirk* and *nirS* are responsible for the catalysis of nitric oxide from nitrite, which is a key step during denitrification (Jones et al. 2008). Thus, primers targeting these specific genes provide a quantitative measure for the abundance of denitrifying communities in the water column (Wei et al. 2015). Denitrification genes abundance of the same magnitude have been reported for other permanently and seasonally stratified lakes were denitrification has occurred (Wenk et al. 2013; Pajares et al. 2017).

In June, *nirK* and *nirS* peaked at a water depth of 17–18 m and remained constant toward the lake bottom. This zone also coincided with the zone where we observed strong ^{15}N and ^{18}O enrichment in the remaining nitrate (Fig. 5) suggesting microbially driven NO_3^- reduction to NO_2^-. In addition, we observed an increase in the denitrifying bacterial abundance in the water layers when aerobic conditions changed to anaerobic conditions (Fig. 4a1 in 14 m to Fig. 4b1 in 14 m), strongly indicating the growth of bacterial species carrying *nirK* and *nirS* genes. Although abundance of the genes *nirK* and *nirS* alone demonstrate only the potential for denitrification, several lines of complementary qualitative and quantitative evidence from chemical and isotopic data indicate that denitrification is taking place in the anoxic water column.

The abundance of the *hzsB* gene characteristic for anammox bacteria (Harhangi et al. 2012) reached values of 10^2 copies per mL^{-1} in the oxic–suboxic zone during the month of June (Fig. 4), where isotopic and water chemistry data also suggested that anammox might have occurred. In contrast, gene abundance for anammox was markedly lower (mostly $\sim 10^1$ copies per mL^{-1}) in samples collected in August. For comparison, the anammox bacteria abundance reported in the water column where anammox was inferred in the permanently stratified lakes Lugano, Tanganyika, and Lake Rassnitzer were as high as 10^4 copies per mL^{-1} (Schubert et al. 2006; Robert Hamersley et al. 2009; Wenk et al. 2013), which is up to 4 orders of magnitude higher compared to our study. The moderate (June) to low numbers of *hzsB* genes (August) observed are consistent with the modeled low anammox rates in June but would require further in situ measurements. Starting in late July, Fohnsee developed a sulfidic zone near the lake bottom where nitrate had been completely reduced and bacterial sulfate reduction had taken place producing H_2S (Fig. 2d–f). Thus, the presence of H_2S in the water column at Fohnsee might have inhibited the growth of anammox bacteria directly above the bacterial sulfate-reduction zone after the end of July. A decline in anammox activity in sulfidic waters has been previously observed in the anoxic basin of Golfo Dulce and in the central Baltic Sea (Dalsgaard et al. 2003; Hannig et al. 2007) and laboratory experiments performed in bioreactors also showed a decrease in anammox rates in the presence of H_2S (Russ et al. 2014). Anammox bacteria have very low doubling times (Kuenen 2008), and thus, they may be unable to adapt to fast changing environmental conditions. Changes in the redox conditions during stratification periods at Fohnsee and during turnover periods may limit microbial growth of anammox bacteria with more favorable conditions in June and July prior to onset of eth occurrence of H_2S in the deeper portions of the water column.

In September, anammox gene copies peaked at a water column depth of 14 m, 6 m above the sulfidic zone. This suggests that anammox might occur in the anoxic water column where sulfide is not present and fluxes of NH_4^+ and NO_2^- are sufficient to maintain anammox activity. This observation is comparable to a study conducted by Wenk et al. (2013), who observed that anammox bacteria peaked under nonsulfidic and suboxic conditions, above the sulfate-reduction zone in Lake Lugano.

Conclusions

We have investigated nitrogen-transformation processes at the seasonally stratified lake Fohnsee during the spring–summer stratification period using water chemistry data, stable isotope parameters ($\delta^{15}N$ and $\delta^{18}O$ of nitrate, $\delta^{15}N$ of ammonium), modeling of concentrations and N isotope ratios of ammonium, and the abundance of marker genes *nirK*, *nirS*, and *hszB* for denitrification and anammox, respectively.

Water chemistry data, the isotopic composition of nitrate, and qPCR data showed that nitrate reduction to nitrite and NO and subsequently to N_2 was a dominant N-removal process once a suboxic zone developed below the oxycline. Concentration and isotope trends for NH_4^+ were dominated by mixing and diffusive processes in the water column. Additional isotope effects likely occurred due to the activity of anammox bacteria, the consumption of NH_4^+ due to nitrification at nanomolar O_2 levels and ammonium assimilation, and the occurrence of ammonium production during DNRA, but were most likely masked by the dominant mixing and diffusion processes in the water column. Modeling and qPCR results suggested that nitrate reduction to nitrite and NO and anammox have the potential to co-occur in the water column after thermal stratification during the month of June.

Bacterial sulfate reduction was observed from the end of July to September in the water column near the lake sediment. The presence of sulfide might have inhibited the activity of anammox bacteria in the water column immediately above the sulfate-reduction zone. But an increase in the gene number of anammox bacteria from August to September suggested that anammox bacteria may have been active in a limited zone below the oxycline where sulfide was not present. This study demonstrated that the combination of chemical, isotopic, modeling, and molecular microbiological approaches is suitable for revealing novel insights into N-transformation and N-removal processes in aquatic ecosystems such as seasonally stratified lakes.

Data availability statement

The data that support the findings of this study are available from the corresponding author [FE] upon reasonable request.

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Conflict of Interest

None declared.

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