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Denitrification, anaerobic ammonium oxidation, and dissimilatory nitrate reduction to ammonium in an East African Great Lake (Lake Kivu)

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Abstract

We investigated anaerobic nitrogen (N) cycling in the water column of Lake Kivu, a deep meromictic tropical lake in East Africa. Data were collected at one station in the Northern Basin and one in the Southern Basin, during two sampling campaigns (June 2011—dry season, and February 2012—rainy season). Short-term incubations of sulfide-free water with ^{15}N-labeled substrates revealed high potential denitrification and dissimilatory nitrate reduction to ammonium (DNRA) rates (up to 350 and 36 nmol N produced L^{-1} h^{-1}, respectively), while anaerobic ammonium oxidation (anammox) was lower (up to 3.3 nmol N produced L^{-1} h^{-1}). However, anammox rates were 15 nmol N produced L^{-1} h^{-1} when $^{15}NH_4^+$ was added at depths where NH_4^+ concentrations were very low (< 1 μmol L^{-1}). With the addition of 5 μmol L^{-1} of $^{15}NO_3^-$ and 10 μmol L^{-1} of H_2S, denitrification and anammox were stimulated in the Northern Basin, while the increase of DNRA rates was less notable. In the Southern Basin, the addition of H_2S decreased denitrification rates, probably because of competition with DNRA, which increased, while no effect was observed on anammox. This study puts into evidence the co-occurrence of denitrification, anammox and DNRA, for the first time in a great tropical lake, and underlines the spatial heterogeneity of these processes. Contrary to numerous reports in literature, we show that anammox can significantly occur in presence of H_2S, suggesting that the contribution of anammox in the N cycle may be underestimated.

As an element required for life, the availability of nitrogen (N) can limit biological growth and ecosystem productivity. N is cycled through the biosphere via a number of microbial-mediated processes including biological fixation of N_2 gas, nitrification, denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonium (DNRA). Human activities, notably the use of fertilizers and farming of N-fixing crops (e.g., soybeans), have disrupted the pre-Anthropocene N-cycle by increasing total global fixed N pool as well as nitrous oxide (N_2O) emissions to the atmosphere (IPCC 2013). N_2O is both a potent greenhouse gas and an ozone depleting agent, and its atmospheric concentrations had risen by 20% in 2011 compared to 1750 (IPCC 2013). Effective prediction and management of ecosystem responses to perturbations of the N-cycle requires detailed knowledge of the processes responsible for N-cycling and the ultimate removal of fixed N back to the atmosphere.

Three anaerobic metabolisms are responsible for nitrate (NO_3^-) or nitrite (NO_2^-) reduction: canonical denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory reduction of nitrate to ammonium (DNRA). These three processes have markedly different impacts on the N-cycle: denitrification and anammox lead to N_2 production and ecosystem N-loss, denitrification produces N_2O as an intermediate in the chemical reaction chain; DNRA leads to N retention as NH_4^+, promoting N recycling. Knowledge on the regulation of these competing processes is crucial for predicting, modeling, and managing the N-cycle.

Canonical denitrification is commonly observed in rivers, streams, estuarine and marine sediments, and in anoxic lake

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Additional Supporting Information may be found in the online version of this article.

waters (Seitzinger 1988). It can be heterotrophic, with organic matter as electron donor, or chemolithotrophic with iron, sulfur compounds, methane, or hydrogen as electron donors (Kirchman et al. 2008). While heterotrophic denitrification is generally assumed to operate ubiquitously in anoxic marine and freshwater sediments, observation of chemolithotrophic denitrification with H_2S come mostly from marine sediments (Jensen et al. 2009; Lavik et al. 2009; Canfield et al. 2010), and some marine oxygen minimum zones such as the Chilean upwelling system and the Baltic Sea (Canfield et al. 2010; Dalsgaard et al. 2013). Nevertheless, some studies suggest that chemolithotrophic denitrification could account for 25–40% of the N loss from streams, wetlands and lakes (Burgin and Hamilton 2008), and though direct measurements of the process exist (e.g., Lake Lugano; Wenk et al. 2013) they are to-date limited. While heterotrophic denitrification has been widely described, the co-occurrence and regulation of denitrification and other anaerobic metabolisms, such as anammox and DNRA, remains uncertain across freshwater and marine environments. Anammox is an autotrophic process, constituting a competitive advantage compared to heterotrophic denitrification (Hulth et al. 2005), and is known to be more oxygen tolerant (Kuypers et al. 2005; Jensen et al. 2008), allowing it to occur in a wider variety of environments. However, the efficiency of the process is reduced, since anammox bacteria have a slow growth rate (Jetten et al. 1998, 2001; Hulth et al. 2005). DNRA competes with denitrification for NO_3^-, with DNRA expected to dominate in environments with a high organic matter : fixed N ratio, whereas higher NO_3^- concentrations may favor denitrification (Kelso et al. 1997; Silver et al. 2001; Dong et al. 2011). As anaerobic processes, the three processes are regulated, in part, by O_2 concentration, as well as the availability of the different substrates (organic matter, NO_2^-, NO_3^-, NH_4^+, HS^-), both determining the competitive relationships.

Denitrification, anammox, and DNRA are all known to be enhanced at high temperatures (Saad and Conrad 1993; Van Hulle et al. 2010; Dong et al. 2011). Tropical lakes represent only a areal small fraction of lakes globally (Lewis 2000) but are characterized by high mean annual temperatures that may support a disproportionally large role in global biogeochemical cycling (Lewis 1987). The N-cycle in tropical freshwaters, and in particular in tropical great lakes, however, remains understudied. The co-occurrence of denitrification, anammox, and DNRA, and their regulation have been poorly examined. We investigated one of the East-African Great Lakes, Lake Kivu, located at the border between Rwanda and the Democratic Republic of Congo. Lake Kivu is characterized by deep waters rich in NH_4^+, carbon dioxide, and methane (Schmid et al. 2005). Compared to temperate lakes, tropic lakes exhibit minimal seasonality of their physico-chemical parameters. In Lake Kivu, the dry season (from June to September) is accompanied by a deeper mixing of the oxygenated surface waters (i.e., the mixolimnion), compared to the rainy season. During the rainy season, NO_3^- accumulates at the base of the mixed-layer, forming a nitrogenous zone (also described as a nitracline) (Llirós et al. 2010; Pasche et al. 2011) where N_2O accumulates (Roland et al. 2016), indicating active N-cycling. A zone of sulfate (SO_4^{2-}) reduction is present below the oxic-anoxic interface, leading to HS^- accumulation to concentrations up to 200 μmol L^{-1} in the anoxic waters (Pasche et al. 2011; Morana et al. 2016). Fluxes of HS^- from these deeper waters into the nitrogenous zone could support denitrification coupled to HS^- oxidation. Furthermore, as the deep waters of Lake Kivu are also rich in NH_4^+, fluxes of NH_4^+ into the nitrogenous zone could fuel anammox. To test these hypotheses, and bring new data and knowledge on N-cycling in tropical lakes, we conducted a suite of process rate measurements, as well as geochemical and microbiological analyses over two seasons and at two stations in Lake Kivu. Only the water column was sampled, since most anaerobic processes are expected to occur in the water column close to the oxic-anoxic interface where terminal electron acceptors are generated (NO_3^-, SO_4^{2-}, etc.). Indeed, the shores of Lake Kivu are very steep, consequently most of the lake is very deep (> 80% of lake has a depth > 200 m, average depth is 244 m). The sedimentary anaerobic processes other than methanogenesis are thus expected to be negligible and to mainly occur in the upper anoxic water column.

Material and methods

Sampling and physico-chemical parameters

Sampling campaigns were conducted during the dry season (June 2011) and rainy season (February 2012), at two stations; one in the Northern Basin (–1.72504°N, 29.23745°E) and one in the Southern Basin (–2.3374°N, 28.9775°E) (Fig. 1). The maximum depths at the sampled stations are 250 m and 120 m in the Northern and Southern Basins, respectively.

Water column was sampled with a vertical 7 L Niskin-type bottle (Hydro-Bios) at 5 m intervals from the surface to 80 m for chemical water analyses. For N stable isotope labeling experiments, water was collected with a higher spatial resolution (at 2.5 m intervals) in a 10 m zone, located 5 m above and 5 m below the oxic–anoxic interface.

Vertical profiles of temperature, conductivity, pH, and oxygen were obtained with a Yellow Springs Instrument 6600 V2 multiparameter probe. The pH probe malfunctioned in June 2011. Therefore, the pH data presented for this campaign were measured with a portable pH meter and a Metrohm pH electrode on water sampled from the Niskin bottles.

N stable isotope labeling experiments

Water was collected in duplicate in amber 250 mL borosilicate bottles from the Niskin bottle with tubing, left to overflow three times the bottle volume, and sealed with Teflon-coated

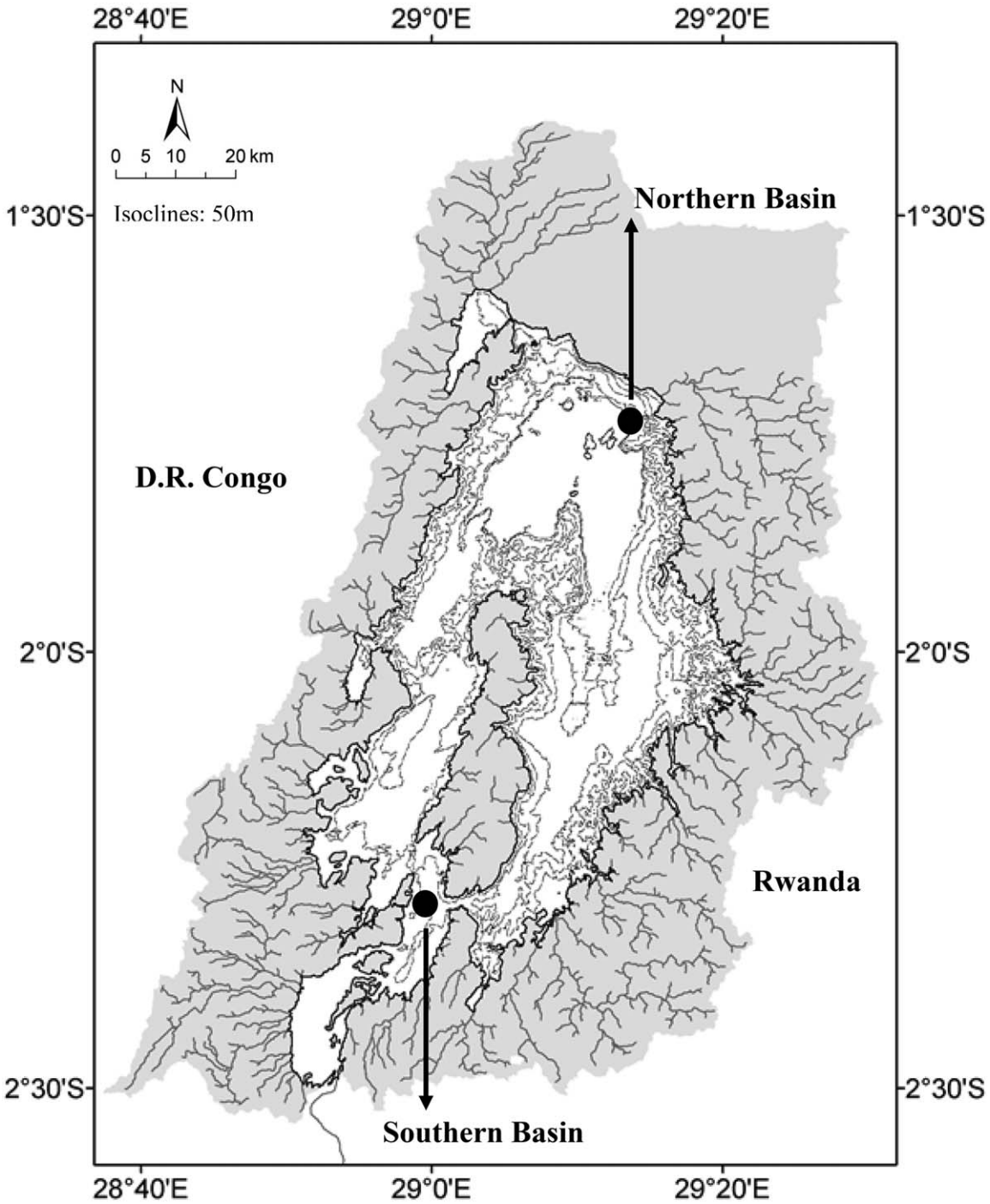

Fig. 1. Map of Lake Kivu, showing sampling sites in the Northern and Southern Basins.

screw caps. Before the injection of ^{15}N-labeled solutions, a 12 h pre-incubation period in dark and at ambient temperature ($\sim$ 25°C) was observed in order to allow the consumption of oxygen inadvertently introduced to the bottles during sampling.

N stable isotope labeling experiments were based on Thamdrup and Dalsgaard (2002). Denitrification, anammox, and DNRA were determined by amending water with $^{15}NO_3^-$ to a final concentration of 5 μmol L^{-1}. During the rainy season, anammox was also determined by amendment with $^{15}NH_4^+$ to a final concentration of 5 μmol L^{-1}. Amendments were made by injecting stock label solutions (concentration of 2.5 mmol L^{-1}) through the septa of the amber glass vials. Six 12 mL vials (Labco Exetainer) were then filled with a

tube from the bottom of each of the duplicate bottles, and placed in the dark in an incubator at ambient temperature (~ 25°C), which was close to the in situ temperature (~ 24°C). Exetainers vials were overfilled to avoid exposure to oxygen. Microbial activity in two Exetainers was immediately stopped through the addition of 500 μL 20% zinc acetate (ZnAc). A time course was established by arresting two further Exetainers at 6 h, 12 h, 18 h, 24 h, and 48 h. In order to test the effect of HS^- on N transformations, experiments were conducted with the supplementary addition of HS^- to amber bottles with $^{15}NO_3^-$ (for final concentration of 10 μmol L^{-1} during the rainy season only). In the Northern Basin, during the rainy season, $^{15}NO_3^-$ enrichment experiments were also conducted at 55 m, 60 m, and 65 m, with $^{15}NO_3^-$ final concentrations of 0.5 μmol L^{-1}, 1 μmol L^{-1}, 2 μmol L^{-1}, 5 μmol L^{-1}, and 10 μmol L^{-1}. These experiments were conducted on water collected 3 d after the other labeling experiments.

$^{29}N_2$ and $^{30}N_2$ concentrations in the Exetainer vials were measured with a gas source isotope ratio mass spectrometer (delta V plus, ThermoScientific) after creating a 1 mL helium headspace (volume injected in the mass spectrometer: 50 μL). Denitrification and anammox rates (detection limits of 2.7 nmol L^{-1} h^{-1} and 0.07 nmol L^{-1} h^{-1}, respectively) in the incubations with $^{15}NO_3^-$ were calculated according to Eqs. 1 and 2, and anammox rates in the incubation with $^{15}NH_4^+$ were calculated according to Eq. 3 (Thamdrup and Dalsgaard 2002; Thamdrup et al. 2006):

$$N_{2\,\text{denitrification}\,15NO3} = {}^{15}N^{15}N_{\text{excess}} * (F_{NO3})^{-2} \quad (1)$$

$$N_{2\,\text{anammox}\,15NO3} = (F_{NO3})^{-1} * ({}^{14}N^{15}N_{\text{excess}} + 2 * (1-(F_{NO3})^{-1}) * {}^{15}N^{15}N_{\text{excess}}) \quad (2)$$

$$N_{2\,\text{anammox}\,15NH4} = {}^{15}N^{14}N_{\text{excess}} * (F_{NH4})^{-1} \quad (3)$$

where $N_{2\ \text{denitrification}\ 15NO3}$ and $N_{2\ \text{anammox}\ 15NO3}$ are the production of N_2 by denitrification and anammox, respectively, in the incubations with $^{15}NO_3^-$ and $N_{2\ \text{anammox}\ 15NH4}$ is the production of N_2 by anammox in the incubations with $^{15}NH_4^+$. $^{15}N^{15}N_{\text{excess}}$ is the production of excess $^{15}N^{15}N$, $^{14}N^{15}N_{\text{excess}}$ is the production of excess $^{14}N^{15}N$, F_{NO3} is the fraction of $^{15}NO_3^-$ in the NO_x pool and F_{NH4} is the fraction of $^{15}NH_4^+$ in the NH_4^+ pool. $^{15}N^{15}N$ and $^{14}N^{15}N$ excess is the excess relative to mass 30 and 29, respectively, in the time zero gas samples.

$^{29}N_2$ and $^{30}N_2$ concentrations from N_2O were also measured in the incubations with the mass spectrometer. N_2O peaks appeared after their respective N_2 peaks. Total N_2O production rates in the incubations were calculated by the sum of the $^{15}N^{15}N_{\text{excess}}$ and the $^{14}N^{15}N_{\text{excess}}$.

DNRA was only measured during the rainy season and was determined as the accumulation of $^{15}N_{\text{excess}}$ from NO_3^- into the NH_4^+ pool, in the same incubations than for denitrification and anammox measurements. When denitrification and anammox measurements were completed, the water samples were flushed with helium in order to evacuate all the N_2 present. Measurements of $^{15}N\text{-}NH_4^+$ were conducted by converting NH_4^+ to N_2 following oxidation by hypobromite, as previously described by Knowles and Blackburn (1993). N_2 was then analyzed as described above.

$$NH_4{}^+{}_{DNRA} = {}^{15}NH_4{}^+{}_{\text{excess}} * (F_{NO3}) \quad (4)$$

where $^{15}NH_4{}^+{}_{\text{excess}}$ is the production of excess $^{15}NH_4{}^+$ in the NH_4^+ pool.

While injecting ZnAc solution to stop the incubations of the Exetainers, the excess water was collected in 2 mL-Eppendorf vials, and stored frozen, to determine the evolution of the NO_x concentrations through time. NO_x were then analyzed by chemiluminescence, after reduction with vanadium chloride (VCl_3), with an NO_2^-, NO_3^-, and NO_x analyzer (Thermo Environmental Instruments), according to the method described by Braman and Hendrix (1989) (detection limit: 0.03 μmol L^{-1}).

Water-column chemical analyses

Samples for determination of vertical profiles of NO_x concentrations were collected in 2 mL-Eppendorf vials, stored frozen and analyzed as described above.

Samples for the determination of NH_4^+, NO_3^-, and NO_2^- concentrations in vertical profiles were collected in 50 mL plastic vials after being filtered through a 0.22 μm syringe filter. 200 μL of H_2SO_4 (5N) were added to each vial for preservation, and samples were stored frozen. NH_4^+ and NO_2^- concentrations were quantified by spectrophotometry, using a 5-cm light path on a spectrophotometer Thermo Spectronic Genesys 10vis, according to the dichloroisocyanurate-salicylate-nitroprussiate colorimetric method (Westwood 1981) and the sulfanilamide coloration method (APHA 1998), respectively. NO_3^- concentrations were determined after vanadium reduction to NO_2^- and quantified with a Multiskan Ascent Thermo Scientific multi-plates reader (APHA 1998; Miranda et al. 2001). The detection limits for these methods were 0.3 μmol L^{-1}, 0.03 μmol L^{-1}, and 0.15 μmol L^{-1}, for NH_4^+, NO_2^-, and NO_3^-, respectively.

Samples for H_2S concentrations were collected directly from the Niskin-type bottle in 60 mL plastic syringes. Water was filtered through a 0.22 μm encapsulated syringe filter in 50 mL plastic vials, without contact with the atmosphere, and was rapidly preserved with 200 μL of 20% ZnAc. Samples were stored frozen. H_2S concentrations were quantified using a 1-cm light path on a spectrophotometer, according to the method described by Cline (1969) (detection limit: 0.25 μmol L^{-1}). Samples for SO_4^{2-} concentrations were filtered through a 0.22 μm syringe filter and collected in 5 mL cryotube. Samples were preserved with 20 μL of 20% ZnAc and were stored frozen. SO_4^{2-} concentrations were determined by ion chromatography (Dionex ICS-1500, with an autosampler

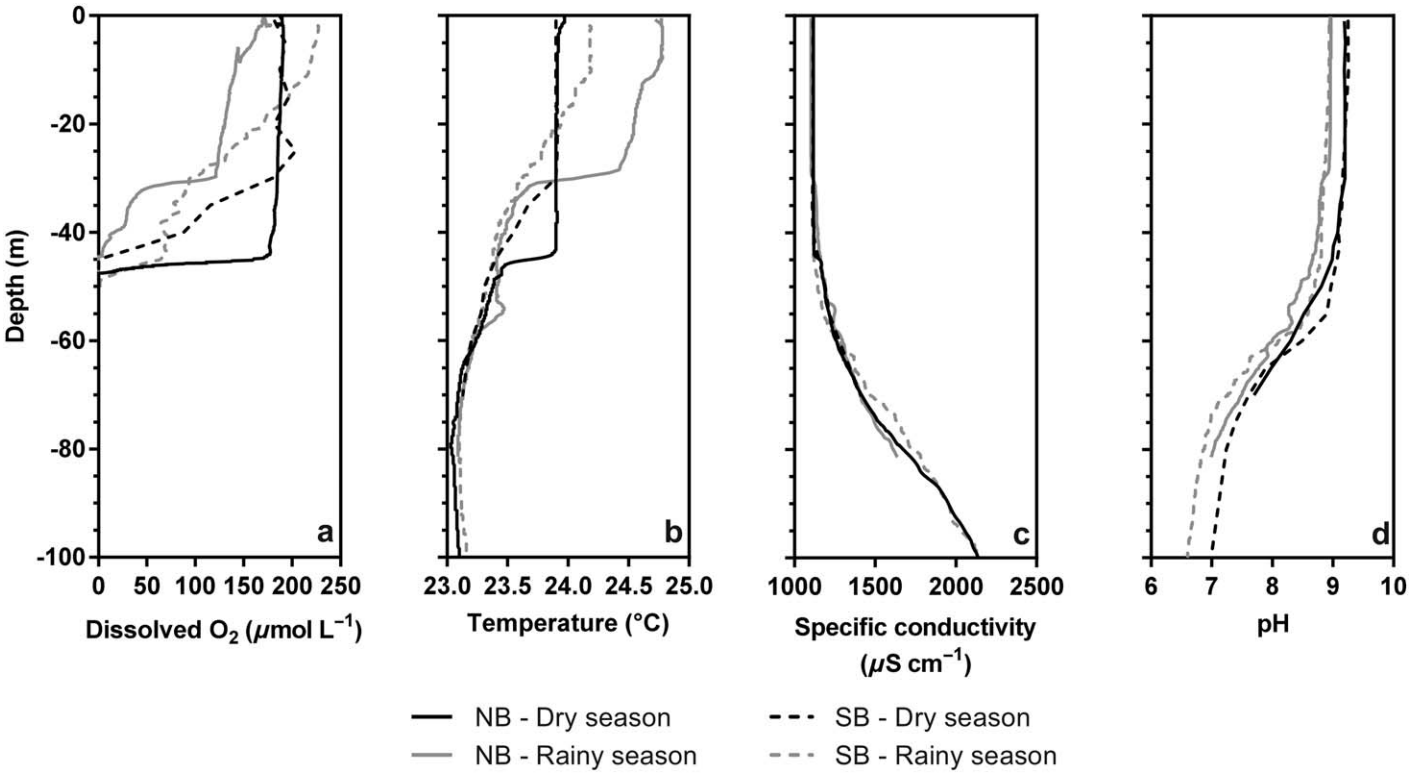

Fig. 2. Physico-chemical parameters (a: dissolved oxygen (μmol L^{-1}); b: temperature (°C); c: specific conductivity (μS cm^{-1}); d: pH) in the Northern Basin (NB; full lines) and Southern Basin (SB; dashed lines), during the dry season (June 2011; black lines) and rainy season (February 2012; grey lines).

Dionex AS50, a guard column Dionex AG22 and an analytical column Dionex IonPac AS22; detection limit: 0.5 μmol L^{-1}).

Samples for determination of N_2O concentrations in vertical profiles were collected in 50 mL borosilicate serum bottles from the Niskin bottle with a tube, left to overflow, poisoned with 100 μL of saturated $HgCl_2$ and sealed with butyl stoppers and aluminum caps. Concentration of N_2O was determined via the headspace equilibration technique and measured by gas chromatography as described by Borges et al. (2015).

Results and discussion

Physico-chemical parameters and Lake Kivu vertical structure description

Lake Kivu is a large (2370 km^2) and deep (maximum depth of 485 m) meromictic lake with permanent anoxic waters below 70 m, but with fluctuations in the depth of the oxycline between the dry and the rainy season (oxygen is mixed to deeper waters in the dry season). It can be divided into a Southern Basin that is smaller and shallower (maximum depth of 180 m) than the Northern Basin (also called main basin, maximum depth of 485 m) and both are connected at a depth of 130 m (Descy et al. 2012). Due to its smaller size, the Southern Basin is less influenced by wind driven mixing. Episodic fluctuations of the stratification are thought to be less frequent in the Southern basin due to sheltering by the surrounding hills (Darchambeau et al. 2014). During our study, differences in the vertical structure of the water column between the Northern and Southern basins were observed (Fig. 2). In the Northern Basin, during the dry season, the water column was anoxic below 47.5 m, while it was anoxic below 45.0 m during the rainy season. In the Southern Basin, the water column was anoxic below 45.0 m during the dry season and below 50.0 m during the rainy season. Primary thermoclines in the Northern Basin strongly differed between seasons and were coincident with the oxycline. In the Southern Basin, the difference between the thermocline between seasons was less notable.

NH_4^+ concentrations reflected the stratification in both basins and were low (< 2 μmol L^{-1}) in the oxic waters, increasing to concentrations up to 90 μmol L^{-1} and 152 μmol L^{-1} at 70 m, in the Northern and Southern Basins, respectively (Fig. 3). The accumulation of NH_4^+ in anoxic waters reflects ammonification during the degradation of the organic matter and the absence of nitrification in the absence of O_2. HS^- concentrations were also higher in anoxic waters, while SO_4^{2-} concentrations were relatively high (100–200 μmol L^{-1}) in oxic waters and started to decrease in the top part of the anoxic waters, where HS^- increased.

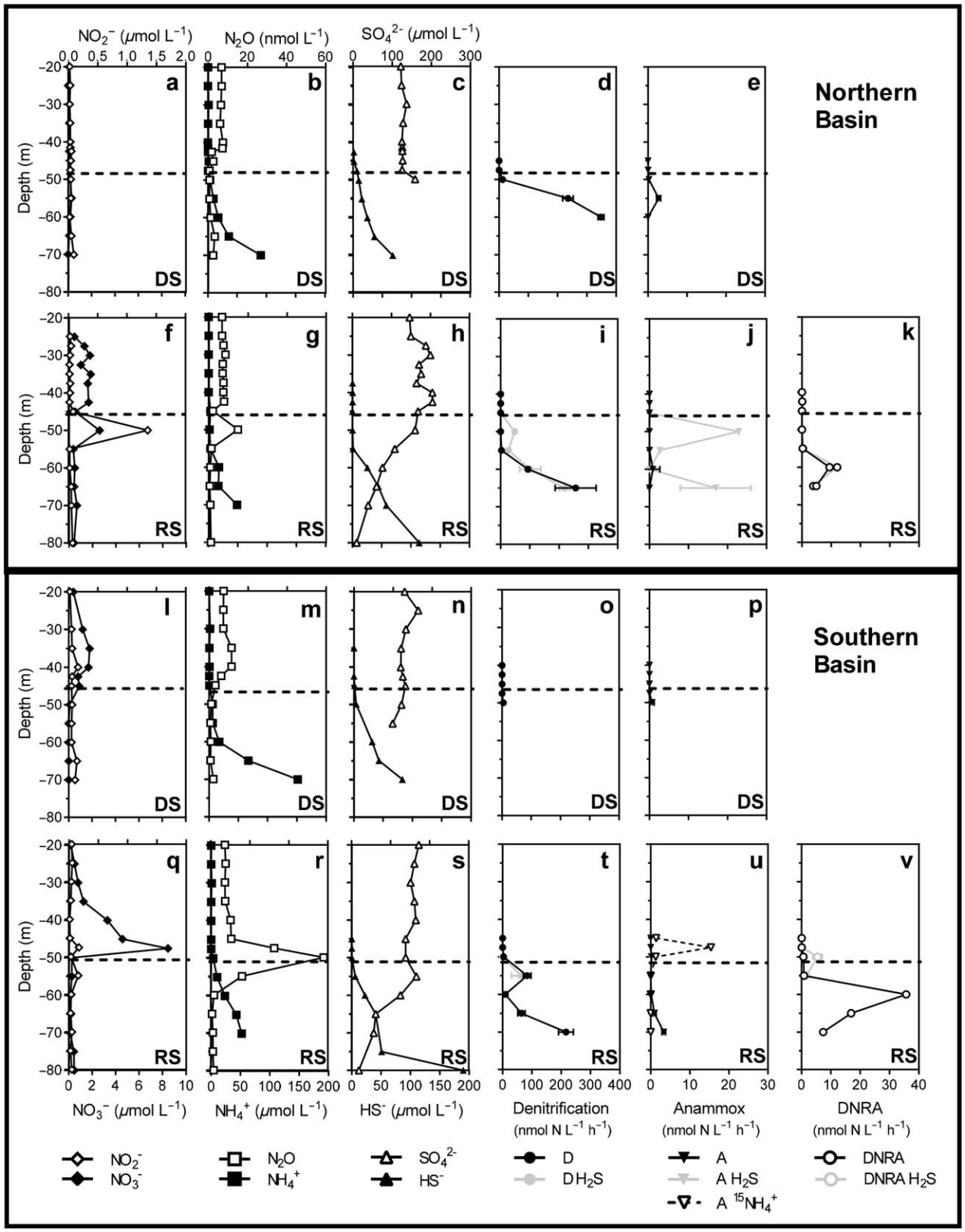

Fig. 3. Vertical profiles of NO_3^- and NO_2^- (a, f, l, q; μmol L^{-1}), N_2O and NH_4^+ (b, g, m, r; nmol L^{-1} and μmol L^{-1}, respectively), SO_4^{2-} and HS^- (c, h, n, s; μmol L^{-1}) concentrations, and rates of Denitrification (d, i, o, t), Anammox (e, j, p, u) and DNRA (k, v) (nmol N produced L^{-1} h^{-1}) without (D, A, and DNRA, respectively) and with (D H_2S, A H_2S, and DNRA H_2S, respectively) H_2S added in the incubations with $^{15}NO_3^-$ added, during both seasons (RS: Rainy season; DS: Dry season) and in both stations (Northern Basin: a–k; Southern Basin: l–v), and Anammox rates in the incubations with $^{15}NH_4^+$ added (A $^{15}NH_4^+$; u) in rainy season.

NO_2^- and NO_3^- concentrations were low throughout most of the water column (Fig. 3), for both seasons and both stations, but accumulation up to 1.5 μmol L^{-1} (in the Northern Basin) and 8.0 μmol L^{-1} (in the Southern Basin), respectively, was observed during the rainy season in proximity to the boundary between oxic and anoxic waters. NO_3^- and NO_2^- accumulation generally co-occurred with peaks in N_2O concentrations (15 nmol L^{-1} in the Northern Basin, and 58 nmol L^{-1} in the Southern Basin). Low oxygen concentrations within these depth intervals suggest redox conditions favorable both to denitrification and to high N_2O yields (Codispoti et al. 1992). During the dry season, N_2O concentrations were higher in oxic waters (around 10 nmol L^{-1}) than in anoxic waters (below 5 nmol L^{-1}), at both stations. N_2O concentrations were near the atmospheric equilibrium in superficial oxic waters (Supporting Information Table S1), while the water column was clearly undersaturated in deep anoxic waters, and oversaturated near the oxic–anoxic interface.

Co-occurrence of denitrification, anammox, and DNRA in the water column of Lake Kivu

Results of time course incubations (29_{excess} and 30_{excess} production) are reported in Supporting Information Fig. S1. Irrespective of seasons and stations, the increase of 29_{excess} and 30_{excess} tended to be low at the beginning of the incubation, then strongly increased, and finally tended to stabilize at the end of the incubation. The initial time lag observed before the production of N_2 could be explained by the time required for the community to recover from the perturbation of the sampling. The plateau observed at the end of the incubations could be due to a bacterial community saturation or substrates limitation (NO_3^- or organic matter).

All the rates reported are statistically significant ($p < 0.05$). Rates reported in this section should be considered as potential rates due to the fact that the addition of the ^{15}N labeled compounds increased substrate concentrations relative to their in situ values. Rates and pathways differed between seasons and stations (Fig. 3). Denitrification rates were higher in the Northern Basin than in the Southern Basin for both seasons. The maximum denitrification rate of 348 nmol N produced L^{-1} h^{-1} was observed at 60 m depth during the dry season. In the Southern Basin, the maximum denitrification rate was 216 nmol N produced L^{-1} h^{-1} and was observed at 70 m during the rainy season. During the dry season, almost no denitrification was observed in the Southern Basin, probably due to shallow sampling (denitrification seemed to start at 50 m depth, and was maybe present deeper). In contrast to denitrification, rates of anammox tended to be higher in the Southern Basin. The maximum anammox rate of 3.3 nmol N produced L^{-1} h^{-1} was observed in the Southern Basin at 70 m during the rainy season. The maximum anammox rate was less than 1% of the maximum denitrification rate, suggesting it played a small role in N_2 production in Lake Kivu. DNRA was also observed in the water column of Lake Kivu during our study. In contrast to denitrification, but like anammox, rates of DNRA were higher in the Southern Basin, with a maximum rate of 36 nmol N produced L^{-1} h^{-1} observed at 60 m. In the Northern Basin, the maximum rate of DNRA was 9 nmol N produced L^{-1} h^{-1} at 60 m.

Schubert et al. (2006) used the same method to quantify denitrification and anammox in the water column of Lake Tanganyika. The vertical structure of Lake Tanganyika water column shares characteristics with Lake Kivu water column: anoxic waters rich in NH_4^+, oxic surface waters depleted in nutrients, NO_3^- accumulation ($\sim$ 10 μmol L^{-1}) near the oxic–anoxic interface and very low NO_2^- concentrations. Schubert et al. (2006) reported maximum denitrification rates of 200 nmol N produced L^{-1} h^{-1}—the same magnitude as the rates observed in Lake Kivu. In Lake Rassnitzer, Hamersley et al. (2009) measured maximum denitrification rates of only 6 nmol N produced L^{-1} h^{-1}. These two studies also measured anammox rates from $^{15}NO_3^-$-labelling experiments, and obtained rates of 20 and 1.4 nmol N produced L^{-1} h^{-1} in Lake Tanganyika and Rassnitzer, respectively. In marine environments, denitrification was estimated to 0–216 nmol N produced L^{-1} h^{-1} (e.g., Brettar and Rheinheimer 1991; Thamdrup et al. 2006; Dalsgaard et al. 2013). If we compare in terms of relative contribution of anammox to N_2 (Table 1), we estimated it to be potentially up to 13% in Lake Kivu, exactly like in Lake Tanganyika (Schubert et al. 2006), while it was estimated to up to 50% in Lake Rassnitzer (Hamersley et al. 2009). In the anoxic water column of Golfo Duce, anammox accounted for 19–35% in the formation of N_2 (Dalsgaard et al. 2003). In Lake Kivu, anammox was not measurable in the main basin (Northern Basin), and can be thus considered as of little importance in the water column of Lake Kivu. However, we must note that our anammox rates could be underestimated, since they were measured indirectly from the addition of $^{15}NO_3^-$, instead of addition of $^{15}NO_2^-$ and $^{15}NH_4^+$. As denitrification can also produce $^{29}N_2$, we firstly considered that $^{29}N_2$ produced was due to denitrification. When $^{29}N_2$ production was too high to be explained by denitrification only, we attributed it to anammox. Moreover, it is still unclear if anammox bacteria can use NO_3^- to oxidize NH_4^+. The study of Kartal et al. (2007) showed that anammox bacteria were capable to reduce NO_3^- in NO_2^-, but at a rate 10% lower than the anammox rate with NO_2^-. The low anammox rates observed during this study can thus also be due to the need to firstly reduce $^{15}NO_3^-$ to $^{15}NO_2^-$, which seems to be an unfavorable process for anammox bacteria.

The differences in denitrification and anammox rates between the different environments can be attributed to the different bacterial communities and environment characteristics, such as substrate availability, physico-chemical parameters (pH, oxygen, salinity, temperature), and the presence

Table 1. Contribution (%) of denitrification and anammox in the formation of N_2, in both basins and during both campaigns, with and without H_2S added.

	Without H_2S (%)			With H_2S (%)		
	Denitrification	**Anammox**	**SD**	**Denitrification**	**Anammox**	**SD**
Northern Basin						
Dry season						
45	0	0	0	n.d	n.d	
47.5	0	0	0	n.d	n.d	
50	98	2	0	n.d	n.d	
55	99	1	0	n.d	n.d	
60	100	0	1	n.d	n.d	
Rainy season						
40	0	0	0	0	0	0
42.5	0	0	0	0	0	0
45	0	0	0	0	0	0
50	100	0	0	68	32	39
55	100	0	0	91	9	5
60	99	1	0	100	0	0
65	100	0	0	93	7	4
Southern Basin						
Dry season						
40	0	0	0	n.d	n.d	
45	0	0	0	n.d	n.d	
47.5	0	0	0	n.d	n.d	
50	87	13	9	n.d	n.d	
Rainy season						
45	0	0	0	0	0	0
47.5	100	0	0	0	0	0
50	94	6	5	100	0	0
55	100	0	0	100	0	0
60	99	1	1	n.d	n.d	
65	99	1	0	n.d	n.d	
70	98	2	0	n.d	n.d	

n.d., not determined; SD, standard deviation.

of inhibitors (e.g., high concentrations of NH_4^+, NO_2^-, organic matter) (Jin et al. 2012). For example, temperature in Lake Rassnitzer was around 5°C at depths sampled during the study of Hamersley et al. (2009), while it was around 23°C in Lake Kivu, strongly influencing anammox and denitrification processes (Saad and Conrad 1993; Van Hulle et al. 2010; Dong et al. 2011). Also, the abundance and diversity of bacterial communities play an important role. Currently, all anammox bacteria identified belong to the order *Planctomycetales* (Strous et al. 1999). The study of İnceoğlu et al. (2015*b*) focused on the identification of bacterial and archaeal communities in the water column of Lake Kivu, at the same stations and during the same field campaigns. These authors showed that *Planctomycetes* were present in the water column, but not well represented (< 1%), in agreement with low anammox rates we observed during this study. On the contrary, in Lake Tanganyika, anammox bacteria seemed to be better represented (up to 1.4%) and overall, were highly active (Schubert et al. 2006), which may explain higher anammox rates observed. High denitrification rates observed in Lake Kivu can also be linked to the abundance of the denitrifying bacterial community. Indeed, İnceoğlu et al. (2015*b*) also revealed the presence of a diversified community of *Proteobacteria*, among which *Betaproteobacteria*. Numerous nitrogen cycle-related bacteria belong to this class, including well-known denitrifiers, such as *Thiobacillus* sp. and *Denitratisoma* sp. (Claus and Kutzner 1985; Tiedje 1994; Ghosh and Dam 2009). In addition to the presence of these bacteria, İnceoğlu et al. (2015*a*) also put in evidence their activity by the identification of specific genes. They thus showed the presence of functional genes involved in denitrification, strongly supporting the occurrence of

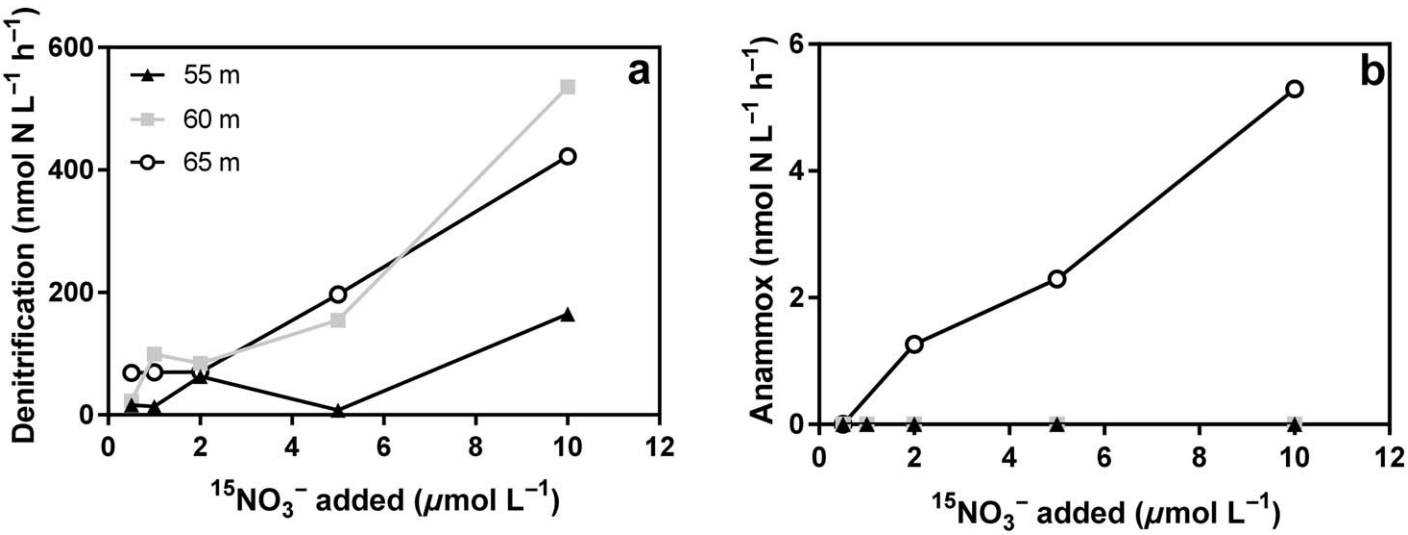

Fig. 4. Potential denitrification (**a**) and anammox (**b**) rates (nmol N produced L^{-1} h^{-1}) with different $^{15}NO_3^-$ concentrations added (0.5 μmol L^{-1}, 1 μmol L^{-1}, 2 μmol L^{-1}, 5 μmol L^{-1}, and 10 μmol L^{-1}), in the Northern Basin, during the rainy season, at the depths of 55 (black triangles), 60 (grey squares), and 65 m (white circles). Experiments were conducted during rainy season only (08 February 2012).

denitrification in the water column of Lake Kivu. Also, the water column of Lake Kivu seems to be a favorable environment for DNRA, since *Epsilonproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria*, among which we can find bacteria capable of DNRA, such as *Wolinella* sp., *Desulfovibrio* sp., *Geobacter* sp., or *Vibrio* sp. (e.g., Bokranz et al. 1983; Dalsgaard and Bak 1994; Strohm et al. 2007) were well represented (İnceoğlu et al. 2015*b*).

Bacterial diversity data thus confirm that denitrification and DNRA naturally occur in the water column of Lake Kivu. The conditions for the occurrence of these processes are encountered during the rainy season, since non negligible NO_x concentrations were observed. Moreover, in the Northern Basin, the location of the N_2O peak in the anoxic part of the water column suggests that N_2O could have been produced through denitrification. On the contrary, the water column of Lake Kivu first might seem to be unfavorable for anammox, since *Planctomycetes* were seldom observed (İnceoğlu et al. 2015*b*). As shown by Fig. 4, the scales of the rates measured are linear in our range of NO_3^- concentrations. We thus calculated natural rates ($^{14}N^{14}N$) in our incubations with $^{15}NO_3^-$ added according to following equations:

$$\text{Natural } N_{2\ \text{denitrification}} = \text{Potential } N_{2\ \text{denitrification}} * (1 - F_{NO3}) \quad (5)$$

$$\text{Natural } N_{2\ \text{anammox}} = \text{Potential } N_{2\ \text{anammox}} * (1 - F_{NO3}) \quad (6)$$

$$\text{Natural } {NH_4}^{+}{}_{\text{DNRA}} = \text{Potential } {NH_4}^{+}{}_{\text{DNRA}} * (1 - F_{NO3}) \quad (7)$$

Supporting Information Figure S2 reports natural rates measured in our incubations, i.e., rates recomputed without substrates added. Denitrification was naturally present in the water column of Lake Kivu, in particular during the rainy season, where a maximum rate of 93 nmol N produced L^{-1} h^{-1} was observed at 65 m in the Northern Basin. During the dry season, natural denitrification rates were lower, due to low NO_3^- concentrations. Almost no natural anammox was observed (maximum rate of 0.01 nmol N produced L^{-1} h^{-1}), and natural DNRA rates were also clearly lower (maximum rate of 0.7 nmol N produced L^{-1} h^{-1}).

The maximum depth-integrated natural denitrification rate was observed in the Northern Basin and can be estimated to 0.01 mmol N m^{-2} d^{-1} (Supporting Information Table S2), while the depth-integrated natural DNRA rate observed in the Northern Basin was estimated to only 0.0001 mmol N m^{-2} d^{-1} (integration from 40 m to 65 m depth). The maximum depth-integrated natural DNRA rate was observed in the Southern Basin and was estimated to 0.0002 mmol N m^{-2} d^{-1}. Dalsgaard et al. (2013) also calculated depth-integrated in situ denitrification rates in the Baltic Sea, which ranged from 0.06 mmol N m^{-2} d^{-1} to 2.11 mmol N m^{-2} d^{-1}. Schubert et al. (2006) calculated depth-integrated denitrification rates of 2.4 mmol N m^{-2} d^{-1} in Lake Tanganyika, but their calculations were based on potential denitrification rates and they admit that their calculations should be taken with caution, since they only performed one measurement. Depth-integrated potential denitrification rates in Lake Kivu were higher than natural rates, but remained low, with a maximum of 0.05 mmol N m^{-2} d^{-1} (in the Northern Basin).

We investigated if substrates fluxes were sufficient to explain natural and potential denitrification rates observed. As the nitracline is spatially limited, it is difficult to precisely calculate NO_3^- fluxes, and would have required dedicated

nitrification measurements. We can calculate NH_4^+ diffusion fluxes, which in turn sustain NO_3^- concentrations through nitrification. As shown in Supporting Information Table S2, upward NH_4^+ fluxes were always sufficient to explain the denitrification rates observed during this study.

Denitrification and anammox processes are limited by substrate availability

We determined the effect of substrate availability on NO_3^- reduction. In the Northern Basin, during the rainy season, $^{15}NO_3^-$ labeling experiments were conducted with amendments of $^{15}NO_3^-$ of different initial concentrations. These experiments were conducted at depths of 55 m, 60 m, and 65 m (Fig. 4). Denitrification rates increased with increasing $^{15}NO_3^-$ concentrations, at all depths measured. The maximum denitrification rate (536 nmol N produced L^{-1} h^{-1}, $N_2 + N_2O$) was observed at 60 m depth with a final $^{15}NO_3^-$ concentration of 10 μmol L^{-1}. No anammox was observed at the depths of 55 m and 60 m, while rates of anammox increased with increasing $^{15}NO_3^-$ concentrations up to 5.2 nmol N produced L^{-1} h^{-1} with 10 μmol L^{-1}, at 65 m. These results strongly suggest that denitrification in Lake Kivu is limited by NO_3^- concentrations, and likewise, that anammox is probably limited by the supply of NO_2^-, through partial denitrification. Experiments amended with $^{15}NH_4^+$ suggest that anammox in the Southern Basin is co-limited by NH_4^+. Indeed, these experiments revealed high rates of anammox in the Southern Basin (Fig. 3u), while no anammox was observed in the Northern Basin. Anammox rates were higher (up to 15 nmol N produced L^{-1} h^{-1}) than those measured with $^{15}NO_3^-$, and were located at shallower depths (at 47.5 m), where NH_4^+ concentrations were very low (less than 1 μmol L^{-1}).

Competition occurs between denitrification, anammox, and DNRA

Denitrification, anammox, and DNRA all compete for NO_3^-, and competition may thus appear between the different processes. During our study, we observed a competitive relationship between denitrification and DNRA, since for both stations, higher denitrification rates corresponded to the lower DNRA rates (Fig. 5a). Also, competition between anammox and DNRA seemed to occur, since anammox rates tended to be lower when DNRA rates were higher (Fig. 5b). Although DNRA can, in theory, fuel the anammox bacterial community in NH_4^+, they can also enter in competition for NO_2^- and NO_3^-. At depths where the different processes were measured, NH_4^+ was not limiting with concentrations ranging between 2 μmol L^{-1} and 43 μmol L^{-1} (so the supply of NH_4^+ by DNRA was not required for anammox), contrary to the low NO_2^- (0.02–1 μmol L^{-1}), and NO_3^- (0.2–3 μmol L^{-1}) concentrations, explaining the competitive relationship. On the contrary, anammox seemed not to enter in competition with denitrification for substrates, since anammox rates tended to be higher when denitrification rates were higher

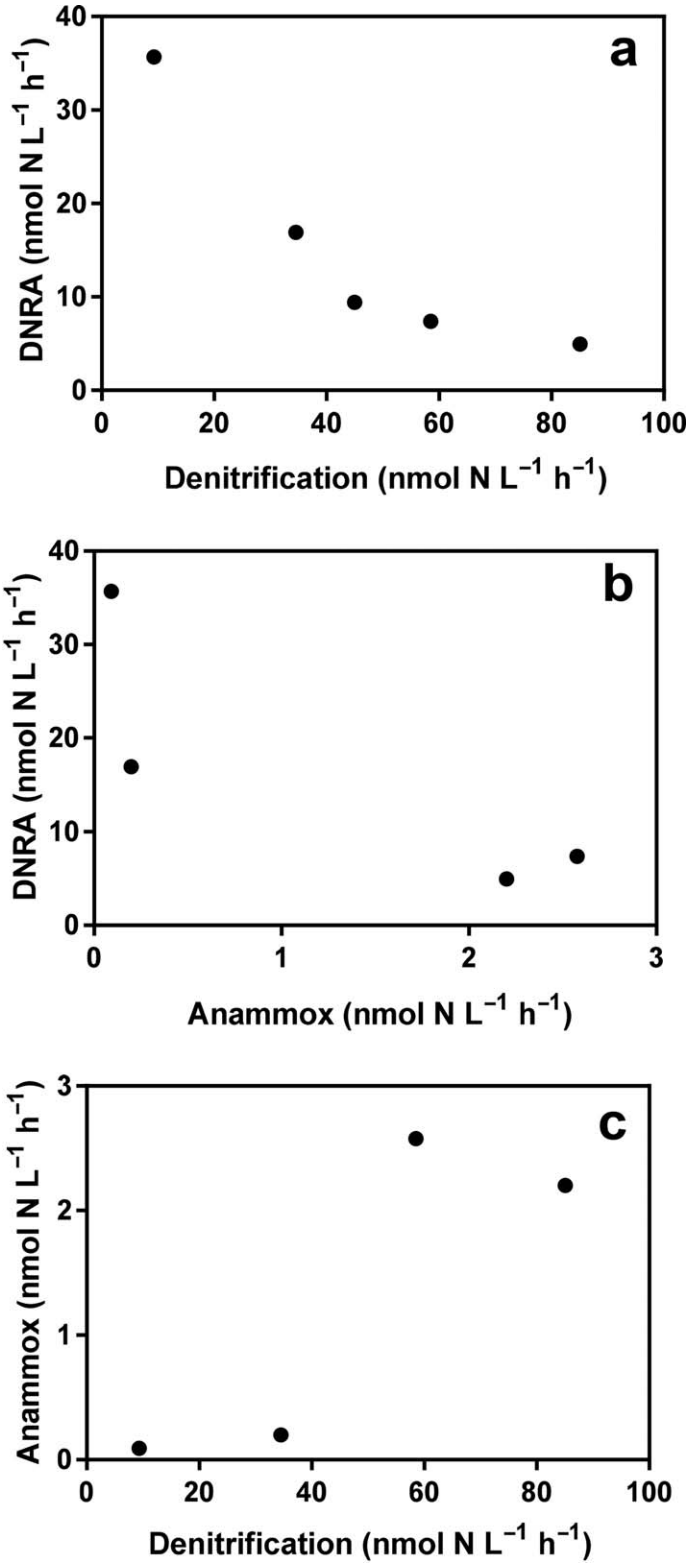

Fig. 5. Correlation between (**a**) DNRA and denitrification rates, (**b**) DNRA and anammox rates and (**c**) Anammox and denitrification rates (nmol N produced L^{-1} h^{-1}) at both stations and during both seasons.

(Fig. 5c). This suggests that anammox bacteria may gain benefit from NO_2^- produced as intermediate during the denitrification process.

The effect of H_2S on denitrification, anammox, and DNRA

All rates reported in this section are potential rates, measured in the incubations with $^{15}NO_3^-$ and H_2S added, during the rainy season.

In the Northern Basin, the addition of H_2S was followed by an increase of denitrification, anammox, and DNRA rates at almost all depths measured, except at 65 m where denitrification and DNRA rates slightly decreased. In particular, anammox rate increased up to 24 nmol N produced L^{-1} h^{-1} at 50 m, where the contribution of anammox to N_2 production reached 32% (Table 1). Some studies in marine anoxic environments have suggested an inhibitory effect of H_2S on anammox (Dalsgaard et al. 2003; Jensen et al. 2008, 2009). However, other studies conducted in wastewater bed reactors and in laboratory cultures showed that anammox bacteria tolerate H_2S and can even be stimulated by H_2S (Kalyuzhnyi et al. 2006; Jung et al. 2007; Russ et al. 2014). The study of Wenk et al. (2013) on Lake Lugano, which used the same incubation method, also showed that anammox was stimulated by the addition of H_2S. To explain anammox activity in the presence of H_2S, they suggested that anammox bacteria lived in aggregates with chemolithotrophic denitrifying bacteria and thus in the presence of lower concentrations of H_2S following its consumption by the denitrifying bacteria. The latter would also produce NO_2^-, which would in turn stimulate anammox. In our study, the addition of H_2S also stimulated denitrification in the Northern Basin, suggesting the occurrence of chemolithotrophic denitrification. We also observed an increased production of N_2O with the addition of H_2S (Table 2), suggesting the occurrence of chemolithotrophic denitrification, which stimulates NO_3^- and NO_2^- reduction relative to N_2O reduction, leading to a higher N_2O production, but a T-student test showed that these increases were not significant ($p = 0.06$). Bacterial communities potentially capable to perform chemolithotrophic denitrification seem to be present in the water column of Lake Kivu. Indeed, İnceoğlu et al. (2015*b*) put in evidence the presence of *Epsilonproteobacteria* and *Gammaproteobacteria*, two classes among which bacteria capable of HS^- oxidation, such as *Sulfurimonas* sp., *Sulfuricurvum* sp., *Thiothrix* sp., and *Thiomicrospira* sp. (e.g., Larkin and Strohl 1983; Friedrich et al. 2005; Ghosh and Dam 2009). İnceoğlu et al. (2015*b*) also showed that *Betaproteobacteria* were well represented, among which *Thiobacillus* sp., capable to perform denitrification coupled to H_2S oxidation.

In the Southern Basin, H_2S experiments were only performed at four depths (45 m, 47.5 m, 50 m, and 55 m) for denitrification and anammox, and at three depths (47.5 m, 50 m, and 55 m) for DNRA. In contrast to the Northern Basin, the addition of H_2S tended to decrease denitrification rates, while no effect was observed on anammox rates, which remained below detection. On the contrary, the addition of H_2S tended to stimulate DNRA rates. An experimental error, such as an inhibition of denitrification by the addition of small quantities of oxygen, seems very unlikely, since a pre-incubation period of 12 h after filling the incubation vials was respected before the start of the experiment, to allow the consumption of the potential external oxygen artificially introduced. Furthermore, the same sampling protocol was use in stations in both basins and all sampling expeditions. Alternatively, denitrifiers can compete for NO_3^- and NO_2^- with DNRA and anammox bacteria. The inhibition of denitrification in presence of H_2S has been frequently reported (e.g., Jorgensen 1989; Joye and Hollibaugh 1995; An and Gardner 2002) but it is now established that denitrification can be coupled with H_2S oxidation (e.g., Brettar and Rheinheimer 1991; Burgin and Hamilton 2008; Jensen et al. 2009). So, it seems that the apparent inhibition of denitrification (actually the inhibition of N_2O reduction to N_2) at high H_2S concentrations could be due to a competition with DNRA for substrates. Indeed, several studies suggest that DNRA can be enhanced at high H_2S concentrations (e.g., Brunet and Garcia-Gil 1996; Rysgaard et al. 1996; Sayama et al. 2005) and becomes more competitive than denitrification. During our measurements, DNRA rates tended to increase when H_2S was added. The fact that denitrification rates decreased with the addition of H_2S only in the Southern Basin could be explained by the higher importance of DNRA in the Southern Basin (reflected by higher DNRA rates in "normal" conditions, without H_2S added), and so by a stronger competition.

Table 2. Relative contribution (% ± standard deviation) of N_2 production compared with N_2+N_2O production without and with H_2S added, in rainy season, for depths with significant ($p < 0.05$) rates of denitrification.

	Without H_2S	With H_2S
Northern Basin		
50	100 ± 0	92 ± 6
55	100 ± 0	100 ± 0
60	96 ± 3	88 ± 5
65	99 ± 3	47 ± 24
Southern Basin		
47.5	100 ± 0	Not detected
50	100 ± 0	100 ± 0
55	99 ± 2	100 ± 2
60	74 ± 27	n.d
65	84 ± 2	n.d
70	94 ± 1	n.d

n.d., not determined.

Spatial heterogeneity

During this study, we put in evidence important differences between the Northern and the Southern Basin of Lake Kivu. Without H_2S added, denitrification tended to be higher in the Northern Basin, while anammox and DNRA tended to be more important in the Southern Basin. The treatment with $^{15}NH_4^+$ added also showed important potential anammox rates only in the Southern Basin. However, differences in bacterial communities between both basins are not significant, since relative abundances of bacterial community capable of DNRA was estimated to be up to 8% and 13% (means of both campaigns) in the Northern and Southern Basins, respectively, while relative abundance of denitrifying community was estimated to 35% and 44%. It seems unlikely that the small difference in term of the bacterial community composition is the only factor determining the different process rates observed in the Northern and Southern Basin. The two basins are morphometrically different: the water column in the Southern Basin seems to be more stable than the Northern Basin, explaining why anammox was more widespread in the Southern Basin, since anammox is a slow process requiring stable environmental conditions (Strous et al. 1999). Also, based upon NH_4^+ vertical profiles, it seems that NH_4^+ concentrations tend to be higher in the Southern Basin, probably linked to higher DNRA rates, what can influence anammox. Concerning heterotrophic denitrification and DNRA, it is difficult to explain differences observed with available data. We can hypothesize that organic matter supply is different between both basins, with higher supply in the Southern Basin, due to its smaller size. Indeed, due to higher proximity of surrounding land, allochthonous organic matter supply may be higher, especially during the rainy season. This would favor DNRA over denitrification (Kelso et al. 1997; Silver et al. 2001; Dong et al. 2011).

Conclusions

The present study reports the occurrence of denitrification, DNRA and anammox for the first time in Lake Kivu, and to our best knowledge, in the water column of a large tropical lake. We showed that these three processes can co-occur in the anoxic water column, although competition for substrates occurred. As in Lake Lugano (Wenk et al. 2013), we showed the co-occurrence of chemolithotrophic denitrification and anammox. Further studies are required to determine if aggregates between denitrifying bacteria and anammox bacteria are possible and if they are present in Lake Kivu, and to elucidate the competitive relationships between the three processes. During this study, we stimulated denitrification, anammox and DNRA rates by the addition of $^{15}NO_3^-$ and $^{15}NH_4^+$. Even if agriculture around Lake Kivu is still traditional, population growth might lead to increased use of fertilizers, and thus of a higher N supply. Also, methane extraction from deep waters to produce electricity has started at industrial scale, and raising the problem of the disposal of the extracted nutrients-rich deep waters. The data of the processes reported in this manuscript could be thus used as baseline study and to calibrate models that address the future evolution of the lake.

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Conflict of Interest

None declared.

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