Constraining the role of iron in environmental nitrogen transformations: Dual stable isotope systematics of abiotic NO_2^- reduction by Fe(II) and its production of N_2O

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ABSTRACT

Despite mounting evidence for biogeochemical interactions between iron and nitrogen, our understanding of their environmental importance remains limited. Here we present an investigation of abiotic nitrite (NO_2^-) reduction by Fe(II) or ‘chemodenitrification,’ and its relevance to the production of nitrous oxide (N_2O), specifically focusing on dual (N and O) isotope systematics under a variety of environmental conditions. We observe a range of kinetic isotope effects that are regulated by reaction rates, with faster rates at higher pH (~8), higher concentrations of Fe(II) and in the presence of mineral surfaces. A clear non-linear relationship between rate constant and kinetic isotope effects of NO_2^- reduction was evident (with larger isotope effects at slower rates) and is interpreted as reflecting the dynamics of Fe(II)-N reaction intermediates. N and O isotopic composition of product N_2O also suggests a complex network of parallel and/or competing pathways. Our findings suggest that NO_2^- reduction by Fe(II) may represent an important abiotic source of environmental N_2O , especially in iron-rich environments experiencing dynamic redox variations. This study provides a multi-compound, multi-isotope framework for evaluating the environmental occurrence of abiotic NO_2^- reduction and N_2O formation, helping future studies constrain the relative roles of abiotic and biological N_2O production pathways.

1. INTRODUCTION

Evidence is mounting for the environmental importance of interactions between iron (Fe) and nitrogen (N) in biogeochemistry. For example, the reduction of Fe(III) coupled to the oxidation of ammonium ('feammox') has been recently demonstrated in soils (Li et al., 2015; Shrestha et al., 2009; Yang et al., 2012), while the reduction of nitrate or nitrite coupled to the oxidation of Fe(II) – or chemodenitrification – has been demonstrated across studies of both soils and bacterial cultures and/or enrichments (Klueglein and Kappler, 2012; Kopf et al., 2013; Melton et al., 2014; Picardal, 2012b; Straub et al., 1996; Weber et al., 2006). Despite the potential importance of these processes in the fate of nitrogen, our understanding of their significance remains limited. In particular, a number of these reactions may be catalyzed both chemically and biologically and the resulting nitrogenous products vary widely, including ammonium (NH_4^+), nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2). The relative contribution of these reaction pathways, therefore, has wide implications for ecosystem function (e.g., N retention/loss) and production of potent greenhouse gases. Furthermore, distinguishing between biologically and chemically catalyzed pathways has important implications for geobiology, including an improved understanding of the evolution of iron and nitrogen based metabolic systems and a mechanistic understanding of biologically mediated transformations of nitrogen, iron and carbon across a host of modern and historical environments (Falkowski et al., 2001).

Natural abundance isotopes of nitrogen and oxygen have proven useful as tools for disentangling complex networks of environmental nitrogen transformations (Casciotti and McIlvin, 2007; Kendall et al., 2007; Rafter and Sigman, 2016; Sigman et al., 2005; Wankel et al.,

2009). In large part, these efforts rely on information gained from environmental samples and/or from experimental cultures grown under environmentally relevant conditions aimed at carefully constraining kinetic isotope effects ($^{15}\epsilon$ and $^{18}\epsilon$ for N and O, respectively), as well as establishing the nature of coupling between isotope effects ($^{18}\epsilon$: $^{15}\epsilon$). Nevertheless, there remain important gaps in our understanding, in particular with respect to important isotope effects involving key nitrogen intermediates including nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and hydroxylamine (NH_2OH). For example, although the dual isotope systematics for biologically mediated oxidative processes involving NO_2^- have been characterized (e.g., NO_2^- formation by NH_4^+ oxidation (Casciotti et al., 2010) and NO_2^- oxidation to NO_3^- (Buchwald and Casciotti, 2010)), information on the dual isotope systematics of reductive processes involving NO_2^- , whether biological or abiotic, is more limited (Brunner et al., 2013; Bryan et al., 1983; Casciotti et al., 2002; Jones et al., 2015).

Specifically, there has been little investigation into the kinetic isotope effects of N and O catalyzed by abiotic chemical reduction of NO_2^- by Fe(II) – or ‘chemodenitrification’ (Jones et al., 2015; Samarkin et al., 2010). These types of reactions may represent an important control on the dual NO_3^- and NO_2^- isotopic composition in reducing environments high in iron but low in organic carbon (e.g., aquifers) as well as in environments experiencing dynamic redox fluctuations (coastal sediments, estuaries, rivers). Moreover, although abiotic reactions are thought to contribute significantly to the production of the potent greenhouse gas N_2O , little is known about the controls on the N and O isotope composition of its production by these pathways, hindering their use in constraining global sources of N_2O . Thus, there is a need for a systematic investigation of N and O isotope effects catalyzed by chemical reactions with Fe

before they can be used to constrain biological/abiotic interactions between N and Fe in the environment.

To this end, we investigated the stable N and O isotope dynamics of the abiotic reduction of NO_2^- by Fe(II), including characterization of the yield and isotopic composition of the product N_2O . As more studies suggest a potential for anaerobic (a)biotic nitrogen transformations coupled with iron cycling, the N and O isotope effects determined here will aid in the application of dual isotopes of nitrite and N_2O for deciphering the underlying biogeochemical mechanisms controlling the fate of N across a host of environments including aquatic systems, aquifers, soils, sediments and wastewater treatment plants.

2. MATERIALS AND METHODS

2.1 Nitrite reduction experiments

Batch experiments were conducted anaerobically under reaction conditions outlined in Figure 1 and Table 1 with 200 M nitrite and aqueous Fe(II) ranging from 0.5-8.1 mM in the presence and absence of goethite at room temperature. Experiments varied three primary parameters: Fe(II) concentration, solution pH, and the presence/absence of the iron-oxide goethite. All experiments were initiated in sterilized anoxic HEPES buffer (30mM) adjusted to the desired pH using NaOH. 140 mL of buffer was added to 160 mL serum bottles and purged with N_2 gas for 30 minutes to remove any trace oxygen. Bottles were then transferred into an anaerobic glove box (5% H_2 / 95% N_2), where Fe(II) was added from a concentrated anoxic FeCl_2 stock solution (~1M). Bottles were then pre-incubated by shaking for 3 days and any Fe precipitates were removed by filtration. In a subset of bottles, the Fe(III) oxyhydroxide goethite

(FeOOH) (as synthesized and fully characterized previously – see (Ekstrom et al., 2010)) was added to a final concentration of 250 μ M Fe (in bottles with 1mM Fe(II)) and 1mM Fe (in bottles with 5 or 9mM Fe(II)). The nitrite stock was made in sterilized buffer at 1M and pipetted into bottles to start experiments with 200 μ M nitrite. Following nitrite addition, bottles were sampled ~4 times within the first 6 hours and less frequently thereafter. Between sampling time points, the crimp-sealed bottles were incubated on an orbital shaker at 150 rpm. Samples were measured for Fe(II) and nitrite concentration using standard spectrophotometric methods. In preliminary experiments samples were screened for ammonium production using the fluorometric method described by Holmes et al., (Holmes et al., 1999), and no ammonium was detected. Subsamples were also measured for nitrite N and O isotopes as described below. Finally, 5ml samples of headspace gas composition were taken at the end of the experiment and injected into pre-evacuated headspace vials (20 ml) for N₂O concentration and isotopic composition analysis.

In addition to these batch incubations, a subset of these conditions was also run as a parallel ‘N₂O time-series’ experiment (pH 8, no mineral addition, Fe(II) 1, 5 and 9mM), in which headspace N₂O measurements were made over time using the autosampler of the IRMS system. For each condition, ten 20ml headspace vials were loaded with solutions as described above and placed immediately onto the headspace purge and trap system coupled to the IRMS. The amount and N and O isotopic composition of evolving N₂O was monitored over time.

2.2 Chemical Analyses

2.2.1 Nitrite

Nitrite concentration was measured using the Greiss-Islovay spectrophotometric method (Pai et al., 1990). Reactions were prepared in 1cm cuvettes in the glove box and diluted as

needed with anoxic HEPES buffer. NaNO_2 standards of 0, 25, 50, 250 μM were run in parallel. All samples and standards received 100 μL sulfanilamide (SAN) and 100 μL naphthyl ethylene diamine (NED). Absorbance was recorded at a wavelength of 543 nm.

2.2.2 Fe(II)

Fe(II) concentration measurements were made using ferrozine (Stookey, 1970). All reactions were conducted within the glove box followed by immediate measurement on the spectrophotometer at 562 nm. Reactions were done in 1cm cuvettes with 2.7 mL of ferrozine, and 0.3 mL of sample. At higher Fe concentrations samples were diluted as necessary with anoxic HEPES buffer.

2.2.3 Isotopic Analyses

Nitrogen and oxygen isotope ratios of nitrite (where $\delta^{15}\text{N} = [({}^{15}\text{R}_{\text{sample}}/{}^{15}\text{R}_{\text{Air}})-1]*1000$ in units of ‰, and ${}^{15}\text{R} = {}^{15}\text{N}/{}^{14}\text{N}$ and where $\delta^{18}\text{O} = [({}^{18}\text{R}_{\text{sample}}/{}^{18}\text{R}_{\text{VSMOW}})-1]*1000$ in units of ‰, and ${}^{18}\text{R} = {}^{18}\text{O}/{}^{16}\text{O}$) samples were measured by chemically converting 20 to 40 nmoles of NO_2^- to N_2O using the azide method in 20ml headspace vials (McIlvin and Altabet, 2005). The evolved N_2O was purified and collected on a modified TraceGas (IsoPrime, Inc.) purge and trap coupled with a Gilson autosampler before isotopic analysis on an isotope ratio mass spectrometer (IsoPrime 100, Elementar Inc.). Internal nitrite isotope standards (WILIS 10, 11 and 20) were run in parallel at 3 different sizes to correct for any variations in sample size and instrumental drift. Based on calibrations against isotope standards USGS 32, 34 and 35 for $\delta^{15}\text{N}$ (Böhlke et al., 2003) and N23, N7373, and N10129 for $\delta^{18}\text{O}$ (Casciotti et al., 2007), the values of internal standards WILIS 10, 11, and 20 are -1.7, +57.1, and -7.8‰ for $\delta^{15}\text{N}$ and +13.2, +8.6 and +47.6‰

for $\delta^{18}\text{O}$, respectively. All isotopic values are reported against the VSMOW (for $\delta^{18}\text{O}$) or Air (for $\delta^{15}\text{N}$) reference scales. Typical reproducibility for $\delta^{15}\text{N}$ is $\pm 0.2\text{‰}$ and for $\delta^{18}\text{O}$ is $\pm 0.2\text{‰}$.

Headspace N_2O isotopic composition was measured in duplicate by direct comparison against the N_2O reference tank on the IRMS system. The composition of this tank ($\delta^{15}\text{N}^{\text{bulk}} = -0.7\text{‰}$; $\delta^{18}\text{O} = +39.1\text{‰}$; site preference (SP) = -5.3‰ , where $\text{SP} = \delta^{15}\text{N}(\alpha) - \delta^{15}\text{N}(\beta)$ and α and β refer to the central and outer N atoms in the linear N_2O molecule, respectively) was calibrated directly against aliquots of two previously calibrated N_2O tanks from the Ostrom Lab at Michigan State University. The molar amount of N_2O in the headspace of each experimental bottle was calculated using the linear relationship between IRMS peak area at m/z 44 and injections of known amounts of N_2O (derived from azide conversion of NO_2^-). Reported values have been corrected for any size linearity of isotopic ratios (31/30, 45/44 and 46/44) by using a series of reference tank subsamples injected into 20ml headspace vials using a gastight syringe. Precision for replicate analyses of our reference gas analyzed as samples for $\delta^{15}\text{N}$ is $\pm 0.3\text{‰}$, for $\delta^{18}\text{O}$ is $\pm 0.4\text{‰}$ and for SP is $\pm 0.8\text{‰}$.

2.3 Mineral Analysis

The speciation of Fe was determined using synchrotron-based X-ray absorption spectroscopy (XAS) (Hansel et al., 2003). Samples were anaerobically mounted on a Teflon plate and sealed with Kapton polyimide film to prevent moisture loss and oxidation while minimizing X-ray absorption. XAS was performed at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 11-2 using a He-purged sample chamber. Spectra were acquired from -200 to approximately 1000 eV around the K-edge of Fe (7111 eV). The mineralogical composition of the sediments was obtained using the extended region of the XAS

spectra (EXAFS region). Percentages of various Fe phases were determined by linear combination fitting (LCF) of k^3 -weighted EXAFS (LC-EXAFS) spectra with a set of reference standards as described in detail previously (Hansel et al., 2003) using the fitting program SIXPack (Webb, 2005).

3. RESULTS

3.1 Nitrite reduction experiments

Reaction between ferrous Fe and nitrite led to complete removal of 200 μ M nitrite when starting Fe(II) levels were greater than $\sim$ 4 mM, but incomplete at lower Fe(II), between 0.6 and 1.0mM (Figure 1). In comparison to changes in NO_2^- , only a small percentage of dissolved Fe (II) was removed by Fe(II) oxidation in the majority of incubations (Figure 2).

The rate of reaction varied greatly in all treatments. The slowest experiments were measured over 100 hours (at pH 7 and 0.6 and 0.8mM Fe(II); Figure 1), and the fastest had complete nitrite reduction in less than 2 hours (pH 8, 6.3 and 8.1 mM Fe(II); Figure 1). Initial reduction (within the first 6 hours) of nitrite by Fe(II) was linear with initial rates varying from 0.1 to 50 $\mu\text{M h}^{-1}$ at pH 7 and 5.9 to 160 $\mu\text{M h}^{-1}$ at pH 8. Corresponding Fe(II) loss, a combination of both oxidation by nitrite and sorption/precipitation, exhibited initial rates ranging from 1 to 343 $\mu\text{M h}^{-1}$ at pH 7 and 11 to 2300 $\mu\text{M h}^{-1}$ at pH 8 (see Figure 2). In all conditions the rate of reaction was increased when goethite was added (Figure 2).

The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of the remaining nitrite increased during the reaction in all incubations, reflecting normal isotope effects for both N and O during nitrite reduction (Figure 3). Using a closed system Rayleigh model, the ^{15}N isotope effect for nitrite reduction by Fe(II) (hereafter, $^{15}\epsilon_{\text{NIR}}$) ranged from 6‰ to 45‰, while the ^{18}O isotope effect (hereafter, $^{18}\epsilon_{\text{NIR}}$) ranged between

6‰ and 33‰ (Table 2). The ratio of $^{18}\epsilon_{\text{NIR}}:^{15}\epsilon_{\text{NIR}}$, was often lower than 1 (Table 2, Figure 4), reflecting a smaller isotope effect for oxygen compared to nitrogen.

3.2 N₂O production experiments

N₂O production was measured in samples at pH 8 having Fe(II) concentrations of 0.9, 4.7 and 9.1mM. N₂O was produced over the first 6 hours during the bulk of nitrite reduction, slowing substantially during the next 6-12 hours of measurement. In general, lower amounts of N₂O accumulated under conditions promoting slower rates of nitrite reduction (not shown). Molar yields of N₂O (e.g., the percentage of NO₂⁻ converted to N₂O) ranged from ~11 to 52%, reflecting considerable variation in the relative magnitudes of reaction mechanisms involved in chemodenitrification.

N and O isotopic composition of the final N₂O ranged from -19.8 to -3.0‰ for $\delta^{15}\text{N}_{\text{N}_2\text{Obulk}}$ and from +29.3 to +46.4‰ for $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ and were strongly correlated, with all but one outlier clustering between -7.4 to -3.0‰ and +38.4 to +46.4‰, respectively (Figure S1). The single outlier N₂O composition corresponds to the only case in which nitrite reduction was not complete, reflecting N₂O produced from only a partially reduced pool of NO₂⁻.

4. DISCUSSION

4.1 Reaction stoichiometry, kinetics and mineral formation

Our findings are consistent with previous studies demonstrating the ability of Fe(II) to chemically reduce nitrite over a large range of experimental conditions (Kampschreur et al., 2011; Picardal, 2012b; Sorenson et al., 1981; van Cleemput and Baert, 1983). Specifically, rapid reaction between high levels of nitrite (~2-43 mM) and ferrous Fe (~5-43 mM) has been

documented over a wide pH range (4 to 8) (Jones et al., 2015; Kampschreur et al., 2011; Moraghan and Buresh, 1977; Nelson and Bremner, 1970; Tai and Dempsey, 2009).

Despite complete loss of nitrite, production of ammonium was never observed and thus nitrite was converted to gaseous products in all cases. Indeed, we observed production of N₂O in all experiments (see below); yet, not enough to account for all of the reduced NO₂⁻ likely pointing to N₂ as an additional product under our experimental pH range. As such, the primary net reactions operative in our experiments are represented by the following equations:

Assuming the stoichiometries of reactions R1 and R2, Fe(II) was in excess of nitrite in the majority of our experiments. An exception was the experiment conducted at pH 8 and lower Fe(II) ≤ 1 mM, where nitrite was in excess and Fe(II) was almost completely oxidized over 100 hours. Consistent with the above reactions, the oxidation of Fe(II) led to rapid Fe(III) precipitation in all conditions except for the lowest initial Fe(II) level (0.5 mM) at pH 7. EXAFS spectral analyses (Figure S2) identified these minerals as goethite, ferrihydrite, and magnetite, which were present at varying proportions depending on conditions (Figure 5). At pH 7, dominant phases were goethite and magnetite with relative contribution of magnetite increasing at higher Fe(II) (Figure 5a). This trend was inversed at higher pH (pH 8), with goethite and ferrihydrite increasing as Fe(II) increased. Interestingly, incubations amended with exogenous goethite initially led to the inhibition of magnetite formation under similar initial Fe(II) levels (Figure 5b). Ferrihydrite and goethite were also identified as secondary products in similar batch

incubations but with higher nitrite and aqueous Fe(II) levels at pH 7 (Jones et al., 2015). In contrast to our findings, however, Jones and colleagues (Jones et al., 2015) did not observe formation of magnetite, and instead found precipitation of lepidocrocite, particularly when nitrite was provided in excess of the initial aqueous Fe(II) concentration. This variability in precipitation patterns is most likely due to differences in the geochemical conditions of the incubations. In particular, when comparing pH 7 incubations the initial aqueous Fe(II) to nitrite ratio in our incubations ranged from 3 to 44 in contrast to a ratio of 0.13 to 4 in Jones et al., (2015). These lower ratios are primarily due to the considerably lower nitrite concentrations used in our incubations (200 μ M versus 2.5-40 mM), leading to lower rates of reaction and likely allowing for more extensive Fe(II)-induced ripening and conversion of ferrihydrite to magnetite.

Indeed, the rate of aqueous Fe(II) loss and corresponding nitrite reduction varied as a function of pH, initial aqueous Fe(II) concentration, and exogenous goethite addition (Figure 6, Table 1). Our observed nitrite reduction and Fe(II) loss rates at pH 7 are generally comparable with previous studies using similar reaction conditions (e.g., (Dhakal et al., 2013; Sorensen and Thorling, 1991; Tai and Dempsey, 2009)), while lower than those rates documented at higher nitrite and Fe(II) levels (albeit at lower Fe(II):NO₂⁻; (Jones et al., 2015; Kampschreur et al., 2011)). For the 4 different scenarios employed here (pH 7 and 8, with/without goethite; Table 1), we observed a linear relationship between Fe(II) concentration and the pseudo-first order rate constant (h^{-1}) (Figure 6; Table 1), highlighting the role of Fe(II) concentrations in regulating nitrite reduction rates.

Addition of goethite consistently led to higher reaction rates between Fe(II) and nitrite at both pH values and regardless of initial Fe(II) concentration (Figure 6; Table 1). Multiple studies have shown that the presence of Fe(III) oxyhydroxides and the sorption of Fe(II) to mineral

surfaces or ligands increases rates of nitrite reduction by Fe(II), including reactions with 2-line ferrihydrite, goethite, biogenic magnetite, lepidocrocite, green rust (GR), siderite and wüstite (Coby and Picardal, 2005; Dhakal et al., 2013; Hansen et al., 1994; Kopf et al., 2013; Rakshit et al., 2007; Sorensen and Thorling, 1991; Tai and Dempsey, 2009). These studies consistently indicate faster kinetics for heterogeneous than homogenous Fe(II)-nitrite reactions.

For the homogenous reaction conditions between aqueous Fe(II) and nitrite (absence of exogenous goethite), heterogeneous reactions likely also contributed to the observed reaction rates. The rapid formation of Fe(III) minerals upon Fe(II) and nitrite reaction provided a secondary and presumably faster nitrite reduction pathway. Thus, heterogeneous reactions would also be operative with ferrous Fe bound within magnetite (Figure 5) and/or Fe(II) adsorbed onto the secondary precipitates goethite and ferrihydrite.

4.2 Nitrite Isotope Systematics

The N and O stable isotope systematics of abiotic nitrite reduction can potentially be useful for discerning nitrite reduction mechanisms in the environment. Oxygen isotopes of nitrite may also readily equilibrate with water as a function of pH and temperature, with faster equilibration occurring at lower pH and higher temperatures (Casciotti et al., 2007). It is therefore possible that the observed oxygen isotope effect of NO_2^- reduction was influenced by oxygen isotope equilibration with water, which would drive the $\delta^{18}\text{O}$ to ~14‰ higher than the water. No difference in $^{15}\epsilon_{\text{NIR}}:^{18}\epsilon_{\text{NIR}}$ was observed between experiments at pH 7 and pH 8, suggesting perhaps this factor was not important in our experiments (Table 2). However, given the rapid consumption rates of our relatively small levels of NO_2^- it is also possible that our experiments did not capture this dynamic. Although, we would not predict high rates of oxygen

exchange at pH 8, the very high $\delta^{18}\text{O}$ values under high extents of NO_2^- consumption would be particularly sensitive to even a small amount of isotopic exchange and may contribute to slightly lower observed $^{18}\epsilon_{\text{NIR}}$ relative to that of $^{15}\epsilon_{\text{NIR}}$ (Table 2, Figure S3).

Although very little NO_2^- isotope data exist for chemical reduction, our data exhibit a wider range of $^{15}\epsilon_{\text{NIR}}$ and $^{18}\epsilon_{\text{NIR}}$ values than other recent studies (e.g., (Heil et al., 2015; Jones et al., 2015)). While $^{15}\epsilon_{\text{NIR}}$ and $^{18}\epsilon_{\text{NIR}}$ varied from 6 to 45‰ (Table 2) in our experiments, these values were consistent and reproducible under similar experimental conditions. Indeed, differences in the isotope effects correlate well with factors that directly influence reaction rate, including substrate concentration, pH and interactions with minerals (Figure 3, Table 2). In particular, variations in nitrite isotopic fractionation were inversely related to the reaction rate, with higher rates producing lower $^{15}\epsilon$ values (Figure 7).

In our experiments, differences in isotope effects between heterogeneous and homogenous reactions could not be explicitly addressed since even our ‘homogenous’ reactions exhibited rapid mineral formation (Figures 3), promoting heterogeneous surface reactions. Even at the lowest levels of mineral precipitation (pH 7) rates were also slower and it is therefore unclear whether the lower isotope effect stemmed from lower overall reaction rate or from reduced interaction with surface-bound Fe(II). If we assume that the rapid reactions were mostly catalyzed by interactions with surface associated Fe(II) and that the slower reactions were less influenced by surface-bound Fe(II), then it is possible that the heterogeneous reaction exhibits a smaller isotope effect for this process, leading to the lower observed net isotope effects at high reaction rates. Future studies should aim to tease apart the relative influence of rate versus mechanism in order to better understand the reaction mechanism.

The observed kinetic isotope effects in the reacting NO_2^- pool are governed by the combination of chemical reactions (e.g., bond forming/breaking) occurring during nitrite reduction. As such, changes in the relative proportions of different mechanisms/pathways (including back reactions) can change the observed isotope effects of the NO_2^- (and other N bearing intermediate) pools. While the net reaction results in the reduction of NO_2^- to N_2O (and and/or N_2 (reactions [1] and [2] above)), the reaction proceeds through one or more nitrogenous and likely Fe-bound nitrogen species (e.g., nitrosyl ($\text{Fe}(\text{NO})^+$ or dinitrosyl ($\text{Fe}(\text{NO})_2^{2+}$) intermediates, which may have limited stability under these reaction conditions (Bonner and Pearsall, 1982; Kopf et al., 2013; Kustin et al., 1966; Pearsall and Bonner, 1982). Specifically, we consider the involvement of an Fe(II) intermediate and isotope fractionation occurring at each of the reaction steps given below (reaction 3-5): the reduction of NO_2^- to NO [R3], the complexation of NO with Fe(II) [R4] and the reduction of the Fe-NO complex to N_2O (or N_2) [R5].

While the forward reactions resulting in the formation of NO (k_3 in R3) or the formation of a nitrosyl complex (k_4 in R4) may occur with a particular isotope effect, the backward reactions (k_{-3} or k_{-4}) may occur with distinctively different isotope effects. Indeed, recent evidence indicates that the initial reduction of NO_2^- to NO [R3] may largely be controlled by an equilibrium reaction (Kampschreur et al., 2011), in which case the forward/backward reactions give rise to an equilibrium isotope effect. Additionally, NO_2^- $\delta^{18}\text{O}$ could be significantly affected

by the incorporation of a ‘new’ O atom (from H₂O) during the reverse reaction [R3] in the reverse direction. In fact, the observation of a lower ¹⁸ε than ¹⁵ε (Table 2, Figure 3), supports a possible influx of ‘new’ O atoms from water, lowering the observed isotope effect for ¹⁸O. We observed that slower rates of reduction were associated with even lower ¹⁸ε¹⁵ε (Table 2), which appears at least consistent with incorporation of O atoms from water – possibly reflecting the influence of reaction reversibility, an equilibrium isotope effect between NO₂⁻ and NO or even chemically catalyzed equilibration between NO₂⁻ and water, the effect of which would become more apparent at slower reaction rates. The influence of this type of pathway reversibility together with O atom equilibrium isotope effects has been well documented in the sulfate reduction system (Bradley et al., 2011; Brunner et al., 2011; Holler et al., 2011; Rees, 1973; Wankel et al., 2014) – may be influential in this system as well. Thus, we suggest that the relative ratio of forward to backward reactions plays the key role in regulating our observed isotope effects in the reactant NO₂⁻ pool.

Finally, to the degree that the net reaction is multi-step and/or proceeds through multiple (and likely transient) intermediate species and/or through parallel pathways (e.g., reaction R6a vs. R6b), changes in the relative rates of each step will contribute to changes in the net isotope effect observed in the NO₂⁻ and product N₂O pools. Likely, all of these factors contribute to our observed relationship between the kinetic isotope effect and reaction rate (Figure 7). Future work involving ¹⁸O labeled water could prove useful for further disentangling such complexity.

4.3 Relationship between NO_2^- reduction and N_2O isotope dynamics

The final amount and N and O isotopic composition of the N_2O accumulated in the batch experiment bottles were measured, including the intramolecular nitrogen isotopic composition, or site preference (SP). Complementary to these end product measurements, N_2O formation and isotopic composition was also monitored over time for a subset of these conditions (pH 8, no goethite, and starting Fe(II) concentrations of 0.9, 4.7 and 9.1mM). The endpoint composition of the N_2O in these ‘time series’ experiments (Figure S4) was consistent with the endpoint measurements of the batch experiment bottles (Figure S1).

Comparison of the starting composition of the reactant NO_2^- with the product N_2O offers important insights into reaction mechanisms. The elevated $\delta^8\text{O}_{\text{N}_2\text{O}}$ values relative to NO_2^- , reflect the influence of branching effects by the preferential abstraction of ^{16}O during reduction steps of both NO_2^- and NO (Casciotti et al., 2002). Together with the strong correlation between the $\delta^5\text{N}$ and $\delta^8\text{O}$ of product N_2O (Figure S1) this indicates a strong coupling of the kinetic isotope effect on N and the combined kinetic and branching isotope effects on oxygen during the formation of N_2O . The final $\delta^5\text{N}$ of the accumulated N_2O was on average $\sim 8\text{‰}$ lower than the $\delta^5\text{N}$ of the starting NO_2^- , with higher N_2O yields exhibiting smaller differences (excluding the case in which NO_2^- did not fully react – Figure 1).

As all of the reacted NO_2^- was not accounted for in the product N_2O , another N-bearing pool must represent the mass balance complement to the N_2O pool, having a higher $\delta^5\text{N}$ than the starting NO_2^- . Initial product N_2O $\delta^5\text{N}$ values during the time series experiments were lower than the N isotope effects calculated from the NO_2^- pool (Figure S4) also reflecting production of a separate N bearing pool. At high N_2O yields ($\sim 30\text{-}50\%$), end point $\delta^5\text{N}$ values were on average

373 ~4 to 8‰ lower than starting NO_2^- $\delta^{15}\text{N}$ values – and by mass balance imply production of an N-
374 bearing pool at least several permil higher than the starting NO_2^- $\delta^{15}\text{N}$ values. Jones and others
375 (Jones et al., 2015) observed similar results, suggesting that isotopically heavier N must have
376 accumulated in the NO pool. Under similar reaction conditions (e.g. pH 7, very high NO_2^- and
377 Fe(II)), Kampschreur and colleagues (Kampschreur et al., 2011) observed complete recovery of
378 NO_2^- as NO and N_2O – suggesting that the missing mass balance complement to the N_2O is
379 likely to be found as NO. Under the higher pH conditions of our time series experiment (pH 8),
380 however, NO was only observed at low levels (qualitatively observed as separate peaks while
381 monitoring masses 30 and 31 on the IRMS during N_2O analyses) and only under low Fe(II)
382 conditions (0.9mM) suggesting that, while possibly an important transient intermediate, gas
383 phase NO did not appear to have been a significant end product ($< \sim 5\%$). A lack of observed NO
384 accumulation under our higher Fe(II) conditions also appears to highlight the role of Fe(II) in
385 providing the forward kinetic drive (and/or complexation of NO) and likely promoting formation
386 of Fe-bound nitrosyl species (as in reaction R4 above). In comparing the likelihood of an NO vs
387 N_2 pool as the missing mass balance complement of the low $\delta^{15}\text{N}$ N_2O , Jones and others (2015)
388 suggested an NO product pool having a higher $\delta^{15}\text{N}$ as a more parsimonious explanation since it
389 is less reduced than N_2O (making the assumption that an N_2 pool derived from an N_2O precursor
390 should be isotopically lower than the N_2O). However, based on the apparently low observed
391 accumulation of NO in our time series measurements at pH 8, together with the assumption that
392 a high $\delta^{15}\text{N}$ N_2 pool deriving from N_2O reduction would be unlikely, we suggest instead that the
393 production of N_2 and N_2O may be occurring in parallel, competing reactions (as in R6a and
394 R6b), under our experimental conditions (with the production of N_2 having a smaller isotope
395 effect than the production of N_2O). Alternatively, some proportion of NO may have remained

bound in a nitrosyliron complex under the higher dissolved Fe(II) conditions. Indeed the difference in proportion of end products is related to differences in the formation kinetics and stability of (di)nitrosyl intermediates, which are sensitive to pH (Bonner and Pearsall, 1982; Pearsall and Bonner, 1982). Either way, whether the missing N pool is comprised of NO, as observed and inferred by others (Jones et al., 2015; Kampschreur et al., 2011), or is comprised primarily of N₂ as appears to be the case in our experiments, the N isotopic composition of the N₂O offers a useful perspective on the source of N and the isotope systematics of N₂O release by chemodenitrification.

The intramolecular ¹⁵N site preference of the product N₂O also reflects differences in the governing reaction mechanisms. N₂O SP values both from the batch experiments and the time series measurements ranged from -0.4 to +26.0‰ with endpoint values correlating with final concentration and yield of N₂O (Figure 8). In our time series experiments, higher levels of Fe(II) lead to more consistently elevated SP values starting ~ +14.5‰ and increasing to ~ +26.0‰ (Figure S4) – similar to previous observations of N₂O SP by chemodenitrification falling between +10 and +22‰ (Jones et al., 2015). In contrast, under lower Fe(II) conditions (~0.9mM) initial SP values were as low as 0‰, although eventually increased to 26.0‰ before reaching a final value of 15.2‰ (Figure S4). Compared to $\delta^{15}\text{N}_{\text{N}_2\text{O}_{\text{bulk}}}$ and $\delta^{18}\text{O}_{\text{N}_2\text{O}}$, which reflect the combined influence of both the composition of reactants (e.g., NO₂⁻) and the kinetic isotope effects associated with the reaction pathways, SP is thought to be independent of the $\delta^{15}\text{N}$ of the N source and instead reflect only formation pathway. Specifically, the combination of two NO precursor molecules to form N₂O in a singly catalyzed reaction should result in very little difference between the $\delta^{15}\text{N}$ of the beta (outer) and alpha (inner) N atoms, such as is observed for nitric oxide reductases in denitrifying bacteria (Toyoda et al., 2005; Yamazaki et al., 2014). In

contrast, mechanisms whereby combination of two NO molecules proceeds via formation of an O-N=N-O intermediate favors breaking of $^{14}\text{N-O}$ bond over a $^{15}\text{N-O}$ bond – and promotes the ^{15}N enrichment of the alpha position (e.g., elevated SP value), as is thought to occur during NH_2OH decomposition and N_2O production by ammonia oxidizing bacteria (Frame and Casciotti, 2010; Sutka et al., 2003) and denitrifying fungi (Rohe et al., 2014; Sutka et al., 2008; Yang et al., 2014). Elevated SP values (+35‰) have in fact also been observed in chemical reactions involving NH_2OH and NO_2^- including in the presence of Fe catalysts – although the decomposition of NH_2OH may play the primary role rather than NO_2^- (Heil et al., 2015). Results of chemical reduction of NO_2^- in experiments using other reductants (e.g., trimethylamine-borane) have also yielded elevated SP values – suggesting an important role for an intermediate species (Toyoda et al., 2005). Studies investigating reduction of NO_2^- by either aqueous Fe(II) or Fe(II)-containing primary minerals have also noted production of N_2O having positive SP values up to 22‰ (Jones et al., 2015; Peters et al., 2014), although low SP values have also been reported (Samarkin et al., 2010). We suggest that the range of SP values observed in field studies (Peters et al., 2014) and in lab studies such as ours and those of others (Jones et al., 2015; Samarkin et al., 2010), reflects shifts in the balance of at least two mechanisms of N_2O formation and specifically the relative involvement of intermediate nitrosyl and dinitrosyl species (and the factors regulating their formation and stability; (Bonner and Pearsall, 1982; Kopf et al., 2013; Pearsall and Bonner, 1982)). Indeed, in our experiments, higher N_2O yields correspond with higher reduction rates and in turn higher concentrations of Fe(II). Thus, in summary, the high Fe(II) conditions of our experiments apparently favor the formation of nitrosyl-iron complexes as reaction intermediates and precursors for reactions yielding elevated SP values for product N_2O .

On the other hand, lower levels of Fe(II) and the correspondingly slower reduction of NO_2^- apparently produce N_2O having generally lower SP values (Figure 8; Figure S4).

5. Environmental Implications

Here we have shown that abiotic reduction of nitrite by Fe(II) is rapid under a range of circumneutral conditions, in agreement with previous studies (Picardal, 2012a) (and references therein), specifically examining reactant concentrations, surface interactions and pH. Further, these factors appear to control the relative proportions of reaction pathways, with strong implications for the isotopic evolution of reactant NO_2^- as well as the isotopic composition and yield of product N_2O . Specifically, even though elevated levels of Fe(II) increase reaction rate, the homogeneous reaction of NO_2^- with aqueous Fe(II) is kinetically slow under our experimental conditions compared to biological reduction (e.g., (Betlach and Tiedje, 1981)). Nevertheless, in most natural environments at circumneutral pH, aqueous Fe(II) is found adsorbed onto mineral surfaces and/or bound as ligands. Thus, while the homogenous reaction of aqueous Fe(II) with NO_2^- may be kinetically inhibited, the heterogeneous reaction will most likely drive environmental chemodenitrification. In fact, our data also reflect dramatically increased reactivity of NO_2^- with surface associated Fe(II). In all cases exhibiting Fe-oxide formation, both the rates of nitrite reduction (Figure 6) as well as corresponding yields of N_2O (Figure 8) were dramatically higher. Thus, under redox conditions promoting production of Fe(II) and conditions enabling sorption of Fe onto mineral surfaces (e.g., soils, porewaters, permeable sediments, riparian zones), the kinetic drive for abiotic NO_2^- reduction by Fe(II) is substantial – as is the potential for its significance as an abiotic source of N_2O to the atmosphere. Notably, recent work with a culture of nitrate-reducing iron oxidizing bacteria also suggests that

anaerobic Fe oxidation actually occurs as a chemical side reaction upon the intracellular production of NO_2^- and Fe oxides, raising the possibility that anaerobic Fe oxidation by other nitrate reducing microbes may also stem primarily from chemical interactions rather than direct enzymatic catalysis (Klueglein and Kappler, 2012; Kopf et al., 2013).

While this study helps to lay the framework for improving our understanding of chemodenitrification and its stable isotope systematics, the sensitivity of the nitrite isotope effects and the product N_2O to reaction conditions will complicate interpretation of natural abundance isotope values for detecting reactions with Fe(II). Additionally, the range of isotope effect measurements made here for chemodenitrification overlaps previously measured isotope effects for biological nitrite reduction. However, the much higher isotope effects observed at slower rates of chemodenitrification are far higher than those observed for biological nitrite reduction, such that under certain conditions distinction between the two may be evident. Furthermore, biological nitrite reduction catalyzed by the copper-containing form of nitrite reductase (nirS), was recently shown to impart a very low ratio of coupled isotope effects for nitrite reduction ($^{18}\epsilon^{15}\epsilon \sim 0.1$) (Martin and Casciotti, 2016) – far lower than that observed for chemodenitrification in our experiments exhibiting values much closer to 1. Accordingly, environmental studies should account for factors including pH and Fe(II) concentrations/fluxes, abundance and form of Fe-bearing minerals, concentrations/fluxes of nitrite as well as the abundance and activity of genetic indicators of biological nitrite reduction. Further experimental constraints for nitrite reduction by Fe(II) can build from these results to improve prediction of rates – and based on relationships described here – also make predictions of the nitrite dual isotope effects for the reaction. Constraining pH will be especially crucial to account for potential oxygen isotope exchange with water, which is rapid at pH values < 7 (Casciotti et al.,

2007). Many of the ecosystems in which nitrite accumulation may be important such as groundwater, estuaries and coastal sediments may also exhibit dynamic changes in pH (during tidal flushing of an estuary, draining of intertidal porewater spaces or even storm runoff events, for example). Currently, rates of nitrite-water oxygen isotope equilibration have only been quantified in seawater (Buchwald and Casciotti, 2013). Thus, in order to fully exploit NO_2^- oxygen isotopes in other ecosystems, future work on exchange rates across a range of salinity will be necessary.

With this improved understanding of controls on N_2O production by abiotic nitrite reduction, future studies should focus on establishing the importance of chemodenitrification in the environment especially under environmental conditions in which it compete with biological nitrite reduction. For example, in environments exhibiting rapidly fluctuating redox conditions, such as estuarine sediments and permeable coastal and shelf sediments, high fluxes of Fe(II) released by iron-reducing bacteria and/or by abiotic reduction by sulfur intermediates are often closely juxtaposed with elevated nitrogen concentrations in overlying water. Such conditions could represent prime hotspots for abiotic reactions between NO_2^- and Fe(II) and the abiotic formation of N_2O .

Table 1. First-order rate constants for nitrite reduction at pH 7 and pH 8

pH	Starting [Fe(II)] (mM)	Goethite added	k_1 (h ⁻¹)	Initial Rate (M h ⁻¹)
7	0.6	0	0.0007	0.1
7	4.7	0	0.02	4.0
7	8.7	0	0.10	19
8	0.6	0	0.03	5.9
8	4.2	0	0.23	41
8	6.3	0	0.35	60
7	0.8	0.25	0.005	1.0
7	4.8	1	0.15	28
7	7.9	1	0.29	50
8	1.0	0.25	0.05	9.8
8	4.5	1	0.69	99
8	8.1	1	1.68	160

Table 2. Observed N and O isotope effects for abiotic reduction of nitrite by Fe(II). In some cases reactions were too fast for reliable measurement of NO_2^- isotopes. ND = not enough nitrite detected.

pH	Starting [Fe(II)] (mM)	Goethite (mM)	$^{15}\epsilon$ (‰)	$^{18}\epsilon$ (‰)	$^{18}\epsilon: ^{15}\epsilon$
7	0.6	0	33.9±24.8	24.8±15.9	0.7
7	4.7	0	25.1±2.5	18.1±2.1	0.7
7	8.7	0	6.1±1.0	7.8±0.2	1.3
8	0.6	0	22.6±1.0	14.4±1.6	0.6
8	4.2	0	6.6±1.1	5.7±1.3	0.9
8	6.3	0	N.D.	N.D.	N.D.
7	0.8	0.25	44.8±9.7	33.0±8.3	0.7
7	4.8	1	11.8±0.6	11.2±0.3	0.9
7	7.9	1	5.9	5.2	0.9
8	1.0	0.25	15.1±0.5	11.2±0.6	0.7
8	4.5	1	N.D.	N.D.	N.D.
8	8.1	1	N.D.	N.D.	N.D.

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FIGURE CAPTIONS

Figure 1. Reaction of nitrite with aqueous Fe(II) as a function of time. Rates of nitrite reduction were faster at higher Fe(II) concentrations, higher pH and in the presence of exogenous goethite. Note the time scale change at higher iron and high pH.

Figure 2. Dissolved Fe(II) concentration as a function of time, shown in panels by pH and Fe(II) concentration (note the time scale change at higher iron and high pH). Circles refer to experiments in which incubations were amended with goethite.

Figure 3. Closed system Rayleigh plots illustrating differences in the observed isotope effects of nitrite reduction by Fe(II) for $\delta^{15}\text{N-NO}_2^-$ (top) and $\delta^{18}\text{O NO}_2^-$ (bottom) at pH 7 (right) and pH 8 (left). Dotted lines indicate experiments containing amended goethite.

Figure 4. $\delta^{18}\text{O-NO}_2$ and $\delta^{15}\text{N-NO}_2$ in all incubations. The dotted line indicates a 1:1 relationship.

Figure 5. Summary of the final secondary minerals formed at the end of the Fe(II)-nitrite incubations. Mineral proportions were obtained via linear combination EXAFS shown in Figure S2.

Figure 6. Pseudo-first order rate constants (with respect to NO_2^-) varied linearly with starting Fe(II) concentration, with faster rates at higher pH and in the presence of an FeOOH (goethite) mineral surface. Rate constants were calculated assuming a pseudo-first order rate with Fe(II) in excess of nitrite, and exponential fitting of nitrite concentration over time.

Figure 7. The relationship between kinetic isotope effect ($^{15}\epsilon$ (top) and $^{18}\epsilon$ (bottom)) and pseudo-first order rate constant. Changes in observed kinetic isotope effects as a function of reaction rate likely reflect complex shifts in reaction mechanisms, pathways and the formation and stability of intermediates.

Figure 8. Site preference as related to the N_2O yield from reduction of NO_2^- by Fe(II). Changes in SP reflect differences in N_2O production mechanisms and likely reflect the formation and reactivity of nitrosyl-iron intermediates.

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Figure 1

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Figure 8