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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 N₂ or retained as NH₄⁺. These processes can potentially be driven by oxidation of reduced inorganic compounds at oxic-anoxic interfaces. Several studies have investigated the use of Fe²⁺ 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 Fe²⁺ oxidation. Anaerobic sediment slurries were supplemented with ¹⁵N-substrates and electron donors (Fe²⁺ 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 Fe²⁺ addition in 7 of 10 slurry experiments and in some cases, denitrification was concomitantly reduced. The determined kinetic parameters (Vₘₐₓ and Kₘ) for Fe²⁺-driven DNRA were 4.7 μmol N L⁻¹ d⁻¹ and 33.8 μmol Fe²⁺ L⁻¹, respectively and reaction stoichiometry for Fe²⁺:NH₄⁺ (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 Fe²⁺ concentrations may be exploited by DNRA organisms and have an inhibitory effect on denitrification, thus Fe²⁺ 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.

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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 NOₓ) 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 N₂O to N₂. Alternatively, N₂ 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
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 NO₃⁻-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 anaerobic and n-damo to NO₃⁻ reduction in freshwater sediments on average appear relatively minor (Thamdrup, 2012; Norði et al., 2013), with either denitrification or DNRA being the dominating NO₃⁻ 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 nitrate and nitrite, 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, NO₃⁻ 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 NO₃⁻. 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 Fe²⁺ 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 Fe²⁺ as electron donor for nitrate reduction have been inferred to produce N₂ as the end product based on reaction stoichiometry (e.g. Straub et al., 1996; Muehe et al., 2009; Chakraborty and Picardal, 2013)

\[
\text{NO}_3^- + 5\text{Fe}^{2+} + 12\text{H}_2\text{O} \rightarrow 1/2\text{N}_2\text{(g)} + 5\text{Fe(OH)}_3 + 9\text{H}^+ \tag{1}
\]

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 Fe²⁺ (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 Fe²⁺ oxidizers in batch incubations of marine sediment, but the nitrogenous product was not explicitly identified (Laufè et al., 2016b). A smaller number of studies have shown the reduction of nitrate to NH₄⁺ in sediments enrichments (Weber et al., 2006; Coby et al., 2011).

\[
\text{NO}_3^- + 8\text{Fe}^{2+} + 21\text{H}_2\text{O} \rightarrow \text{NH}_4^+ + 8\text{Fe(OH)}_3 + 14\text{H}^+ \tag{2}
\]

Although molecular investigations have identified phylogenetically diverse groups as potentially contributing to nitrate reduction with Fe²⁺ oxidation in nature (Straub et al., 2004; Laufè et al., 2016a), Geobacter metallireducens is, to our knowledge, the only isolate so far demonstrated to couple Fe²⁺ 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 Fe²⁺ oxidizing nitrate reducers in freshwater lake sediments (Melton et al., 2012, 2014); indicating that these organisms are likely to be subject competition for Fe²⁺ 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 (Laufè, Roy, et al., 2016; Laufè, 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 Fe²⁺-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 Fe²⁺ 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 Fe²⁺ 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 Fe²⁺ oxidation was linked to DNRA while denitrification was unaffected or, in some cases, reduced in response to increases in Fe²⁺ availability (Robertson et al., 2016).
The investigations in the Yarra estuary suggest that Fe\textsuperscript{2+} availability may influence the retention or removal of dissolved inorganic N in sediments, however the quantitative contribution of these Fe\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+}-fueled NO\textsubscript{3}\textsuperscript{-}-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\textsuperscript{2} and a maximum depth of approximately 20 m (Jorgensen 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\textsubscript{3}, NO\textsubscript{2}, NH\textsubscript{4} and SO\textsubscript{4}\textsuperscript{2-}) samples were filtered (0.2 \(\mu\)m) in the field and frozen upon return to the laboratory until analysis. Samples for dissolved Fe\textsuperscript{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 environmental N and Fe cycling 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 out in an anaerobic glove bag (N\textsubscript{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\textsubscript{3}, NO\textsubscript{2}, NH\textsubscript{4} and SO\textsubscript{4}\textsuperscript{2-}, Fe\textsuperscript{2+}, H\textsubscript{2}S, 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 g, 5 min). The supernatant was removed and filtered (0.2 \(\mu\)m) into samples for nutrients (frozen), Fe\textsuperscript{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\textsubscript{2}S (fixed in 20 \(\mu\)L 20% zinc acetate solution mL\textsuperscript{-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\textsuperscript{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 \(\mu\)m), 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\textsubscript{3}. 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\textsuperscript{IICl\textsubscript{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 \(\mu\)L, 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\textsubscript{2} and \(^{15}\)N-N\textsubscript{2}O isotopes (see below). Samples for nutrients were filtered (0.2 \(\mu\)L) and frozen until measurement and samples for Fe\textsuperscript{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.
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., 2005; Muehe et al., 2009). To investigate the use of alternative electron donors to Fe\(^{2+}\), in 2013 every Fe\(^{2+}\) concentration 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).

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

<table>
<thead>
<tr>
<th>Experimental setup</th>
<th>Treatment</th>
<th>(^{15})N substrate</th>
<th>Fe(^{2+}) addition</th>
<th>Acetate</th>
<th>Replicates</th>
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<tr>
<td>Reactors 2013</td>
<td>(^{15})NO(_3)</td>
<td>25 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(^{15})NO(_3) + Fe(^{2+})</td>
<td>25 (\mu)mol L(^{-1})</td>
<td>250 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>1</td>
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<tr>
<td></td>
<td>EN (^{15})NO(_3)</td>
<td>1000 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
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<tr>
<td></td>
<td>EN (^{15})NO(_3) + Fe(^{2+})</td>
<td>1000 (\mu)mol L(^{-1})</td>
<td>5000 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>1</td>
</tr>
<tr>
<td>Serum Vials 2013</td>
<td>(^{15})NO(_3)</td>
<td>30 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(^{15})NO(_3) + Fe(^{2+})</td>
<td>30 (\mu)mol L(^{-1})</td>
<td>200 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(^{15})NO(_3) + Acetate</td>
<td>30 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>100 (\mu)mol L(^{-1})</td>
<td>3</td>
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<tr>
<td></td>
<td>(^{15})NO(_3) + Acetate + Fe(^{2+})</td>
<td>30 (\mu)mol L(^{-1})</td>
<td>200 (\mu)mol L(^{-1})</td>
<td>100 (\mu)mol L(^{-1})</td>
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<td></td>
<td>EN (^{15})NO(_3)</td>
<td>1000 (\mu)mol L(^{-1})</td>
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<td>EN (^{15})NO(_3) + Fe(^{2+})</td>
<td>1000 (\mu)mol L(^{-1})</td>
<td>5000 (\mu)mol L(^{-1})</td>
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<td>1000 (\mu)mol L(^{-1})</td>
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<tr>
<td></td>
<td>EN (^{15})NO(_3) + Fe(^{2+}) + Acetate</td>
<td>1000 (\mu)mol L(^{-1})</td>
<td>5000 (\mu)mol L(^{-1})</td>
<td>1000 (\mu)mol L(^{-1})</td>
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<tr>
<td>Serum Vials 2014</td>
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<td>30 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(^{15})NO(_3) + Low Fe(^{2+})</td>
<td>30 (\mu)mol L(^{-1})</td>
<td>100 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
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<tr>
<td></td>
<td>(^{15})NO(_3) + High Fe(^{2+})</td>
<td>30 (\mu)mol L(^{-1})</td>
<td>500 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
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<td>0 (\mu)mol L(^{-1})</td>
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<tr>
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<td>500 (\mu)mol L(^{-1})</td>
<td>0 (\mu)mol L(^{-1})</td>
<td>3</td>
</tr>
</tbody>
</table>

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\(_2\)O 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\(_2\)O were injected into a Gas Chromatograph, passed through a reduction oven (600 °C), which reduces N\(_2\)O 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 N₂ 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, NO₃⁻, NO₂⁻, SO₄²⁻ 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 ammonia 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 Fe²⁺ and Fe³⁺ were determined using the Ferrozine assay after extraction. Sulfide concentrations in sediment were determined photometrically using Cline reagents (Cline, 1969). DIC was measured by mass 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 Fe²⁺ in low pH brine formed during freezing. This was shown by an incomplete total ¹⁵N recovery during time points where nitrite accumulated in slurries, whereas following nitrite consumption, almost all (~90%) ¹⁵N 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 μmol L⁻¹ throughout the water column in 2013, while in 2014 oxygen declined below 12 m depth to less than 10 μmol L⁻¹ in the bottom-water. Sulfate concentrations were ~200 μmol L⁻¹ throughout the water column in both 2013 and 2014. In 2013, nitrate was detectable throughout the water column, increasing from ~3 μmol L⁻¹ to ~8 μmol L⁻¹ in deeper water layers. In 2014, dissolved Fe²⁺ was undetectable throughout the water column until 19 m depth where ~25 μmol 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 (Jorgensen 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 μmol L⁻¹ between 0.5 and 3 cm depth and was depleted at 4.5 cm. Nitrite was undetectable in sediment. Dissolved Fe²⁺ in pore water samples were ~250 μmol L⁻¹ in surface layers and increased with depth to >500 μmol L⁻¹ at 5 cm depth. Highly reactive solid-phase Fe(III) extracted with dithionite increased from ~75 μmol cm⁻³ in the upper 0.5 cm to over 150 μmol cm⁻³ at 3 cm depth. Dithionite-extractable Fe (III) remained below 30 μmol cm⁻³ throughout the upper 5 cm of sediment. Sulfate concentrations in surface sediment were on average 45 μmol L⁻¹ and were depleted to ~10 μmol L⁻¹ below 3 cm. Free sulfide was always <0.5 μmol L⁻¹ at the sediment depths investigated.

3.2. Sediment slurry incubations: effect of Fe²⁺ addition on NO₃⁻ 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 ¹⁵N (and additional) substrates (Fig. 1). Nitrate consumption began immediately after addition, accompanied by linear increases in ¹⁵N-N₂ and ¹⁵NH₄⁺ and consumption of Fe²⁺. In experiments with low initial nitrate concentrations (25–30 μmol L⁻¹), nitrite typically accumulated over the first 48 h of experiments before being entirely consumed. Under these conditions, ¹⁵N-N₂O was also observed to accumulate in slurry experiments before being consumed. Production of ¹⁵N₂O and ¹⁵NH₂ continued as long as nitrate/nitrite/N₂O 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 ¹⁵N-N₂ (measured as ¹⁴N¹⁵N and ¹⁵N¹⁵N) matched that predicted from random isotope pairing through denitrification (data not shown), which excludes a significant contribution of anammox to N₂ production (Thamdrup and Dalsgaard, 2002). Thus we rule out potential complications with the application of ¹⁵N 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 ¹⁵N-substrates were added. At the time of ¹⁵N-substrate addition, Fe²⁺ concentrations were ~35 μmol Fe²⁺ L⁻¹ (Fe²⁺ from sediment addition) in control experiments with only


\[ 15N-\text{nitrate}, \text{ while those supplemented with additional } Fe^{2+}, \text{ initial concentrations were } \sim 220 \mu mol \text{ Fe}^{2+} \text{ L}^{-1}. \text{ In the reactor with added } Fe^{2+}, \text{ the DNRA rate was enhanced by 76\% relative to control reactors, while denitrification was suppressed by 47\% (Table 2). } \]

\[ \text{Fe}^{2+} \text{ addition also increased rates of } \text{Fe}^{2+} \text{ removal, nitrate reduction, and nitrite accumulation while } N_2O \text{ accumulation was reduced.} \]

\[ \text{In serum vial experiments conducted in 2013, similar patterns were observed as in bioreactor experiments, although no significant differences were observed between control and } \text{Fe}^{2+}-\text{amended treatments. Rates of DNRA and } \text{Fe}^{2+} \text{ removal were increased while denitrification was reduced. The lack of significant differences in these serum vial experiments may be due to differences in initial } \text{Fe}^{2+} \text{ concentrations between control (} \sim 45 \mu mol \text{ L}^{-1} \text{) and } \text{Fe}^{2+}-\text{amended (} \sim 80 \mu mol \text{ L}^{-1} \text{) vials being comparatively smaller than in bioreactor experiments.} \]

\[ \text{In 2014, serum vial experiments were carried out with } 15N-\text{nitrate and two different } \text{Fe}^{2+} \text{ amendments (Table 1; Fig. 2). Dissolved } \text{Fe}^{2+} \text{ concentrations at the time of } 15N-\text{substrate addition and after addition of } \text{Fe}^{2+} \text{ (doses shown in Table 1) were } \sim 35, \sim 165 \text{ and } \sim 290 \mu mol \text{ L}^{-1} \text{ in control, low and high } \text{Fe}^{2+} \text{ additions, respectively. DNRA rates were significantly enhanced (} p < 0.05 \text{) with the low } \text{Fe}^{2+} \text{ additions relative to the control (Table 2). In these experiments, } \text{Fe}^{2+} \text{ removal was also significantly enhanced and nitrite reduction significantly reduced. In experiments with high } \text{Fe}^{2+} \text{ concentrations, DNRA rates were also enhanced relative to control vials, accounting for 55\% of nitrate reduction end products. Denitrification in experiments with high } \text{Fe}^{2+} \text{ additions was significantly reduced compared to rates in experiments with low } \text{Fe}^{2+} \text{ addition although not compared to controls with no added } \text{Fe}^{2+} \text{ due to high variability in the later (Fig. 2). } \text{Fe}^{2+} \text{ removal was also enhanced relative to control vials. No significant differences were observed between rates of } \text{NOx} \text{ reduction, nitrite accumulation or } N_2O \text{ accumulation between control and high } \text{Fe}^{2+} \text{ addition experiments. All slurries had an initial total ammonium } (^{14}\text{NH}_4^+ + ^{15}\text{NH}_4^+) \text{ concentration of } \sim 50 \mu mol \text{ L}^{-1} \text{ which increased at rates approximately equal to or slightly greater than that of } ^{15}\text{NH}_2 \text{ production (data not shown). This indicates the majority of ammonium} \]

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**Fig. 1.** Typical progression of dissolved and gaseous constituents during serum vial slurry experiments at low (top) and enrichment (bottom) 15N-nitrate concentrations without Fe2+ additions.
Table 2
Average rates (all μmol N L⁻¹ d⁻¹ or μmol Fe²⁺ 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 ¹⁵N-substrate addition. Superscript numbers (¹²³⁴⁵): values with the same numbers are significantly (t-test; p < 0.05) different from each other. ‘acc.’: accumulation. Shaded areas highlight enrichment (‘EN’) treatments.

<table>
<thead>
<tr>
<th>Experimental setup</th>
<th>Treatment</th>
<th>Denitrification to N₂</th>
<th>DNRA</th>
<th>Fe²⁺ removal</th>
<th>NO₃⁻ reduction</th>
<th>Nitrite acc.</th>
<th>¹⁵N-N₂O acc.</th>
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<tbody>
<tr>
<td><strong>Reactors 2013</strong></td>
<td>¹⁵NO₃</td>
<td>1.9</td>
<td>1.7</td>
<td>-3.7</td>
<td>-7.2</td>
<td>-0.3</td>
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</tr>
<tr>
<td></td>
<td>¹⁵NO₃ + Fe²⁺</td>
<td>1</td>
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<td>8.2</td>
<td>1.3</td>
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<tr>
<td></td>
<td>EN ¹⁵NO₃</td>
<td>5</td>
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<td>-61.6</td>
<td>-39.3</td>
<td>19.5</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>EN ¹⁵NO₃ + Fe²⁺</td>
<td>2.4</td>
<td>3.7</td>
<td>-125.6</td>
<td>-13.6</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Serum Vials 2013</strong></td>
<td>¹⁵NO₃</td>
<td>4.7 (1.5)</td>
<td>2.5</td>
<td>-14.5 (7.9)</td>
<td>-14.3 (1.8)</td>
<td>4.4 (1.6)</td>
<td>0.3 (0.3)</td>
</tr>
<tr>
<td></td>
<td>¹⁵NO₃ + Fe²⁺</td>
<td>3.2 (1.6)</td>
<td>2.8</td>
<td>-17.7 (9.6)</td>
<td>-13.2 (2.3)</td>
<td>3.2 (1.4)</td>
<td>1.2 (1.0)</td>
</tr>
<tr>
<td></td>
<td>¹⁵NO₃ + Acetate</td>
<td>5.8 (0.9)</td>
<td>4.3</td>
<td>-3.7 (5.3)</td>
<td>-16.9 (0.9)</td>
<td>0.2 (1.7)</td>
<td>1.7 (0.9)</td>
</tr>
<tr>
<td></td>
<td>EN ¹⁵NO₃</td>
<td>4.8 (1.1)</td>
<td>5.3</td>
<td>-5.1 (2.5)</td>
<td>-15.9 (2.2)</td>
<td>0.5 (0.2)</td>
<td>1.8 (0.3)</td>
</tr>
<tr>
<td></td>
<td>EN ¹⁵NO₃ + Fe²⁺</td>
<td>4.5 (1.5)</td>
<td>1.6</td>
<td>-4.0 (2.3)</td>
<td>-16.6 (2.8)</td>
<td>7.2 (0.3)</td>
<td>-0.03 (0.03)</td>
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<td></td>
<td>EN ¹⁵NO₃ + Fe²⁺ + Acetate</td>
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<td>-81.2 (16.1)</td>
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<td></td>
<td>EN ¹⁵NO₃ + Acetate</td>
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<td>7.5 (9.6)</td>
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<td>EN ¹⁵NO₃ + Fe²⁺ + Acetate</td>
<td>0.2 (0.1)</td>
<td>2.3</td>
<td>-70 (0.2)</td>
<td>-125.1 (31.6)</td>
<td>76.8 (18.5)</td>
<td>7.1 (9.9)</td>
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<tr>
<td><strong>Serum Vials 2014</strong></td>
<td>¹⁵NO₃</td>
<td>4.8 (0.8)</td>
<td>3.7</td>
<td>-1.8 (0.4)</td>
<td>-11.5 (0.1)</td>
<td>1.0 (0.4)</td>
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<tr>
<td></td>
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<td>5.9 (0.4)</td>
<td>4.4</td>
<td>-6.1 (0.3)</td>
<td>-11.7 (0.4)</td>
<td>0.18 (0.1)</td>
<td>0.99 (0.6)</td>
</tr>
<tr>
<td></td>
<td>¹⁵NO₃ + High Fe²⁺</td>
<td>3.5 (0.5)</td>
<td>4.3</td>
<td>-9.0 (1.0)</td>
<td>-11.6 (0.1)</td>
<td>0.70 (0.4)</td>
<td>1.00 (1.0)</td>
</tr>
<tr>
<td></td>
<td>¹⁵NO₂</td>
<td>4.2 (1.0)</td>
<td>3.7</td>
<td>-2.4 (0.5)</td>
<td>-7.9 (0.9)</td>
<td>n/d</td>
<td>1.4 (0.2)</td>
</tr>
<tr>
<td></td>
<td>¹⁵NO₂ + Low Fe²⁺</td>
<td>4.1 (0.4)</td>
<td>3.6</td>
<td>-7.1 (0.7)</td>
<td>-7.8 (0.5)</td>
<td>n/d</td>
<td>1.2 (0.05)</td>
</tr>
<tr>
<td></td>
<td>¹⁵NO₂ + High Fe²⁺</td>
<td>3.2 (0.2)</td>
<td>3.6</td>
<td>-8.0 (0.9)</td>
<td>-6.8 (0.3)</td>
<td>n/d</td>
<td>1.5 (0.2)</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>n/d</td>
<td>n/d</td>
<td></td>
<td>-1.1 (1.1)</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>
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 ($V_{\text{max}}$ 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
(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\(_2\)O and N\(_2\), with production rates increasing after \(\sim 5\)–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 \(\sim 5\)–6 days after an initial production of 15NH\(_4^+\) 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 15NH\(_4^+\) 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\(_2\)O accumulation were enhanced relative to parallel experiments at low substrate concentrations. The addition of 5000 \(\mu\)mol L\(^{-1}\) Fe\(^{2+}\) to bioreactor experiments reduced the denitrification rate and increased DNRA \(\sim 10\)–fold to account for 60% of total nitrate reduction. Fe\(^{2+}\) removal was also enhanced, while nitrate reduction, nitrite- and N\(_2\)O-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 15N-nitrate experiments (74%) than in parallel experiments under low substrate concentrations (65% see above). The addition of Fe\(^{2+}\) to 15N-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
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 N₂O. ¹⁵NH₄⁺ and ¹⁵N-N₂ accumulated approximately linearly and ceased when nitrate, nitrite and N₂O was depleted. Acetate was consumed linearly throughout the experiment and was not fully depleted by the end of experiments. Dissolved Fe²⁺ was initially consumed. However, when nitrate became depleted, Fe²⁺ 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 NO₃ 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). ¹⁵NH₄⁺ 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 ¹⁵N-N₂ showed a similar lag phase as in enrichment experiments without acetate, with maximum rates reached after ~5 days. ¹⁵N-N₂ production ceased once nitrate, nitrite and N₂O were depleted. There was no significant consumption of Fe²⁺ 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 ¹⁵N-nitrate. Weak stimulation of denitrification and a reduction in Fe²⁺ removal was also measured, however these were not deemed significantly different from control rates. The addition of Fe²⁺ to these experiments made no significant changes to process rates relative to acetate-amended vials without Fe²⁺.

In serum vials provided with high substrate concentrations of ¹⁵N-nitrate and acetate, denitrification, nitrate reduction and nitrite- and N₂O-accumulation were stimulated relative to those vials with only ¹⁵N-nitrate (Table 2). DNRA rates slightly increased while Fe²⁺ removal was reduced. The addition of Fe²⁺ to parallel incubations with ¹⁵N-nitrate and acetate caused a large reduction in denitrification while Fe²⁺ removal and nitrite
accumulation increased. Small changes were measured in rates of DNRA and N₂O accumulation.

4. DISCUSSION

4.1. Fe²⁺-fueled DNRA

Nitrate reduction coupled to Fe(II) oxidation has become increasingly well studied in microbial cultures and environmental enrichments since its 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 Fe²⁺ 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 ¹⁵N substrates (nitrate or nitrite) were added, leading to a removal of N as gaseous N₂ (and intermediate N₂O) 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 Fe²⁺ increased rates of DNRA relative to parallel incubations without Fe²⁺ addition, and in some cases it also reduced denitrification rates. The direct stimulation of ¹⁵N-ammonium production by addition of Fe²⁺ is further supported by a reaction stoichiometry corresponding to that expected from Eq. (2). Thus we suggest that in Lake Almind, Fe²⁺ at low concentrations is oxidized through NC⁴ reactions with NO instead of spontaneous abiotic reactions. Secondly, NO is considered an important product of abiotic nitrite reduction by Fe²⁺ (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 ¹⁵N substrates at the end of our experiments (Supplementary Fig. S1), suggesting that NO was not an important product.

Firstly, abiotic reactions between nitrite and Fe²⁺ are expected to proceed more quickly than reactions with nitrate, and thus accumulation of substantial amounts of nitrate in nitrite reducing cultures in the presence of soluble Fe²⁺ would not be expected if abiotic reactions were important (Carlson et al., 2012). However, the progressive accumulation and consumption of intermediates (i.e. nitrite, N₂O; 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 Fe²⁺ (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 ¹⁵N 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 NH₄⁺, as well as gaseous end products (Summers and Chang, 1993; Hansen et al., 1994, 1996). However, the abiotic reduction of NO₃⁻ to NH₄⁺ by green rusts is considered an important product of abiotic nitrite reduction by Fe²⁺ (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 ¹⁵N substrates at the end of our experiments (Supplementary Fig. S1), suggesting that NO was not an important product.

Finally, we only observed changes in the free (dissolved) Fe²⁺ 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 Fe²⁺ at low pH by preserving iron samples with sulfamic acid (as opposed to HCl), which rapidly reduces nitrite to N₂, thereby avoiding artefactual increases in nitrite and Fe²⁺ consumption/production (Kluglein and Kappler, 2013).

Although the involvement of abiotic reactions cannot be entirely ruled out, in our experiments we suggest that Fe²⁺-driven nitrate reduction is most likely to be carried out enzymatically by sediment microorganisms. Accordingly, we now refer to Fe²⁺-fueled DNRA as a microbial process for the following discussion.
4.2. Varying electron donors for NOx 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 N2 production under high acetate availability, and the lack of or even negative response to Fe2+ 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 Fe2+ 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 Fe2+-fueled DNRA does not proceed mixotrophically and that in Lake Almind, Fe2+-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 Fe2+-dependent nitrate reduction have observed that some microbial isolates only achieve a metabolic benefit and cell growth through a mixotrophic 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 Fe2+-fueled DNRA does not proceed mixotrophically and that in Lake Almind, Fe2+-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).

In the present study we repeatedly observed a reduction in N2 production with increasing Fe2+ concentration. This effect was especially pronounced in experiments subjected to enrichment conditions. Soluble Fe2+ 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 Fe2+ 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 Fe2+ availability, while organisms carrying out DNRA may be able to utilize Fe2+ 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 Fe2+ 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 in the present study repeatedly observed a reduction in N2 production with increasing Fe2+ concentration. This effect was especially pronounced in experiments subjected to enrichment conditions. Soluble Fe2+ 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 Fe2+ 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 Fe2+ availability, while organisms carrying out DNRA may be able to utilize Fe2+ 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 Fe2+ availability is high, DNRA organisms may have particular metabolic advantages over denitrifying organisms.

In the present study we repeatedly observed a reduction in N2 production with increasing Fe2+ concentration. This effect was especially pronounced in experiments subjected to enrichment conditions. Soluble Fe2+ 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 Fe2+ 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 Fe2+ availability, while organisms carrying out DNRA may be able to utilize Fe2+ 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 Fe2+ 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 in the present study repeatedly observed a reduction in N2 production with increasing Fe2+ concentration. This effect was especially pronounced in experiments subjected to enrichment conditions. Soluble Fe2+ 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 Fe2+ 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 Fe2+ availability, while organisms carrying out DNRA may be able to utilize Fe2+ 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 Fe2+ availability is high, DNRA organisms may have particular metabolic advantages over denitrifying organisms.

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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 N₂O 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 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$^{2+}$-fueled 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.

REFERENCES


and NH$_4$F.


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