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The fate of nitrogen is linked to iron(II) availability in a freshwater lake sediment

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Abstract

The fate of nitrogen in natural environments is controlled by anaerobic nitrate-reducing processes by which nitrogen is removed as N_2 or retained as NH_4^+. These processes can potentially be driven by oxidation of reduced inorganic compounds at oxic-anoxic interfaces. Several studies have investigated the use of Fe^{2+} as an electron donor in nitrate reduction in bacterial cultures, however current information on this process in the environment is sparse. We aimed to determine whether nitrate-reducing processes in the freshwater Lake Almind (Silkeborg, Denmark) were linked to Fe^{2+} oxidation. Anaerobic sediment slurries were supplemented with ^{15}N-substrates and electron donors (Fe^{2+} and/or acetate) to characterize nitrate-reducing processes under environmentally relevant substrate concentrations and at higher concentrations traditionally used in microbial enrichment studies.

Dissimilatory nitrate reduction to ammonium, DNRA, was stimulated by Fe^{2+} addition in 7 of 10 slurry experiments and in some cases, denitrification was concomitantly reduced. The determined kinetic parameters (V_{max} and K_m) for Fe^{2+}-driven DNRA were 4.7 μmol N L^{-1} d^{-1} and 33.8 μmol Fe^{2+} L^{-1}, respectively and reaction stoichiometry for Fe^{2+}:NH_4^+ (8.2:1) was consistent with that of predicted stoichiometry (8:1). Conversely, under enrichment conditions, denitrification was greatly increased while DNRA rates remained unchanged. Increased Fe^{2+} concentrations may be exploited by DNRA organisms and have an inhibitory effect on denitrification, thus Fe^{2+} may play a role in regulating N transformations in Lake Almind. Furthermore, we suggest enrichment conditions may promote the adaptation or change of microbial communities to optimally utilize the available high substrate concentrations; misrepresenting metabolisms occurring *in situ*.

Keywords: Nitrogen; Denitrification; DNRA; Sediment

1. INTRODUCTION

In anaerobic environments such as aquatic sediments, several dissimilatory processes compete for available nitrate (and nitrite; here together designated NO_x) which is the most favorable electron acceptor following oxygen depletion (Capone and Kiene, 1988). Denitrification reduces nitrate in a stepwise process via nitrite, NO, and N_2O to N_2. Alternatively, N_2 production may be linked to the oxidation of ammonium or methane through the biochemically distinct anammox and nitrite-dependent anaerobic methane oxidation (n-damo) pathways (Strous et al., 1999; Ettwig et al., 2010), which are both active in freshwater sediments (Deutzmann and Schink, 2011; Yoshinaga et al., 2011). These three processes effectively remove bioavailable N from natural and man-made systems, remediating excess N loading and reducing its possible deleterious ecosystem effects such as eutrophication. By contrast, a fourth competing process, dissimilatory nitrate reduction to

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ammonium (DNRA) reduces nitrate via nitrite to ammonium using a distinct, ammonium-producing nitrite reductase (Simon, 2002; Giblin et al., 2013); thereby retaining N in the environment where it may be reused or transported (Burgin and Hamilton, 2007).

Recognizing the factors which influence the partitioning between these enzymatically distinct NO_x-transforming processes (Simon and Klotz, 2013) is especially relevant in light of modern excess N loading, water quality management, and future environmental change. The contributions of anammox and n-damo to NO_x reduction in freshwater sediments on average appear relatively minor (Thamdrup, 2012; Norði et al., 2013), with either denitrification or DNRA being the dominating NO_x sink. The fate of nitrate in continuous cultures of a microbial community from marine sediment was predictable from the substrate C:N ratio, the relative supply of nitrite and nitrate, and the generation time (Kraft et al., 2014). In another study, nitrate limited conditions and high organic carbon availability were also shown to be a major factor promoting DNRA over denitrification in enrichments of activated sludge from wastewater treatment (van den Berg et al., 2015). In general, denitrification is found to dominate under higher nitrate availability (Bonin, 1996; Dong et al., 2009), while DNRA may dominate under more reducing conditions with high labile carbon availability and nitrate limitation (Christensen et al., 2000; Jäntti and Hietanen, 2012; Hardison et al., 2015).

While electron acceptor and organic substrate availability have been identified as regulating factors in organotrophic denitrification and DNRA, NO_x reduction can also be coupled to the oxidation of inorganic substrates including reduced sulfur (Beijerinck, 1904) and iron compounds (Straub et al., 1996). As such, the availability of different types of inorganic electron donors may thus contribute to determining the fate of NO_x. The oxidation of sulfide coupled to nitrate reduction has been well studied in numerous environmental microbial strains for many years (Beijerinck, 1904; Teske and Nelson, 2006) and identified as an important nitrogen cycling process in anoxic water columns (Jensen et al., 2009; Canfield et al., 2010; Wenk et al., 2013) and potentially also in sediments (Brunet and Garcia-Gil, 1996; Sayama et al., 2006; Burgin and Hamilton, 2007). However the possibility of Fe^{2+} as an electron donor for nitrate reduction in the environment had not been investigated until comparatively recently (Straub et al., 1996).

Most bacterial isolates capable of utilizing Fe^{2+} as electron donor for nitrate reduction have been inferred to produce N_2 as the end product based on reaction stoichiometry (e.g. Straub et al., 1996; Muehe et al., 2009; Chakraborty and Picardal, 2013)

$$NO_3^- + 5Fe^{2+} + 12H_2O \rightarrow 1/2N_{2(g)} + 5Fe(OH)_3 + 9H^+ \quad (1)$$

This process is often reported to proceed mixotrophically; requiring an organic co-substrate such as acetate (Kappler et al., 2005; Muehe et al., 2009; Chakraborty et al., 2011). Although some studies have identified autotrophic organisms capable of reducing nitrate with Fe^{2+} (Hafenbradl et al., 1996; Weber et al., 2006; Li et al., 2014) authors report great difficulty in continuously transferring these strains without the addition of organic substrate. One recent study demonstrated autotrophy in a strain of nitrate reducing Fe^{2+} oxidizers in batch incubations of marine sediment, but the nitrogenous product was not explicitly identified (Laufer et al., 2016b). A smaller number of studies have shown the reduction of nitrate to NH_4^+ in sediments enrichments (Weber et al., 2006; Coby et al., 2011).

$$NO_3^- + 8Fe^{2+} + 21H_2O \rightarrow NH_4^+ + 8Fe(OH)_3 + 14H^+ \quad (2)$$

Although molecular investigations have identified phylogenetically diverse groups as potentially contributing to nitrate reduction with Fe^{2+} oxidation in nature (Straub et al., 2004; Laufer et al., 2016a), *Geobacter metallireducens* is, to our knowledge, the only isolate so far demonstrated to couple Fe^{2+} oxidation to DNRA (Lovley et al., 1993; Finneran et al., 2002). *Geobacter* species were also implicated as conveyors of this process in enrichment cultures (Weber et al., 2006; Coby et al., 2011). Members of the genus *Geobacter* are generally known for their ability to reduce metal oxides and are abundant in iron-rich freshwater sediments where they may therefore catalyze a complete anaerobic redox cycle of iron (Weber et al., 2006).

Recent studies have investigated the ecology of Fe^{2+} oxidizing nitrate reducers in freshwater lake sediments (Melton et al., 2012, 2014); indicating that these organisms are likely to be subject competition for Fe^{2+} with photoferrotrophs in illuminated sediments (Melton et al., 2012) and may coexist with heterotrophic denitrifying organisms (Melton et al., 2014). Investigation of iron oxidizing nitrate reducers have also been carried out on shallow (0.5–1 m) brackish sediments (Laufer, Røy, et al., 2016; Laufer, Nordhoff, et al., 2016); adding to our limited knowledge on vertical distribution and potential phylogenies of these organisms in natural environments. Despite the increasing number of studies into Fe^{2+}-driven nitrate reduction, the use of high (milimolar) substrate concentrations in enrichment studies can cause complications in interpreting observations (Edwards, 1970); thus little is known about the quantitative contribution to nitrate reduction in natural environments.

Recent studies have inferred Fe^{2+} as an important controlling factor regulating the partitioning between denitrification and DNRA in sediments of the salt wedge estuary of the Yarra River, Australia (Roberts et al., 2014; Robertson et al., 2016). Here, the varying extent of the salt wedge structure causes fluctuations in bottom-water oxygen concentrations due to changing water column stratification (Roberts et al., 2012; Bruce et al., 2014). DNRA rates were shown to increase under oxic conditions, when porewater Fe^{2+} concentrations peaked (Roberts et al., 2012, 2014); an observation which is in contrast to the common observation of reducing (lower oxygen) conditions favoring DNRA (discussed above). Further investigation using slurry experiments conducted at several sites along the estuarine gradient demonstrated that Fe^{2+} oxidation was linked to DNRA while denitrification was unaffected or, in some cases, reduced in response to increases in Fe^{2+} availability (Robertson et al., 2016).

The investigations in the Yarra estuary suggest that Fe^{2+} availability may influence the retention or removal of dissolved inorganic N in sediments, however the quantitative contribution of these Fe^{2+}-driven processes to environmental N and Fe cycling in other aquatic systems, and their environmental controls are poorly understood. We investigated nitrate-reducing processes in the iron-rich sediments of Lake Almind. Our primary objective was to determine to which extent nitrate reduction is coupled to Fe^{2+} oxidation and how this may influence the fate of nitrate in this freshwater system. We also aimed to further characterize the effect of electron donor availability on nitrate reduction pathways by varying the availability of Fe^{2+} and acetate in slurry experiments at environmentally relevant substrate concentrations as well as at concentrations comparable to previous enrichments studies. Our results provide new insights into the dynamics of Fe^{2+}-fueled NO_x-reducing processes in an iron-rich fresh water system.

2. METHODS

2.1. Study site

Lake Almind is an oligotrophic lake near Silkeborg, Denmark, with an area of 0.52 km^2 and a maximum depth of approximately 20 m (Jørgensen et al., 2011). During summer months, thermal stratification of the water column results in reduced mixing and hypoxia in bottom-waters. Sediment and water samples were collected from the deepest point of Lake Almind in May 2013 and June 2014. Depth profiles of water column temperature and oxygen were determined with an oxygen electrode (YSI 55 Dissolved Oxygen sensor). Water samples were collected in a 2 L Niskin bottle for water column depth profiles and bottom-water for use in incubations. Nutrient (NO_3^-, NO_2^-, NH_4^+ and SO_4^{2-}) samples were filtered (0.2 μm) in the field and frozen upon return to the laboratory until analysis. Samples for dissolved Fe^{2+} were preserved with sulfamic acid (final concentration in samples 40 mM as suggested by Klueglein and Kappler, 2013) and stored at 4 °C until analysis. Following water sampling, sediment was collected in 5 cm (diameter) plastic core liners using a manual corer and returned to the University of Southern Denmark (SDU). Cores that were not processed immediately for sediment depth profiling were stored in the dark in temperature controlled rooms within 2 °C of *in situ* bottom-water temperature. Cores for sediment depth profiles were collected only in 2014.

2.2. Sediment depth profiles

Upon returning to SDU, cores collected in 2014 were sectioned every 0.5 cm in the upper 3 cm of sediment cores and subsequently every 1 cm below 3 cm. Core slicing was carried outin an anaerobic glove bag (N_2 atmosphere) on the day of collection. Samples were taken for pH (duplicate cores), solid-phase iron (triplicate cores; porosity/water content, weak acid extractible iron, organic content) and pore water constituents (triplicate cores; NO_3^-, NO_2^-, NH_4^+ and SO_4^{2-}-, Fe^{2+}, H_2S, DIC). Solid phase samples for analysis of Fe and organic content were stored in 50 mL plastic tubes and sealed inside the glove bag before being frozen until analysis. Samples for pH were sealed, removed from the glove bag and immediately measured with a calibrated pH meter (Meterlab PHM210). Samples for pore water constituents were sealed in 15 mL tubes inside the glove bag and centrifuged (20,000 rcm, 5 min). The supernatant was removed and filtered (0.2 μm) into samples for nutrients (frozen), Fe^{2+} (acidified in the field as described above and stored at 4 °C), DIC (filtered into vials without air space, stored at 4 °C) and H_2S (fixed in 20 μL 20% zinc acetate solution mL^{-1} stored at 4 °C).

2.3. Bio-reactor experiments

For bioreactors and serum vial experiments (Section 2.4), sediment cores were collected and stored at close to bottom-water temperature in the dark for one to two days before use in slurry experiments. Sediment collected in May 2013 was used to create dilute sediment slurries in bioreactors designed for anoxic, headspace-free incubation (described in Dalsgaard et al., 2014) to assess the influence of Fe^{2+} on nitrate reduction processes. Briefly, reactors consisted of a glass cylinder (inner diameter 9 cm) with a piston consisting of a 8.9 cm diameter PVC disk and metal supporting rods with cut-off glass syringe in the center. The piston fitted tightly in the cylinder by means of two o-rings, and the PVC disk was covered in glass to reduce oxygen diffusion from the plastic into the reactor. Reactors were filled to 1 L with filtered (0.2 μm), helium-purged lake bottom-water and any air space was removed. Surface (top 2 cm) sediment was added to water-filled reactors through inlet tubes at the top of the reactor to achieve a slurry of approximately 1:100 sediment to water. Slurries were mixed using glass-coated magnetic stirring bars and kept in darkness in a large water bath in a temperature-controlled room (12 °C) to prevent temperature increases due to the motors in stirring plates. Following sediment addition, slurries were allowed to mix overnight in the dark to remove residual oxygen and NO_x. Substrates and samples were added and removed through the inlet tubes in the top of the reactors. Anoxically prepared solutions of ^{15}N-nitrate and $Fe^{II}Cl_2$ were added to reactors (Table 1). Samples were taken before and immediately after substrate addition and then every ~24 h for ~10 days after substrate addition. Liquid samples were removed for gas measurements by overflowing the sample into 3 mL Exetainers (Labco, UK) and preserving with formaldehyde (50 μL, 37%) to prevent microbial activity before being sealed without headspace. A helium headspace was introduced to gas samples prior to measurement of ^{15}N-N_2 and ^{15}N-N_2O isotopes (see below). Samples for nutrients were filtered (0.2 μL) and frozen until measurement and samples for Fe^{2+} were preserved with sulfamic acid.

2.4. Serum vial experiments

Later experiments were conducted in serum vials in order to increase the number of treatments and replicates

Table 1
Overview of experiments and treatments conducted in 2013 and 2014. Shaded areas highlight enrichment ('EN') treatments.

Experimental setup	Treatment	^{15}N substrate µmol L^{-1}	Fe^{2+} addition µmol L^{-1}	Acetate µmol L^{-1}	Replicates
Reactors 2013	$^{15}NO_3^-$	25	0	0	1
	$^{15}NO_3^- + Fe^{2+}$	25	250	0	1
	EN $^{15}NO_3^-$	1000	0	0	1
	EN $^{15}NO_3^- + Fe^{2+}$	1000	5000	0	1
Serum Vials 2013	$^{15}NO_3^-$	30	0	0	3
	$^{15}NO_3^- + Fe^{2+}$	30	200	0	3
	$^{15}NO_3^-$ + Acetate	30	0	100	3
	$^{15}NO_3^-$ + Acetate + Fe^{2+}	30	200	100	3
	EN $^{15}NO_3^-$	1000	0	0	2
	EN $^{15}NO_3^- + Fe^{2+}$	1000	5000	0	2
	EN $^{15}NO_3^-$ + Acetate	1000	0	1000	2
	EN $^{15}NO_3^- + Fe^{2+}$ + Acetate	1000	5000	1000	2
Serum Vials 2014	$^{15}NO_3^-$	30	0	0	3
	$^{15}NO_3^-$ + Low Fe^{2+}	30	100	0	3
	$^{15}NO_3^-$ + High Fe^{2+}	30	500	0	3
	$^{15}NO_2^-$	30	0	0	3
	$^{15}NO_2^-$ + Low Fe^{2+}	30	100	0	3
	$^{15}NO_2^-$ + High Fe^{2+}	30	500	0	3
	Fe^{2+}	0	500	0	3

in a more manageable way. Sediment collected in May 2013 was also incubated in serum vial experiments to determine the effects of varying electron donors – Fe^{2+} and acetate – on nitrate reduction pathways (Table 1). Previous studies investigating Fe^{2+}-driven nitrate reduction have used enrichment under high substrate conditions (e.g. 10 mM nitrate, 10 mM Fe^{2+}; Klueglein and Kappler, 2013). In order to investigate how these conditions may influence process rates and pathways, we subjected parallel sets of slurry incubations (in bioreactors and in serum vials) to comparable high substrate concentrations. Additionally, several studies investigating these processes have observed that isolated microbial strains utilize a mixotrophic metabolism; where an organic co-substrate is oxidized as well as $Fe^{2+}$by nitrate reducing organisms (e.g. Straub et al., 1996; Kappler et al., 2005; Muehe et al., 2009). To investigate the use of alternative electron donors to Fe^{2+}, in 2013 sediment slurries were also amended with acetate (Table 1). Sediment collected in June 2014 was used to determine the influence of two different Fe^{2+} concentrations on the reduction of ^{15}N-nitrate or -nitrite (Table 1) in serum vial experiments.

Slurry experiments were carried out as described in (Robertson et al., 2016). Briefly, dilute (1 in 100) sediment slurries were made in serum vials (May 2013: 160 mL; June 2014: 120 mL) using surface (0–2 cm) lake sediment and filtered (0.2 µm) site water, leaving a known volume of gas headspace. The slurry was purged with helium and then incubated in the dark overnight on a shaker table within 2 °C of bottom-water temperature to allow organisms to consume residual oxygen and nitrate. Initial pH before iron additions was noted and anoxically prepared $Fe^{II}Cl_2$ solution was added to the final concentrations given in Table 1. The pH was adjusted again to that of control vials without Fe^{2+} addition (pH ~7–7.2) with sterile 0.5 M NaOH. During this preincubation period, concentrations of Fe^{2+} and pH were monitored in slurries to ensure they were stable over time before addition of ^{15}N substrates.

During ^{15}N substrate addition and subsequent samplings, sediment slurries were transferred briefly to an anaerobic chamber (N_2 atmosphere) to prevent oxidation of Fe^{2+} in air. Helium-purged $Na^{15}NO_3$, $Na^{15}NO_2$ and sodium acetate solutions were added to slurries as described in Table 1. Following substrate addition, samples of gas headspace (2 ml) for ^{15}N-N_2 and ^{15}N-N_2O measurement were withdrawn from the headspace using a glass syringe and transferred to prefilled 3 ml Exetainers (LabCo, UK) in exchange for water (He-purged, 5 ml 50% w/v $ZnCl_2$ L^{-1}). For nutrient and iron samples, 4.5 mL He was forced into serum vials and the same volume of sample was withdrawn so as not to leave over- or under-pressure inside vials. Samples for iron were acidified using sulfamic acid as before and measured immediately after sampling. Samples for nutrients were filtered (0.2 µm) and frozen until analysis. The pH of the slurries was monitored following each sampling point and adjusted if necessary with sterile 0.5 M HCl or NaOH. Headspaces of vials were exchanged with Helium before being returned to the shaking table between sampling points.

2.5. Chemical analyses

Samples for ^{15}N-N_2 and ^{15}N-N_2O were injected into a Gas Chromatograph, passed through a reduction oven (600 °C), which reduces N_2O quantitatively to N_2 and measured on an Isotope Ratio Mass Spectrometer (GC-IRMS) as described in Dalsgaard et al. (2013). A cumulative total of ^{15}N-N_2 production was calculated, with measurements being corrected for changing sample and headspace volumes. Any ^{15}N in ammonium was also measured on GC-

IRMS with prior conversion to N_2 by alkaline hypobromite iodine (Risgaard-Petersen et al., 1995; Füssel et al., 2012) with a recovery efficiency of >95%.

In samples from water column profiles, sediment depth profiles and slurry incubations collected in May 2013, NO_3^-, NO_2^-, SO_4^{2-} and acetate were determined on an Ion Chromatograph (Dionex ICS-1500). Nitrite in sediment slurries collected in June 2014 were determined photometrically (modified from Grasshoff et al., 1999). Total ammonium concentrations in sediment depth profiles and slurry experiments (2014 only) were determined photometrically using the salicylate-hypochlorite method (Bower and Holm-Hansen, 1980). Dissolved (filterable) iron samples in water profiles, sediment profiles and slurry incubations preserved with sulfamic acid were measured photometrically using Ferrozine assay (Stookey, 1970; Viollier et al., 2000). Porosity was determined by weighing a known volume of each sediment horizon and drying overnight at 105 °C to calculate water content. Reactive Fe was extracted in dithionite-citrate-acetic acid solution for 2 h on a shaker table (Lord, 1980; Thamdrup et al., 1994). Combined Fe^{2+} and Fe^{3+} were determined using the Ferrozine assay after extraction. Sulfide concentrations in sediment were determined photometrically using Cline reagents (Cline, 1969). DIC was measured by flow injection analysis (Hall and Aller, 1992). Organic content of sediments was determined by combustion of known weights of dried (105 °C as above) sediment at 520 °C for 8 h.

Nutrient samples were filtered and frozen until analysis, however nitrite can undergo abiotic reaction with Fe^{2+} in low pH brine formed during freezing. This was shown by an incomplete total ^{15}N recovery during time points where nitrite accumulated in slurries, whereas following nitrite consumption, almost all (~90%) ^{15}N was recoverable (Supplementary Fig. S1).

Statistical significances between replicates of each treatment were determined using a Student's *t*-test. Michaelis Menten kinetics were calculated by applying a non-linear fit to data from the present study as well as data compiled from Robertson et al. (2016) in GraphPad Prism 7 software.

3. RESULTS

3.1. Site description

Depth profiles of the water column of Lake Almind were sampled in 2013 and June 2014 (data not shown). Bottom-water temperatures were ~6 °C and ~10 °C in 2013 and 2014, respectively. Oxygen was always >300 $\mu mol\ L^{-1}$ throughout the water column in 2013, while in 2014 oxygen declined below 12 m depth to less than 10 $\mu mol\ L^{-1}$ in the bottom-water. Sulfate concentrations were ~200 $\mu mol\ L^{-1}$ throughout the water column in both 2013 and 2014. In 2013, nitrate was detectable throughout the water column, increasing from ~3 $\mu mol\ L^{-1}$ to ~8 $\mu mol\ L^{-1}$ in deeper water layers. In 2014, dissolved Fe^{2+} was undetectable throughout the water column until 19 m depth where ~25 $\mu mol\ L^{-1}$ was measured.

Sediment depth profiles of pore water and solid phase constituents were taken in June 2014. Only values from the surface (0–5 cm) sediment are reported here (data not shown). Typical of Danish lakes of this size (Jørgensen et al., 2011), Lake Almind sediments had a high organic content; decreasing from ~30% in surface sediment to ~20% in deeper layers. Profiles of pH increased from a surface value of ~7.4 to ~7.5 in subsurface layers. DIC increased with depth from a surface concentration ~2.5 to 3 $mmol\ L^{-1}$ at 5 cm. Nitrate increased from undetectable concentrations in surface sediment to 5–10 $\mu mol\ L^{-1}$ between 0.5 and 3 cm depth and was depleted at 4.5 cm. Nitrite was undetectable in sediment. Dissolved Fe^{2+} in pore water samples were ~250 $\mu mol\ L^{-1}$ in surface layers and increased with depth to >500 $\mu mol\ L^{-1}$ at 5 cm depth. Highly reactive solid-phase Fe(III) extracted with dithionite increased from ~75 $\mu mol\ cm^{-3}$ in the upper 0.5 cm to over 150 $\mu mol\ cm^{-3}$ at 3 cm depth. Dithionite extractable Fe (III) remained below 30 $\mu mol\ cm^{-3}$ throughout the upper 5 cm of sediment. Sulfate concentrations in surface sediment were on average 45 $\mu mol\ L^{-1}$ and were depleted to ~10 $\mu mol\ L^{-1}$ below 3 cm. Free sulfide was always <0.5 $\mu mol\ L^{-1}$ at the sediment depths investigated.

3.2. Sediment slurry incubations: effect of Fe^{2+} addition on NO_x reduction

Initial sediment slurry incubations were carried out in bioreactors. However to increase ease of replication and still avoid oxygen contamination in experiments, later incubations were carried out in serum vials sampled within an anaerobic chamber. Rates determined in both of these experimental set ups are shown in Table 1. General trends were observed across all treatments and experiments exposed to lower (≤35 μM) substrate concentrations following the addition of ^{15}N (and additional) substrates (Fig. 1). Nitrate consumption began immediately after addition, accompanied by linear increases in ^{15}N-N_2 and $^{15}NH_4^+$ and consumption of Fe^{2+}. In experiments with low initial nitrate concentrations (25–30 $\mu mol\ L^{-1}$), nitrite typically accumulated over the first 48 h of experiments before being entirely consumed. Under these conditions, ^{15}N-N_2O was also observed to accumulate in slurry experiments before being consumed. Production of ^{15}N-N_2 and $^{15}NH_4^+$ continued as long as nitrate/nitrite/N_2O was measurable in slurries, and ceased after their depletion. All nitrate was typically consumed with ~3–4 days. In all experiments, the isotopic composition of ^{15}N-N_2 (measured as $^{14}N^{15}N$ and $^{15}N^{15}N$) matched that predicted from random isotope pairing through denitrification (data not shown), which excludes a significant contribution of anammox to N_2 production (Thamdrup and Dalsgaard, 2002). Thus we rule out potential complications with the application of ^{15}N stable isotope methods when denitrification, anammox and DNRA cooccur (Song et al., 2013, 2016).

In an initial bioreactor experiment, 10 mL sediment was added to 1 L filtered, helium-purged lake water and ^{15}N-substrates were added. At the time of ^{15}N-subtrate addition, Fe^{2+} concentrations were ~35 $\mu mol\ Fe^{2+}\ L^{-1}$ (Fe^{2+} from sediment addition) in control experiments with only

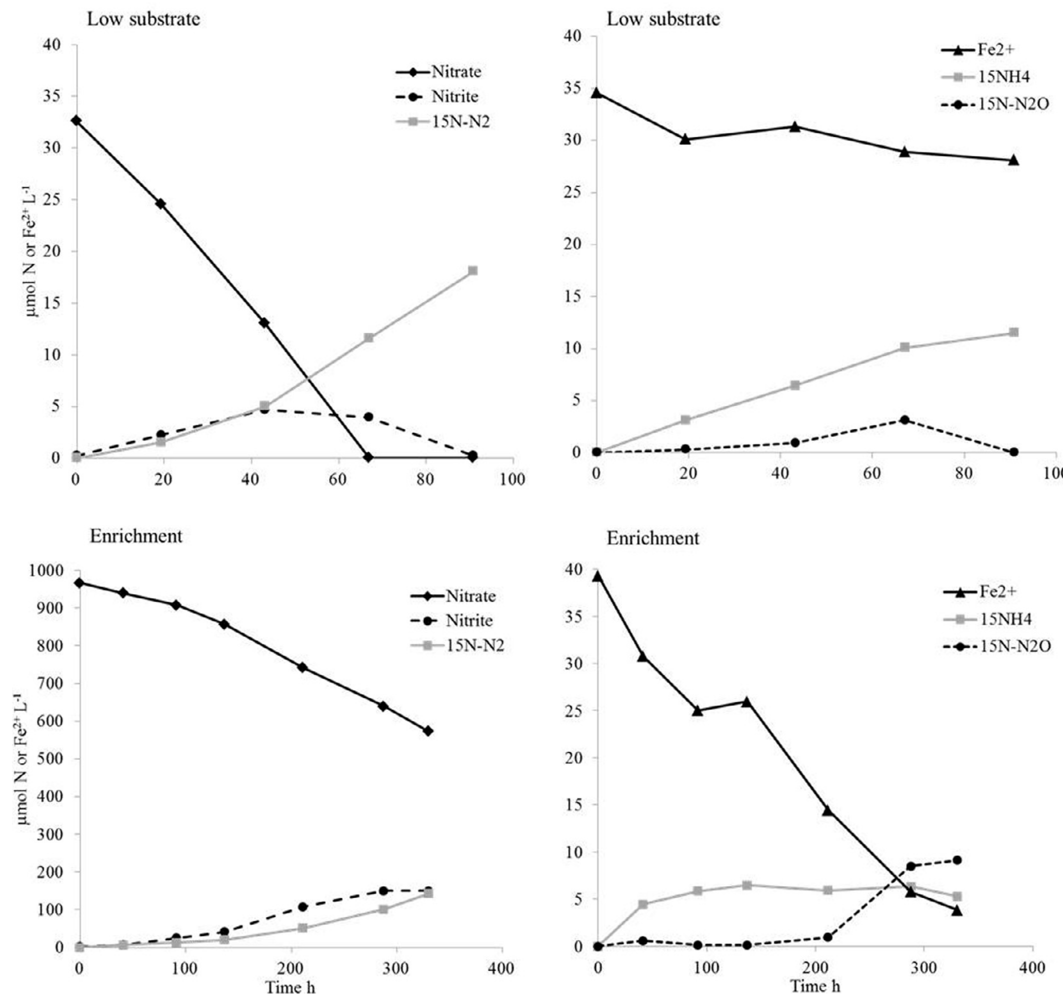

Fig. 1. Typical progression of dissolved and gaseous constituents during serum vial slurry experiments at low (top) and enrichment (bottom) ^{15}N-nitrate concentrations without Fe^{2+} additions.

^{15}N-nitrate, while those supplemented with additional Fe^{2+}, initial concentrations were $\sim$220 µmol Fe^{2+} L^{-1}. In the reactor with added Fe^{2+}, the DNRA rate was enhanced by 76% relative to control reactors, while denitrification was suppressed by 47% (Table 2). Fe^{2+} addition also increased rates of Fe^{2+} removal, nitrate reduction, and nitrite accumulation while N_2O accumulation was reduced. In serum vial experiments conducted in 2013, similar patterns were observed as in bioreactor experiments, although no significant differences were observed between control and Fe^{2+}-amended treatments. Rates of DNRA and Fe^{2+} removal were increased while denitrification was reduced. The lack of significant differences in these serum vial experiments may be due to differences in initial Fe^{2+} concentrations between control ($\sim$45 µmol L^{-1}) and Fe^{2+}-amended ($\sim$80 µmol L^{-1}) vials being comparatively smaller than in bioreactor experiments.

In 2014, serum vial experiments were carried out with ^{15}N-nitrate and two different Fe^{2+} amendments (Table 1; Fig. 2). Dissolved Fe^{2+} concentrations at the time of ^{15}N-substrate addition and after addition of Fe^{2+} (doses shown in Table 1) were $\sim$35, $\sim$165 and $\sim$290 µmol L^{-1} in control, low and high Fe^{2+} additions, respectively. DNRA rates were significantly enhanced ($p < 0.05$) with the low Fe^{2+} additions relative to the control (Table 2). In these experiments, Fe^{2+} removal was also significantly enhanced and nitrite reduction significantly reduced. In experiments with high Fe^{2+} concentrations, DNRA rates were also enhanced relative to control vials, accounting for 55% of nitrate reduction end products. Denitrification in experiments with high Fe^{2+} additions was significantly reduced compared to rates in experiments with low Fe^{2+} addition although not compared to controls with no added Fe^{2+} due to high variability in the later (Fig. 2). Fe^{2+} removal was also enhanced relative to control vials. No significant differences were observed between rates of NO_x reduction, nitrite accumulation or N_2O accumulation between control and high Fe^{2+} addition experiments. All slurries had an initial total ammonium ($^{14}NH_4^+ + {}^{15}NH_4^+$) concentration of $\sim$50 µmol L^{-1} which increased at rates approximately equal to or slightly greater than that of $^{15}NH_4^+$ production (data not shown). This indicates the majority of ammonium

Table 2
Average rates (all μmol N L^{-1} d^{-1} or μmol Fe^{2+} L^{-1} d^{-1}) from slurry experiments (see Table 1 for number of replicates); (SD); (*): data significantly (t-test; $p < 0.05$) different from control experiments with only ^{15}N-substrate addition. Superscript numbers ([1 2 3 4 5]): values with the same numbers are significantly (t-test; $p < 0.05$) different from each other. 'acc.': accumulation. Shaded areas highlight enrichment ('EN') treatments.

Experimental setup	Treatment	Denitrification to N_2	DNRA	Fe^{2+} removal	NO_x reduction	Nitrite acc.	^{15}N-N_2O acc.
Reactors 2013	$^{15}NO_3^-$	1.9	1.7	−3.7	−7.2	−0.3	1.1
	$^{15}NO_3^- + Fe^{2+}$	1	3	−25.2	−8.2	1.3	0.5
	EN $^{15}NO_3^-$	5	0.4	−61.6	−39.3	19.5	2.1
	EN $^{15}NO_3^- + Fe^{2+}$	2.4	3.7	−125.6	−13.6	1.3	0.1
Serum Vials 2013	$^{15}NO_3^-$	4.7 (1.5)	2.5 (0.4)	−14.5 (7.9)	−14.3 (1.8)	4.4 (1.6)	0.3 (0.3)
	$^{15}NO_3^- + Fe^{2+}$	3.2 (1.6)	2.8 (0.5)	−17.7 (9.6)	−13.2 (2.3)	3.2 (1.4)	1.2 (1.0)
	$^{15}NO_3^-$ + Acetate	5.8 (0.9)	4.3 (0.5)	−3.7 (5.3)	−16.9 (0.9)	0.2 (1.7)	1.7 (0.9)
	$^{15}NO_3^-$ + Acetate + Fe^{2+}	4.8 (1.1)	5.3 (1.0)	−5.1 (2.5)	−15.9 (2.2)	0.5 (0.2)	1.8 (0.3)
	EN $^{15}NO_3^-$	4.5 (1.5)	1.6 (0.1)	−4.0 (2.3)	−16.6 (2.8)	7.2 (0.3)	−0.03 (0.03)
	EN $^{15}NO_3^- + Fe^{2+}$	0.3 (0.06)	3.6 (1.3)	−81.2 (16.1)*	−6.7 (1.8)	2.5 (2.0)	0.1 (0.1)
	EN$^{5}NO_3^-$ + Acetate	16.3 (14.4) [1]	2.8 (1.0)	−1.2 (0.06) [4]	−159.7 (7.0)*	24.6 (9.8) [5]	7.5 (9.6)
	EN $^{15}NO_3^- + Fe^{2+}$ + Acetate	0.2 (0.1) [1]	2.3 (0.2)	−70.5 (0.2) [4]	−125.1 (31.6)	76.8 (18.5) [5]	7.1 (9.9)
Serum Vials 2014	$^{15}NO_3^-$	4.8 (0.8)	3.7 (0.1)	−1.8 (0.4)	−11.5 (0.1)	1.0 (0.4)	1.32 (0.2)
	$^{15}NO_3^-$ + Low Fe^{2+}	5.9 (0.4) [2]	4.4 (0.1)*	−6.1 (0.3)*	−11.7 (0.4)	0.18 (0.1)*	0.99 (0.6)
	$^{15}NO_3^-$ + High Fe^{2+}	3.5 (0.5) [2]	4.3 (0.1)*	−9.0 (1.0)*	−11.6 (0.1)	0.70 (0.4)	1.00 (1.0)
	$^{15}NO_2^-$	4.2 (1.0)	3.7 (0.2)	−2.4 (0.5)	−7.9 (0.9)	n/d	1.4 (0.2)
	$^{15}NO_2^-$ + Low Fe^{2+}	4.1 (0.4) [3]	3.6 (0.5)	−7.1 (0.7)*	−7.8 (0.5)	n/d	1.2 (0.05)
	$^{15}NO_2^-$ + High Fe^{2+}	3.2 (0.2) [3]	3.6 (0.2)	−8.0 (0.9)*	−6.8 (0.3)	n/d	1.5 (0.2)
	Fe^{2+}	n/d	n/d	−1.1 (1.1)	n/d	n/d	n/d

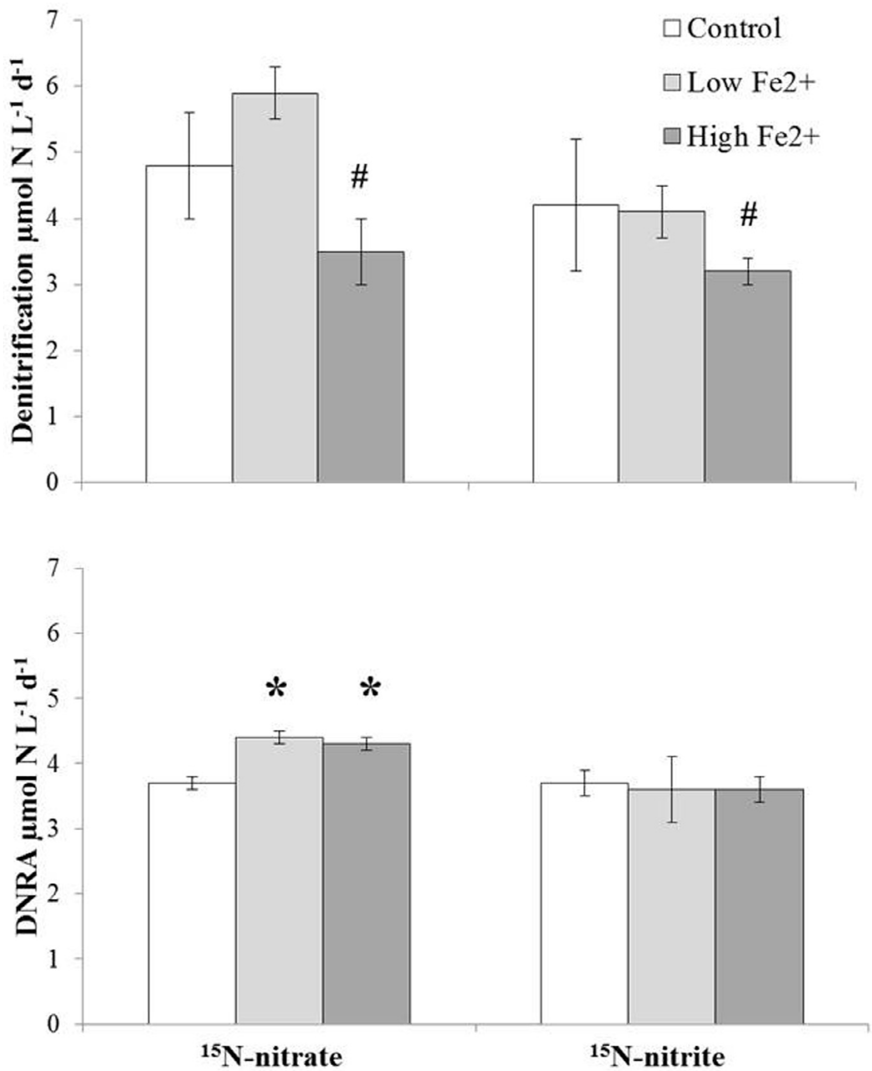

Fig. 2. Rates of denitrification and DNRA from serum vial experiments conducted in 2014 using ^{15}N-nitrate and -nitrite additions. Columns with '*' indicates a significant difference from controls; Columns with '#' indicates a significant difference from experiments with Low Fe^{2+} additions.

formation in slurries was from DNRA and a smaller proportion was produced from organic matter oxidation – most likely coupled to organotrophic denitrification.

Correlations between rates of $^{15}NH_4^+$ production and Fe^{2+} removal in ^{15}N-nitrate and ^{15}N-nitrate + Fe^{2+} incubations carried out in 2014 indicated a stoichiometry of Fe^{2+} oxidation and DNRA of 8.2:1, close to the predicted stoichiometry of 8:1 of Fe^{2+}-driven DNRA (Eq. (2)). The determined kinetic parameters (V_{max} and K_m) for Fe^{2+}-driven DNRA were 4.6 µmol N L^{-1} d^{-1} and 33.8 µmol Fe^{2+} L^{-1}, respectively. In addition, an inhibitory effect of increasing Fe^{2+} concentration on denitrification is also apparent (Figs. 3 and 5). No clear relationships were apparent between nitrate concentrations and denitrification or DNRA rates when kinetic fits were applied (data not shown).

In 2014, parallel slurry experiments were carried out with addition of ^{15}N-nitrite (Table 2). Initial dissolved Fe^{2+} concentrations were ~35, ~170 and ~290 µmol L^{-1} in control, low and high Fe^{2+} additions, respectively. Rates in experiments with only ^{15}N-nitrite were not significantly different to those observed in ^{15}N-nitrate experiments apart from the NO_x reduction rate, which was significantly lower ($p< 0.05$, t-test) with ^{15}N-nitrite than with ^{15}N-nitrate. In ^{15}N-nitrite experiments, Fe^{2+} amendments had no significant effect on DNRA rates, although DNRA on average increased slightly from 46 to 47 and 53% of nitrate reduction from control to low and high Fe^{2+} experiments, respectively. Fe^{2+} removal was enhanced relative to controls in both Fe^{2+} treatments. Denitrification was significantly reduced in high compared to low Fe^{2+} experiments.

3.3. Sediment slurry incubations: effect of enrichment conditions

A typical progression of compounds in the sediment slurry incubations conducted under enrichment conditions

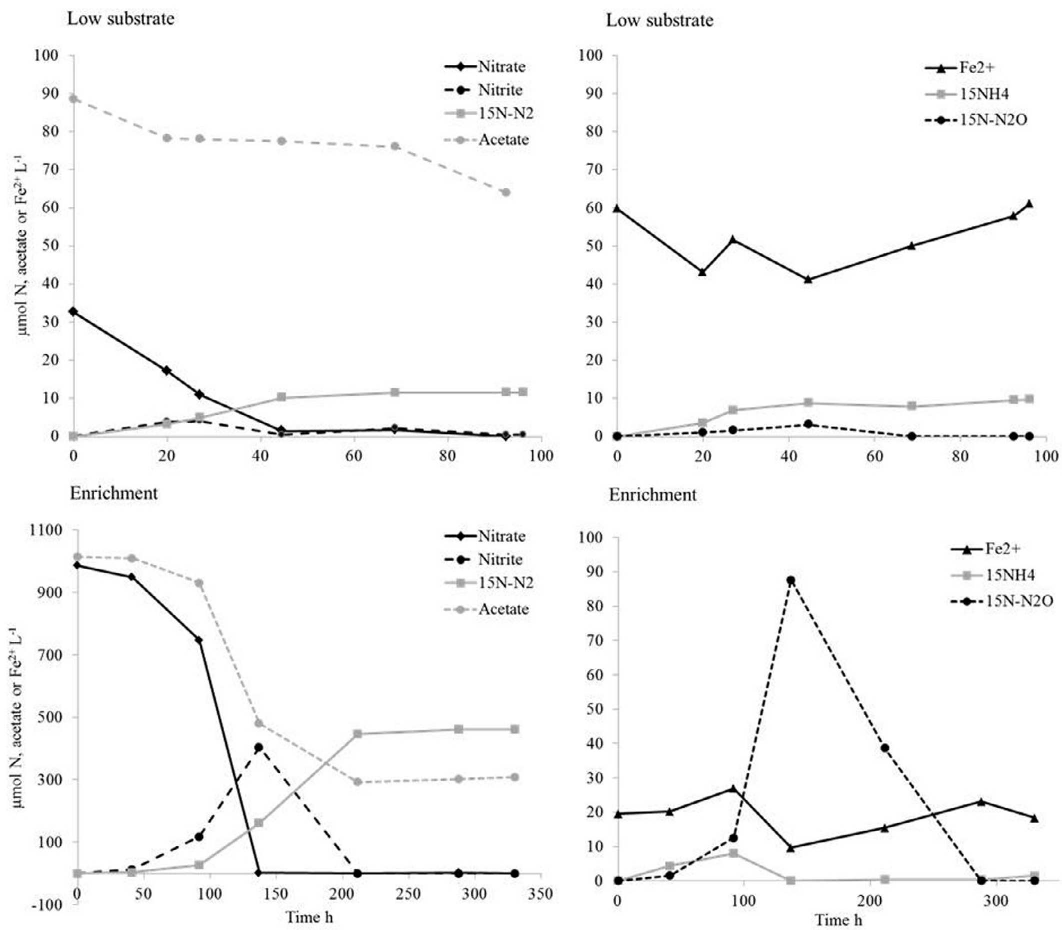

Fig. 3. Typical progression of dissolved and gaseous constituents during serum vial slurry experiments with acetate additions at low (top panel) and enrichment (bottom panel) ^{15}N-nitrate concentrations without Fe^{2+} additions.

(1 mmol L^{-1} NO_3^-, 5 mmol L^{-1} Fe^{2+}; Table 1) is shown in Fig. 1. Nitrate and Fe^{2+} were consumed approximately linearly throughout these experiments, while nitrite accumulated continually throughout incubations without being fully consumed by the end of experiments. An acceleration was observed in the production of both N_2O and N_2, with production rates increasing after ~5–6 days from initial rates that were slightly higher than at low nitrate concentrations. In contrast, DNRA rates slowed after the first 2 days and activity ceased after ~5–6 days after an initial production of $^{15}NH_4^+$ that was also slightly higher than at low nitrate concentrations (Fig. 1). As process rates in enrichment experiments were calculated from the initial four time points (Table 2), which represents a longer time span than in low substrate experiments (i.e. 150 h rather than 65 h), the, DNRA rates in enrichment experiments are likely to be somewhat underestimated due to the deceleration of $^{15}NH_4^+$ accumulation. In the investigations carried out in bioreactors, denitrification was the dominant pathway of nitrate reduction (93%) while DNRA accounted for a much smaller fraction of nitrate reduction than observed under low substrate concentrations (Table 2). Fe^{2+} removal, nitrate reduction, and nitrite and N_2O accumulation were enhanced relative to parallel experiments at low substrate concentrations. The addition of 5000 μmol L^{-1} Fe^{2+} to bioreactor experiments reduced the denitrification rate and increased DNRA ~10-fold to account for 60% of total nitrate reduction. Fe^{2+} removal was also enhanced, while nitrate reduction, nitrite- and N_2O-accumulation rates were reduced.

Serum vial experiments using the same enrichment conditions and sediment showed similar effects as the bioreactor experiment. Thus, denitrification accounted for a greater proportion of nitrate reduction under high ^{15}N-nitrate experiments (74%) than in parallel experiments under low substrate concentrations (65%; see above). The addition of Fe^{2+} to ^{15}N-nitrate experiments at enrichment concentrations resulted in a reduction in denitrification, accounting for only 6% of nitrate reduction, and increases in DNRA and Fe^{2+} removal rates (Fig. 4). Nitrate reduction and nitrite accumulation rates were also reduced.

3.4. Sediment slurry incubations: effect of acetate addition

Results of sediment slurry experiments amended with acetate at both environmentally relevant and high substrate concentrations are shown in Table 1. A typical progression of dissolved and gaseous compounds in experiments at

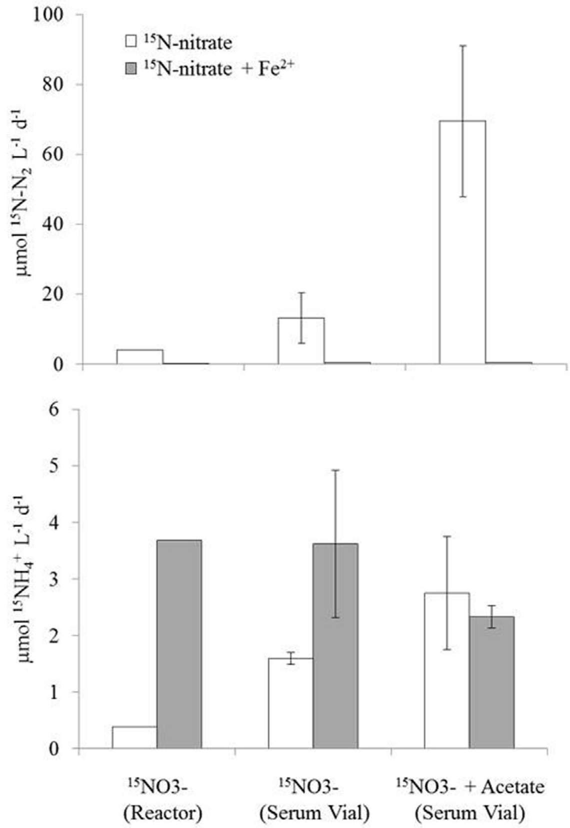

Fig. 4. Production of ^{15}N-N_2 (top panel) and $^{15}NH_4^+$ under enrichment ('EN') conditions in experiments conducted in 2013 in bioreactors ('Reactor'; $n = 1$) and Serum Vials ($n = 2$).

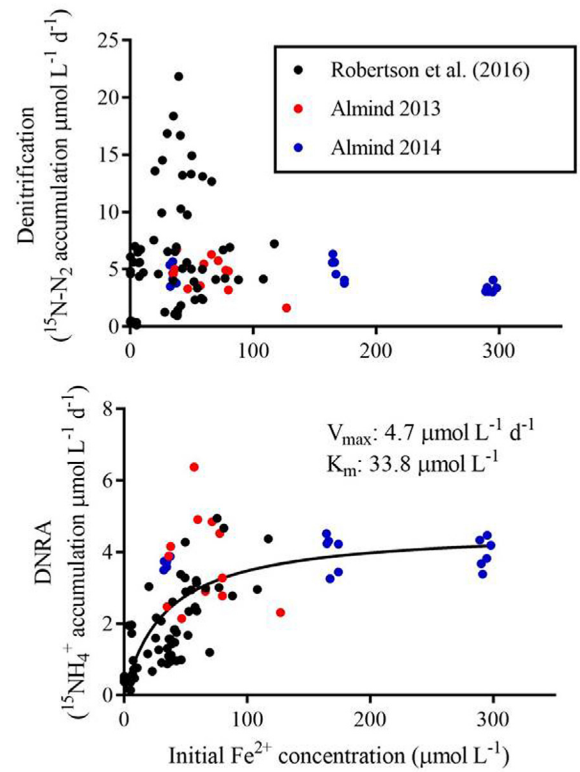

Fig. 5. Individual rates (used for averages in Table 2) denitrification (as ^{15}N-N_2 production; top panel) and DNRA (as $^{15}NH4^+$ production; bottom panel) plotted as a function of initial Fe^{2+} concentrations immediately following ^{15}N-nitrate addition. Data compiled from all non-enrichment serum vial slurry incubations (all $^{15}NO_3^-$, $^{15}NO_2^-$, Fe^{2+} and acetate experiments) carried out in 2013 and 2014 in the present study plotted with data from Fig. 7 in Robertson et al. (2016).

environmentally relevant concentrations and enrichment conditions is shown in Fig. 3. As in experiments without acetate, nitrate was consumed linearly leading to sequential accumulation and depletion of nitrite and N_2O. $^{15}NH_4^+$ and ^{15}N-N_2 accumulated approximately linearly and ceased when nitrate, nitrite and N_2O was depleted. Acetate was consumed linearly throughout the experiment and was not fully depleted by the end of experiments. Dissolved Fe^{2+} was initially consumed. However, when nitrate became depleted, Fe^{2+} concentrations began to increase, most likely due to dissimilatory reduction of Fe(III) with remaining acetate.

In acetate-amended experiments exposed to enrichment conditions (Fig. 3), nitrate consumption was initially slow before a more rapid consumption to depletion after 5–6 days. Acetate consumption also followed this pattern, with acetate and NO_x being consumed at a molar ratio of 0.73:1. In parallel experiments without acetate, less than 50% of the added nitrate (~1000 µmol L^{-1}) had been consumed by the end of the experiment (approximately 14 days; see Figs. 1 and 3). $^{15}NH_4^+$ initially accumulated at rates comparable to low substrate experiments but was then consumed. In this way, DNRA rates were potentially underestimated due to rates being derived from the first four time points (Fig. 3), as discussed above. Production of ^{15}N-N_2 showed a similar lag phase as in enrichment experiments without acetate, with maximum rates reached after ~5 days. ^{15}N-N_2 production ceased once nitrate, nitrite and N_2O were depleted. There was no significant consumption of Fe^{2+} throughout these experiments.

Under low substrate concentrations, significant ($p < 0.05$) changes were observed in DNRA (increase) and nitrite accumulation (decrease) in slurries with added acetate relative to control vials with only ^{15}N-nitrate. Weak stimulation of denitrification and a reduction in Fe^{2+} removal was also measured, however these were not deemed significantly different from control rates. The addition of Fe^{2+} to these experiments made no significant changes to process rates relative to acetate-amended vials without Fe^{2+}.

In serum vials provided with high substrate concentrations of ^{15}N-nitrate and acetate, denitrification, nitrate reduction and nitrite- and N_2O-accumulation were stimulated relative to those vials with only 1000 µmol L^{-1} ^{15}N-nitrate (Table 2). DNRA rates slightly increased while Fe^{2+} removal was reduced. The addition of Fe^{2+} to parallel incubations with ^{15}N-nitrate and acetate caused a large reduction in denitrification while Fe^{2+} removal and nitrite accumulation increased. Small changes were measured in rates of DNRA and N_2O accumulation.

4. DISCUSSION

4.1. Fe^{2+}-fueled DNRA

Nitrate reduction coupled to Fe(II) oxidation has become increasingly well studied in microbial cultures and environmental enrichments since it's discovery (Straub et al., 1996), however twenty years later the contribution of this process to nitrogen and iron cycling in natural environments is still poorly understood. In this study we demonstrate that relatively small changes in Fe^{2+} concentration can cause shifts in the fate of nitrogen in freshwater lake sediment. In slurries of Lake Almind sediment, denitrification was the dominant route of nitrate reduction when only ^{15}N substrates (nitrate or nitrite) were added, leading to a removal of N as gaseous N_2 (and intermediate N_2O) and a smaller proportion being reduced through DNRA. In 7 of 10 types of slurry experiments (Table 2) under either environmental or enrichment conditions, the addition of Fe^{2+} increased rates of DNRA relative to parallel incubations without Fe^{2+} addition, and in some cases it also reduced denitrification rates. The direct stimulation of ^{15}N-ammonium production by addition of Fe^{2+} is further supported by a reaction stoichiometry corresponding to that expected from Eq. (2). Thus we suggest that in Lake Almind, Fe^{2+} at low concentrations is oxidized through DNRA and may be important in controlling partitioning between nitrate reducing processes.

The measured rates of Fe^{2+}-driven DNRA (typically ~3–6 μmol N L^{-1} d^{-1}) and their determined enzyme kinetics were similar to those measured by Robertson et al. (2016) in slurries of sediment from the Yarra Estuary. In that study, Michaelis–Menten kinetics derived from a compilation across experiments indicated a half saturation constant (K_m) and maximum reaction velocity (V_{max}) with respect to Fe^{2+} of 122 μmol Fe^{2+} L^{-1} and 8.1 μmol N L^{-1} day^{-1} for DNRA. As experimental methods and substrate concentrations are directly comparable between studies, we were able to build on the existing data from Robertson et al. and calculate revised kinetic parameters for this process. From the compiled dataset of serum vial experiments, we were able to demonstrate the dependence of DNRA on Fe^{2+} in the individual experiments and calculated the K_m and V_{max} of Fe^{2+}-driven DNRA as 33.8 μmol Fe^{2+} L^{-1} and 4.7 μmol L^{-1} day^{-1}. In addition, the trend of decreasing denitrification rates as a response to Fe^{2+} amendment (Fig. 5) was consistent with the previous study (Robertson et al., 2016). From compiling data from these studies we have shown that the general kinetic response of Fe^{2+}-driven DNRA appears to hold across very different sediment types (see Section 3.1 for site description) and locations.

Nitrate reduction with Fe^{2+} may potentially involve both biotic and abiotic reactions; leading to difficulties in interpretation of results and potential experimental artefacts (Picardal, 2012). The contribution of these interactions between NO_x and Fe^{2+} has been discussed in detail in previous enrichment studies (Picardal, 2012; Carlson et al., 2013), and also for experiments under environmentally relevant substrate concentrations such as those used in the current study, with the conclusion that DNRA was most likely microbially catalyzed (Robertson et al., 2016). Here we briefly elaborate on the potential involvement of abiotic reactions in this study.

Several mechanisms involving both biotic and abiotic reactions have been suggested to explain the reduction of nitrate coupled to Fe^{2+} oxidation (Carlson et al., 2012) at both acidic (e.g. Nelson and Bremner, 1970; Klueglein and Kappler, 2013) and circumneutral pH levels (e.g. Moraghan and Buresh, 1976). Although the precise mechanisms involved in Fe^{2+}-fueled nitrate reduction were not determined in the present study, we can draw some conclusions as to the nature of these processes based on available data and rule out major contributions from some mechanisms proposed by Carlson et al. (2012).

Firstly, abiotic reactions between nitrite and Fe^{2+} are expected to proceed more quickly than reactions with nitrate, and thus accumulation of substantial amounts of nitrite in nitrate reducing cultures in the presence of soluble Fe^{2+} would not be expected if abiotic reactions were important (Carlson et al., 2012). However, the progressive accumulation and consumption of intermediates (i.e. nitrite, N_2O; Fig. 1) which we observed during experiments is typical of microbially catalyzed-processes (Zumft, 1997) and not of spontaneous abiotic reactions. Secondly, NO is considered an important product of abiotic nitrite reduction by Fe^{2+} (Carlson et al., 2012 and references therein). While NO was not measured directly in the current study, we were able to recover the vast majority (~85–90%) of added ^{15}N substrates at the end of our experiments (Supplementary Fig. S1), suggesting that NO was not an important product. Thirdly, the presence (or production) of green rusts in experiments has been shown to cause abiotic reduction of nitrate (or nitrite) to NH_4^+, as well as gaseous end products (Summers and Chang, 1993; Hansen et al., 1994, 1996). However, the abiotic reduction of NO_x to NH_4^+ by green rusts, has been shown to be concentration dependent, increasing with increasing substrate concentration (Hansen et al., 2001). In our study, however, NH_4^+ production did not show 1st-order dependence on Fe^{2+} (Fig. 5). Finally, we only observed changes in the free (dissolved) Fe^{2+} pool and not in the solid-associated Fe(II) component with which green rusts and other Fe(II)-minerals would be associated (data not shown).

In addition to the points discussed above, we sought to reduce the possibility of post-experimental artifacts from abiotic reaction of nitrite and Fe^{2+} at low pH by preserving iron samples with sulfamic acid (as opposed to HCl), which rapidly reduces nitrite to N_2; thereby avoiding artefactual increases in nitrite and Fe^{2+} consumption/production (Klueglein and Kappler, 2013).

Although the involvement of abiotic reactions cannot be entirely ruled out, in our experiments we suggest that Fe^{2+}-driven nitrate reduction is most likely to be carried out enzymatically by sediment microorganisms. Accordingly, we now refer to Fe^{2+}-fueled DNRA as a microbial process for the following discussion.

4.2. Varying electron donors for NO_x reduction

From the experimental manipulations of electron donor availability, it is possible to begin to infer the metabolic nature of nitrate reducing processes in Lake Almind sediments. The substantial increases observed in N_2 production under high acetate availability, and the lack of or even negative response to Fe^{2+} additions, suggest that denitrification in Lake Almind is an organotrophic process. Increases were also observed in DNRA rates under low acetate additions (100 μmol L^{-1}) relative to control vials without acetate. Concurrently a significant reduction in Fe^{2+} consumption rates was observed in vials with added acetate (Table 2). As such it is apparent that when limited in organic substrate, at least some proportion of DNRA organisms may also be able to exploit these compounds through a heterotrophic, or possibly mixotrophic pathway (discussed below). The results suggest, however, that Fe^{2+}-fueled DNRA does not proceed mixotrophically and that in Lake Almind, Fe^{2+}-fueled DNRA may rather be an autotrophic process. Due to very low sulfide concentrations measured in Lake Almind sediments (data not shown), we rule out that nitrate reduction may be coupled to sulfide oxidation as shown in some previous studies in brackish and marine environments (Brettar and Rheinheimer, 1991; Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2008; Dong et al., 2011).

Previous studies into Fe^{2+}-dependent nitrate reduction have observed that some microbial isolates only achieve a metabolic benefit and cell growth through a mixotrophic metabolism (e.g. Straub et al., 1996; Kappler et al., 2005; Muehe et al., 2009; Chakraborty et al., 2011; Laufer et al., 2016a), oxidizing both Fe^{2+} and an organic co-substrate. Although some studies have reported organisms capable of reducing nitrate autotrophically with Fe^{2+} (Hafenbradl et al., 1996; Li et al., 2014; Kanaparthi and Conrad, 2015; Laufer et al., 2016b), the majority of both mixotrophic and autotrophic strains reduce nitrate to gaseous end products (N_2, N_2O). Thus our indication that Fe^{2+}-driven DNRA may be carried out autotrophically provides new insights into metabolisms linking Fe and N cycling, while it is also clear that members of the microbial community can also carry out DNRA using organic substrates. A lack of acetate dependence was also observed for Fe^{2+}-driven DNRA in estuarine sediments (Robertson et al., 2016), suggesting this metabolism may be widespread in sediments with high dissolved Fe^{2+} contents.

Combined addition of acetate and Fe^{2+} with low nitrate concentrations made no significant alterations to process rates relative to experiment with acetate and nitrate only (Table 2). Nitrate reduction was increased in experiments with acetate (with and without Fe^{2+}) relative to slurries with only ^{15}N-nitrate, indicating heterotrophic nitrate reduction, while DNRA remained constant regardless of Fe^{2+}, acetate or nitrate availability. These observations suggest that 1: denitrification is not coupled to Fe^{2+} oxidation in Lake Almind, 2: denitrification may be inhibited by high Fe^{2+} availability and 3: the observed decrease in denitrification is not due to competition for nitrate by DNRA when Fe^{2+} availability increases.

In the present study we repeatedly observed a reduction in N_2 production with increasing Fe^{2+} concentration. This effect was especially pronounced in experiments subjected to enrichment conditions. Soluble Fe^{2+} has been suggested to have deleterious effects on nitrate reducing processes (Carlson et al., 2012), possibly by disrupting electron transport during denitrification or producing toxic intermediates (Brons et al., 1991; Cooper et al., 2003; Carlson et al., 2013). Furthermore, the oxidation of Fe^{2+} was previously suggested to be a detoxification mechanism in some cases, rather than a metabolic strategy (Poulain and Newman, 2009; Carlson et al., 2012). Considering this, it is possible that denitrifying organisms in Lake Almind sediment may be susceptible to toxic effects caused by increased Fe^{2+} availability, while organisms carrying out DNRA may be able to utilize Fe^{2+} oxidation coupled to nitrate reduction as a true metabolic strategy, as suggested by some studies (Muehe et al., 2009; Chakraborty et al., 2011). Therefore, when Fe^{2+} availability is high, DNRA organisms may have particular metabolic advantages over denitrifying organisms.

4.3. Growth under enrichment conditions

The use of high (mmol L^{-1}) substrate concentrations altered the dynamics between nitrate reduction processes substantially relative to the incubations at environmentally relevant concentrations. This emphasizes that the use of high substrate levels may lead to a misrepresentation of metabolisms in natural microbial communities (Edwards, 1970). Exposing sediment communities to enrichment conditions similar to those used in culturing experiments, we found that greatly increased nitrate availability led to substantial increases in denitrification, while DNRA remained relatively unchanged between low (25–30 μmol L^{-1}) and high (~1000 μmol L^{-1}) nitrate concentrations (Figs. 1, 3 and 4).

The acceleration in N_2 production indicates an enrichment of acetate-oxidizing denitrifiers induced by higher substrate concentrations. Microbial growth was also indicated by the, presumably assimilatory, net consumption of ammonium. Increased nutrient availability has been shown to cause shifts in microbial community structure as organisms able to take advantage of higher substrate concentrations become dominant (Ferguson et al., 1984; Schäfer et al., 2001; Haukka et al., 2006). In investigating subtle interactions between elemental cycling, the use of such high concentrations in isolating microorganisms can lead to a misrepresentation of metabolic capacities, selecting for those organisms which are able to grow under enrichment conditions.

A low half saturation constant (K_m) of 5–10 μmol L^{-1} for denitrification is often assumed from kinetic parameters determined in several studies (Tiedje et al., 1982). However, this value may be much higher in some natural sediment communities and can be highly variable between sites investigated. For example, studies determining K_m at a variety of locations have reported values of 1.5–19.8 μmol L^{-1} nitrate (Evrard et al., 2012), 13–640 μmol L^{-1} (Garcia-Ruiz et al., 1998) and 256–1428 μmol L^{-1} (Raymond et al., 1992). A study investigating subtidal sediments determined a high variability in K_m values (2–344 μmol L^{-1}) at just one site, depending on sediment depth and the time of sediment sampling (Joye et al., 1996). One study on soil bacteria also noted that conditioning of sediment organisms to anaerobic conditions greatly increased the apparent K_m of N_2O reduction possibly due to adaptation or alteration of dominant denitrifying organisms (Holtan-Hartwig et al., 2000). High nitrate concentrations have been shown in several studies to increase denitrification rates (Bonin, 1996; Koop-Jakobsen and Giblin, 2010; Dong et al., 2011), thus enrichment conditions may be more likely to favor the growth of denitrifying bacteria rather than organisms carrying out DNRA (Kraft et al., 2011; van den Berg et al., 2015). This may also be reflected in the high number of Fe^{2+}-oxidizing denitrifying microbial strains isolated from natural environments under enrichment conditions relative to strains using DNRA (e.g. Straub et al., 1996; Kappler et al., 2005; Chakraborty & Picardal, 2013). *In situ* where nitrate concentrations are typically much lower, Fe^{2+} availability may be an important factor in governing the fate of N where denitrification may become inhibited or reduced at higher Fe^{2+} concentrations. These results may additionally reflect different life strategies between denitrifiers and ammonifiers in Lake Almind sediments, where denitrifiers are able to take advantage under higher nitrate availability, while Fe^{2+}-fueled DNRA proceeds at a more restricted rate.

5. CONCLUSIONS

In this study we provide one of the first investigations into Fe^{2+}-driven NOx reduction in an environmental context. We have shown that the addition of Fe^{2+} to slurry experiments from a freshwater lake enhanced rates of DNRA rather than denitrification, which has been shown in the majority of previous studies on Fe^{2+}-fueled nitrate reduction. We further show that denitrification in Lake Almind is a heterotrophic process while Fe^{2+}- driven DNRA may be autotrophic rather than mixotrophic with a small proportion of the DNRA community possibly being heterotrophic. In addition we have built upon the limited existing data to provide revised kinetic parameters for Fe^{2+}-driven DNRA, demonstrating the consistency of these parameters across geochemically very different sediments. Further studies will be needed to further elucidate the nature of these pathways. We also show that high substrate concentrations may alter the partitioning between nitrate reducing pathways, possibly causing shifts in microbial communities as they adapt to exploit high substrate availability. Thus, carrying out experiments at environmentally relevant substrate concentrations will provide valuable information on the interaction between biogeochemical processes. Although data available for Fe^{2+}-driven nitrate reduction is very limited, these observations suggest that while Fe^{2+}-fueled nitrate reduction may contribute to N retention in sediments, the availability of substrates (C, N and Fe^{2+}) for competing nitrate reducing processes (e.g. denitrification, anammox) is important in governing the relative contribution of each process to N turnover in iron-rich sediments.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gca.2017.02.014.

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