



15 **ABSTRACT**

16 Natural abundance stable isotopes in nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), and nitrous oxide ( $\text{N}_2\text{O}$ )  
17 have been used to better understand the cycling of nitrogen in marine and terrestrial  
18 environments. However, in order to extract the greatest information from the distributions of  
19 these isotopic species, the kinetic isotope effects for each of the relevant microbial reactions are  
20 needed. To date, kinetic isotope effects for nitrite oxidation and anaerobic ammonium oxidation  
21 (anammox) have not been reported. In this study, the nitrogen isotope effect was measured for  
22 microbial nitrite oxidation to nitrate. Nitrite oxidation is the second step in the nitrification  
23 process, and it plays a key role in the regeneration of nitrate in the ocean. Surprisingly, nitrite  
24 oxidation occurred with an inverse kinetic isotope effect, such that the residual nitrite became  
25 progressively depleted in  $^{15}\text{N}$  as the reaction proceeded. Three potential explanations for this  
26 apparent inverse kinetic isotope effect were explored: 1) isotope exchange equilibrium between  
27 nitrite and nitrous acid prior to reaction, 2) reaction reversibility at the enzyme level, and 3) true  
28 inverse kinetic fractionation. Comparison of experimental data to *ab initio* calculations and  
29 theoretical predictions leads to the conclusion that the fractionation is most likely inverse at the  
30 enzyme level. Inverse kinetic isotope effects are rare, but the experimental observations reported  
31 here agree with kinetic isotope theory for this simple N-O bond-forming reaction. Nitrite  
32 oxidation is therefore fundamentally different from all other microbial processes in which N  
33 isotope fractionation has been studied. The unique kinetic isotope effect for nitrite oxidation  
34 should help to better identify its role in the cycling of nitrite in ocean suboxic zones, and other  
35 environments in which nitrite accumulates.

36

## 36 1. INTRODUCTION

### 37 1.1 Application of Isotopes to Nitrogen Biogeochemistry

38 Stable isotope ratio measurements of nitrogen (N)-containing molecules have long provided  
39 insights into the relative rates of biogeochemical processes comprising the global nitrogen cycle.  
40 The kinetic fractionation factors ( $\alpha_k = {}^{14}\text{k}/{}^{15}\text{k}$ ) and isotope effects ( $\epsilon_k = (\alpha_k - 1) * 1000$ ) for key  
41 microbial processes offer constraints on the mechanisms of enzymatic reactions and provide the  
42 basis for interpretation of natural abundance N isotopic distributions in nature. For example, the  
43 kinetic isotope effects for both nitrogen fixation and denitrification, which add and remove  
44 bioavailable N from the ocean have been used to estimate the relative rates of these fluxes and  
45 thus the residence time of fixed N in the ocean (BRANDES and DEVOL, 2002; DEUTSCH et al.,  
46 2004; ALTABET, 2007).

47 The kinetic isotope effect for nitrogen fixation is small (-2 to +2‰; Table 1), which leads to  
48 addition of bioavailable N to the ocean with a  $\delta^{15}\text{N}$  value near its source value of 0‰. Other  
49 sources of N to the ocean from atmospheric deposition and continental runoff are also believed  
50 to be near 0‰, although this value is highly uncertain (BRANDES and DEVOL, 2002). The  
51 processes that remove bioavailable N from the ocean include denitrification and anammox in  
52 suboxic regions within the water column and marine sediments (recently reviewed by  
53 DALSGAARD et al., 2005; FRANCIS et al., 2007). If the ocean N budget is in steady state, N would  
54 be removed by these processes with a flux-weighted average  $\delta^{15}\text{N}$  value near 0‰ (ALTABET,  
55 2007; BRANDES and DEVOL, 2002; DEUTSCH et al., 2004; DEVOL et al., 2006). While the isotope  
56 effects for anammox are not known at this time, the isotope effects for water column (20-30‰)  
57 and sedimentary (~0‰) denitrification have been used to partition  $\text{N}_2$  losses between the water  
58 column and sediments. The large isotope effect for water column denitrification is a strong

59 constraint on this partitioning and implies that water column denitrification represents a  
60 relatively small proportion of the total N<sub>2</sub> flux (BRANDES and DEVOL, 2002; DEUTSCH et al.,  
61 2004; SIGMAN et al., 2003).

62 The large fractionation factor for nitrate reduction (BARFORD et al., 1999; DELWICHE and  
63 STEYN, 1970; GRANGER et al., 2006) also leads to <sup>15</sup>N enrichment of nitrate in ocean suboxic  
64 zones (BRANDES et al., 1998; CLINE and KAPLAN, 1975; SIGMAN et al., 2005; SIGMAN et al.,  
65 2003; VOSS et al., 2001). Recently, Casciotti and McIlvin (CASCIOTTI and MCILVIN, 2007)  
66 reported δ<sup>15</sup>N<sub>NO<sub>3</sub></sub> values up to +21.7‰ (and δ<sup>18</sup>O<sub>NO<sub>3</sub></sub> values up to +19.0‰) in the suboxic zone of  
67 the eastern tropical North Pacific (ETNP). With a large fractionation factor for nitrate reduction  
68 to nitrite, it would be expected for NO<sub>2</sub><sup>-</sup> to be depleted in <sup>15</sup>N relative to NO<sub>3</sub><sup>-</sup>. However, δ<sup>15</sup>N<sub>NO<sub>2</sub></sub>  
69 values within the suboxic zone were as low as -18.5‰ (CASCIOTTI and MCILVIN, 2007). The  
70 difference in δ<sup>15</sup>N between nitrate and nitrite (Δδ<sup>15</sup>N = δ<sup>15</sup>N<sub>NO<sub>3</sub></sub> - δ<sup>15</sup>N<sub>NO<sub>2</sub></sub>) ranged from +28.4 to  
71 +35.0‰ within the suboxic zone, with maximal values near the top of the secondary nitrite  
72 maximum. These differences in δ<sup>15</sup>N are larger than expected from the fractionation factor for  
73 nitrate reduction during denitrification (20-30‰; Table 1), particularly if there is concomitant  
74 nitrite consumption that acts to increase δ<sup>15</sup>N<sub>NO<sub>2</sub></sub>. These data suggest that either the fractionation  
75 factor for nitrate reduction in the water column is larger than previous estimates, with  
76 confounding effects on isotope-based oceanic nitrogen budgets (above), or that co-occurring  
77 processes act to increase Δδ<sup>15</sup>N. Such processes would need to decrease δ<sup>15</sup>N<sub>NO<sub>2</sub></sub> values relative  
78 to δ<sup>15</sup>N<sub>NO<sub>3</sub></sub>, or increase δ<sup>15</sup>N<sub>NO<sub>3</sub></sub> values while causing a minimal increase in δ<sup>15</sup>N<sub>NO<sub>2</sub></sub>.

79 In the suboxic zone, nitrite can be produced by nitrate reduction (CODISPOTI and  
80 CHRISTENSEN, 1985; CODISPOTI et al., 1986; CODISPOTI and RICHARDS, 1976) or possibly  
81 ammonia-oxidation (LIPSCHULTZ et al., 1990; WARD et al., 1989). The isotope effects for these

82 processes range from +13-38‰ (Table 1), but would be expressed to differing degrees depending  
83 on the extent of reaction completion. Nitrite consumption may be due to numerous processes,  
84 such as nitrite reduction to nitric oxide (NO) by denitrifying bacteria, nitrite reduction to N<sub>2</sub> by  
85 anammox bacteria (DALSGAARD et al., 2003; HAMERSLEY et al., 2007; KUYPERS et al., 2005;  
86 KUYPERS et al., 2003), or nitrite oxidation to nitrate by nitrite oxidizing bacteria (ANDERSON et  
87 al., 1982; CASCIOTTI and MCILVIN, 2007; LIPSCHULTZ et al., 1990; SIGMAN et al., 2005). Nitrite  
88 reduction to NO has been shown to occur with a normal kinetic isotope effect ( $^{15}\epsilon_{k,NIR} \approx +15\text{‰}$ ;  
89 (BRYAN et al., 1983)), which would increase  $\delta^{15}\text{N}_{\text{NO}_2}$  relative to  $\delta^{15}\text{N}_{\text{NO}_3}$  and lead to low  $\Delta\delta^{15}\text{N}$   
90 values. In order to better interpret N (and O) isotopic distributions of nitrate and nitrite in  
91 suboxic zones, the isotope effects for anammox and nitrite oxidation are needed. In this study,  
92 the kinetic isotope effect for nitrite oxidation was determined for pure cultures of the marine  
93 nitrite-oxidizing bacterium *Nitrococcus mobilis*.

94

## 95 **1.2. Isotopic Fractionation**

96 The differential distribution of isotopes between chemical species or phases, known as  
97 isotopic fractionation, has been recognized for many decades and is the basis for many  
98 applications in geochemistry (BIGELEISEN, 1952; BIGELEISEN, 1965; BIGELEISEN and MAYER,  
99 1947; UREY, 1947). Both equilibrium and kinetic isotope effects can lead to isotopic  
100 fractionation between phases or chemical species. The equilibrium fractionation factor,  $\alpha_{\text{eq}} =$   
101  $(A_2/A_1)/(B_2/B_1)$ , defines the isotope ratios that two species, 'A' and 'B', would have at  
102 equilibrium, where '1' and '2' refer to the light and heavy isotopes, respectively. Equilibrium  
103 fractionation arises primarily from differences in the zero point energies of the molecules  
104 undergoing isotopic exchange, leading to enrichment of the heavy isotopes in the more strongly

105 bonded form (BIGELEISEN and MAYER, 1947; SCHAUBLE et al., 2001). This can be shown using  
106 the molecular partition functions, which represent the distribution of the relevant molecules  
107 between their available rotational, translational, and vibrational energy states (UREY, 1947;  
108 BIGELEISEN and MAYER, 1947; RICHERT et al., 1977).

109 Kinetic fractionation factors ( $\alpha_k$ ) arise from small differences in the rates at which  
110 isotopically substituted molecules react, and are often the focus of stable isotope applications in  
111 biochemistry. Kinetic fractionation factors are defined by the instantaneous change in the isotope  
112 ratio of the reaction product ( $R_{Pi}$ ) at a given substrate isotope ratio ( $R_S$ ):  $\alpha_k = R_S/R_{Pi}$  (MARIOTTI  
113 et al., 1981; SCOTT et al., 2004). The kinetic fractionation factor is also often represented by the  
114 ratio of rate constants, assuming first order reaction kinetics apply:  $\alpha_k = k_1/k_2$ , where '1' and '2'  
115 refer to the light and heavy isotopes, respectively (BIGELEISEN and WOLFSBERG, 1958).

116 For a reversible reaction, the ratio of kinetic isotope effects for forward and reverse reactions  
117 is also related to the equilibrium isotope effect:  $\alpha_{eq} = \alpha_{kf}/\alpha_{kr}$ , where  $\alpha_{kf}$  is the kinetic isotope  
118 effect for the forward reaction and  $\alpha_{kr}$  is the kinetic isotope effect for the reverse reaction  
119 (O'LEARY, 1981). Given that the inherent fractionation factors  $\alpha_{eq}$ ,  $\alpha_{kf}$ , and  $\alpha_{kr}$  are constant, this  
120 relation should hold at all times. However, the expression of this equality in the isotope ratios of  
121 substrates and products only holds at equilibrium.

122 Kinetic fractionation factors can also be calculated from first principles using transition state  
123 theory, where the substrate is assumed to be in thermal equilibrium with the transition state of  
124 the reaction (BIGELEISEN, 1949; BIGELEISEN, 1952; BUDDENBAUM and SHINER, 1977). If the  
125 isotopically substituted species is more stably bonded in the substrate than in the transition state,  
126 then molecules containing the heavy isotope will react more slowly. This behavior is termed  
127 "normal" kinetic isotope fractionation, and typically occurs in bond-breaking reactions. On the

128 other hand, if the isotopically substituted species is more stably bonded in the transition state,  
129 which may be the case in some bond-forming reactions, then molecules containing the heavy  
130 isotope may react more quickly. This phenomenon is referred to as “inverse” kinetic isotope  
131 fractionation, and may be expected to occur in some bond-forming reactions (FRY, 1970).

132 In this study, measurements of nitrate and nitrite  $\delta^{15}\text{N}$  in pure culture experiments show that  
133 microbial nitrite oxidation occurs with an inverse kinetic isotope effect. Three potential  
134 mechanisms that could lead to this unusual fractionation are discussed: 1) a reversible reaction  
135 prior to the nitrite oxidation step (‘pre-equilibrium’), 2) reversibility of the nitrite oxidation  
136 reaction itself (‘enzyme reversibility’), and 3) a true enzyme-level inverse kinetic isotope effect.  
137 Examination of each of these mechanisms by comparison of experimental data to equilibrium  
138 and kinetic isotope fractionation theory suggests that this relatively simple bond-forming  
139 reaction proceeds with a true enzyme-level inverse kinetic isotope effect. Although inverse  
140 kinetic isotope effects are unusual in biochemistry, here I argue that its occurrence in nitrite  
141 oxidation should be expected and may have important implications for understanding marine  
142 nitrogen isotope biogeochemistry and nitrite cycling in suboxic zones.

143

## 144 **2. MATERIALS AND METHODS**

### 145 **2.1 Growth and Maintenance of Bacterial Cultures**

146 *Nitrococcus mobilis*, a marine nitrite-oxidizing bacterium (NOB) isolated from the Equatorial  
147 Pacific Ocean near the Galapagos Islands (WATSON and WATERBURY, 1971), was generously  
148 provided by Frederica Valois (Woods Hole Oceanographic Institution). *N. mobilis* is a member  
149 of the gamma-*Proteobacteria* that grows optimally at 25-30 °C and at pH 7.5-8.0 in 70-100%  
150 seawater (WATSON and WATERBURY, 1971). It is an obligate chemolithoautotroph, which fixes

151 carbon dioxide into biomass using  $\text{NO}_2^-$  as an electron source (WATSON and WATERBURY, 1971).  
152 In addition to oxidizing  $\text{NO}_2^-$  to  $\text{NO}_3^-$ , most nitrite oxidizing bacteria can assimilate  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  
153 and  $\text{NH}_4^+$  during growth (STARKENBURG et al., 2006). The physiology of *N. mobilis* has not been  
154 studied exhaustively, but its draft genome contains genes required to transport nitrite, assimilate  
155 nitrite (*nirB*), and reduce nitrite to nitric oxide (*nirK*). No genes have been found in this organism  
156 for ammonium oxidation to nitrite.

157 In this study, *N. mobilis* was maintained in marine NOB medium containing 75% seawater  
158 and 25% distilled water amended with 400  $\mu\text{M}$   $\text{MgSO}_4$ , 30  $\mu\text{M}$   $\text{CaCl}_2$ , 5  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$ , 2.3  $\mu\text{M}$   
159 Fe(III)-EDTA (“Geigy iron”), 0.1  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ , 0.25  $\mu\text{M}$   $\text{MnCl}_2$ , 0.002  $\mu\text{M}$   $\text{CoCl}_2$ , and 0.08  
160  $\mu\text{M}$   $\text{ZnSO}_4$  (WATSON and WATERBURY, 1971). Filter-sterilized  $\text{NaNO}_2$  was added to a final  
161 concentration of 10 mM after autoclaving the base medium. One-liter cultures were inoculated  
162 by transfer of 100 mL cultures (that had exhausted their  $\text{NO}_2^-$ ) into 900 mL NOB medium. The  
163 one-liter maintenance cultures were then grown for approximately one month before transfer or  
164 experimentation.

165

## 166 **2.2 Nitrite Oxidation Experiments**

167 To collect *N. mobilis* cells for isotopic fractionation experiments, 300 mL of maintenance  
168 culture (cell density  $\sim 10^8$  cells/mL) were harvested by centrifugation at 7600 g for 20 minutes in  
169 six 50 mL sterile centrifuge tubes. The cell pellets were each washed with 2 mL sterile marine  
170 NOB medium containing 100  $\mu\text{M}$   $\text{NaNO}_2$ . Washed cells were pooled, then re-aliquotted into  
171 sterile 2 mL centrifuge tubes and collected by centrifugation at 10,000 g. The supernatant was  
172 pipetted away and the cells were resuspended and pooled in 8 mL sterile marine NOB medium  
173 with 100  $\mu\text{M}$   $\text{NaNO}_2$ .



174 Results from three independent experiments are reported here. The first experiment (hereafter  
175 referred to as ‘Experiment 1’) was initiated by adding 0.5 mL of washed cells to flask “A” and  
176 1.0 mL to flask “B”, each containing 100 mL marine NOB medium with 100  $\mu\text{M}$   $\text{NaNO}_2$  at pH  
177 8.2. A third flask received no cells and was incubated with 100  $\mu\text{M}$   $\text{NO}_2^-$  as an uninoculated  
178 control. In the second experiment (hereafter referred to as ‘Experiment 2’), nine 250 mL flasks  
179 were prepared, each with 100 mL of NOB medium at pH 7.8. Experiment 2 was initiated by  
180 adding 0.5 mL of washed cells to triplicate “A” flasks and 1.0 mL to triplicate “B” flasks. The  
181 three final flasks received no cells (uninoculated controls). In the third experiment (hereafter  
182 referred to as “Experiment 3”), three 250 mL flasks were prepared, each with 100 mL of medium  
183 with 100  $\mu\text{M}$   $\text{NaNO}_2$  at pH 8.8. Experiment 3 was initiated by adding 0.5 mL of washed cell  
184 suspension to flask “A” and 1.0 mL to flask “B”. The third flask received no cells and was  
185 incubated as an uninoculated control. In each experiment, final cell densities were estimated to  
186 be on the order of  $10^6$  - $10^7$  cells/mL, with half as many cells in “A” flasks compared with “B”  
187 flasks.

188 Flasks were incubated at room temperature with shaking (Experiments 1 and 2) or without  
189 shaking (Experiment 3). Time course samples were collected immediately after inoculation and  
190 periodically thereafter until approximately all of the  $\text{NO}_2^-$  had been converted to  $\text{NO}_3^-$  (2-6 days,  
191 depending on the experiment). At each time point, 10-15 mL were withdrawn for nitrite and  
192 nitrate concentration analyses, as well as for nitrite and nitrate isotopic analyses; these samples  
193 were passed through a 0.2  $\mu\text{M}$  pore size membrane filter and stored frozen.

194

195 **2.3 Chemical Analyses**

196  $\text{NO}_2^-$  concentrations were analyzed in duplicate using the Greiss-Islovay colorimetric  
197 reaction (STRICKLAND and PARSONS, 1972). Combined  $\text{NO}_2^- + \text{NO}_3^-$  concentrations were  
198 determined from the  $m/z = 44$  peak area obtained by isotopic analyses using the denitrifier  
199 method (SIGMAN et al., 2001; CASCIOTTI et al., 2002). A two-point calibration for  $\text{NO}_2^- + \text{NO}_3^-$   
200 concentration was derived from blank (0  $\mu\text{M}$  added) and 20  $\mu\text{M}$  standards, analyzed after every  
201 nine samples. Most samples were analyzed in duplicate, and uncertainties in  $[\text{NO}_2^- + \text{NO}_3^-]$  were  
202 derived from replicate analyses where possible.  $\text{NO}_3^-$  concentrations were calculated by  
203 difference between  $[\text{NO}_2^- + \text{NO}_3^-]$  and  $[\text{NO}_2^-]$ , and standard deviations for  $[\text{NO}_3^-]$  measurements  
204 were calculated by error propagation from standard deviations of  $[\text{NO}_2^-]$  and  $[\text{NO}_2^- + \text{NO}_3^-]$   
205 measurements.

206

207 **2.4 Isotopic Analyses and Corrections**

208 Nitrite  $\delta^{15}\text{N}$  values ( $\delta^{15}\text{N}_{\text{NO}_2} = ([^{15}\text{N}/^{14}\text{N}]_{\text{NO}_2} / [^{15}\text{N}/^{14}\text{N}]_{\text{AIR}} - 1) \times 1000$ ) from the time course  
209 samples were analyzed by reaction with acetic acid-buffered sodium azide to form nitrous oxide  
210 ( $\text{N}_2\text{O}$ ) via the “azide method” (MCILVIN and ALTABET, 2005). Isotope ratios of the produced  
211  $\text{N}_2\text{O}$  were measured by continuous flow isotope ratio mass spectrometry (CF-IRMS) using an  
212 on-line purge and trap system for extraction and purification of the  $\text{N}_2\text{O}$  analyte (see CASCIOTTI  
213 et al., 2007). Analyses were calibrated to the AIR- $\text{N}_2$  reference scale using sodium nitrite salts  
214 with calibrated  $\delta^{15}\text{N}_{\text{NO}_2}$  values: N-23 (+3.7‰), N-7373 (-79.6‰), and N-10219 (+2.5‰)  
215 (CASCIOTTI et al., 2007). Solutions of these standard nitrite materials were reacted and analyzed  
216 in parallel to nitrite samples, with an aliquot of each standard analyzed initially and after every  
217 nine experimental samples. Sample and standard quantities were matched to achieve 10 nmoles

218 of either sample or standard  $\text{NO}_2^-$  converted to 10 nmoles of  $\text{N}_2\text{O}$  by reaction with sodium azide.  
219 In this technique, one nitrogen atom in the  $\text{N}_2\text{O}$  arises from  $\text{NO}_2^-$  and one from azide (MCILVIN  
220 and ALTABET, 2005). Analyses were conducted only on samples with  $\text{NO}_2^-$  concentrations  
221 greater than 1  $\mu\text{M}$ . Each sample was analyzed in duplicate and the  $\delta^{15}\text{N}$  values are reported as the  
222 average  $\pm$  one standard deviation.

223 Nitrate plus nitrite  $\delta^{15}\text{N}$  values ( $\delta^{15}\text{N}_{\text{NO}_2+\text{NO}_3} = ([^{15}\text{N}/^{14}\text{N}]_{\text{NO}_2+\text{NO}_3}/[^{15}\text{N}/^{14}\text{N}]_{\text{AIR}} - 1) \times 1000$ )  
224 were analyzed by bacterial conversion of nitrogen oxides ( $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) to  $\text{N}_2\text{O}$  via the  
225 “denitrifier method” (CASCIOITI et al., 2002; SIGMAN et al., 2001). The isotopes of  $\text{N}_2\text{O}$  were  
226 analyzed via CF-IRMS, as described above. Analyses were calibrated to the AIR- $\text{N}_2$  reference  
227 scale using potassium nitrate reference materials USGS32 ( $\delta^{15}\text{N} = +180.0\text{‰}$ ), USGS34 ( $\delta^{15}\text{N} = -$   
228  $1.8\text{‰}$ ), and USGS35 ( $\delta^{15}\text{N} = +2.7\text{‰}$ ) (BOHLKE et al., 2003). Standards were reacted and  
229 analyzed in parallel to samples, with an aliquot of each standard analyzed initially and after  
230 every nine experimental samples. Sample and standard quantities were matched to achieve 20  
231 nmoles of sample ( $\text{NO}_2^- + \text{NO}_3^-$ ) or standard ( $\text{NO}_3^-$ ) converted to 10 nmoles  $\text{N}_2\text{O}$  by the  
232 denitrifier method. Most samples were analyzed in duplicate and are reported as average values  
233  $\pm$  one standard deviation.

234 The majority of the time course samples contained a mixture of  $\text{NO}_2^-$  and  $\text{NO}_3^-$ . Therefore, in  
235 order to estimate nitrate  $\delta^{15}\text{N}$  ( $\delta^{15}\text{N}_{\text{NO}_3}$ ) values, the procedure previously described (CASCIOITI  
236 and MCILVIN, 2007) was followed for mass and isotope balance of the mixture components:  
237  $\delta^{15}\text{N}_{\text{NO}_3} = (\delta^{15}\text{N}_{\text{NO}_2+\text{NO}_3} \times [\text{NO}_2^- + \text{NO}_3^-] - \delta^{15}\text{N}_{\text{NO}_2} \times [\text{NO}_2^-]) \div [\text{NO}_3^-]$ . The approximation  
238 required by this equation that  $^{15}\text{N}$  is a minor constituent does not introduce significant error in  
239  $\delta^{15}\text{N}_{\text{NO}_3}$  estimation for the samples described here. Standard deviations for  $\delta^{15}\text{N}_{\text{NO}_3}$  were  
240 calculated by error propagation using the standard deviations for  $\delta^{15}\text{N}_{\text{NO}_2}$ ,  $\delta^{15}\text{N}_{\text{NO}_2+\text{NO}_3}$ , and

241 concentration measurements. For samples with a single  $\delta^{15}\text{N}_{\text{NO}_2+\text{NO}_3}$  analysis, the standard  
242 deviation was assumed to be 0.3‰ (SIGMAN et al., 2001).

243

## 244 **2.5 Fractionation Factor Estimates**

245 The kinetic fractionation factor for nitrite oxidation ( $^{15}\alpha_{\text{k,NXR}}$ ) was calculated from the  
246  $\delta^{15}\text{N}_{\text{NO}_2}$  data using a closed-system Rayleigh distillation model (SCOTT et al., 2004):  $\ln(^{15}\text{R}_{\text{NO}_2})$   
247  $= (1/^{15}\alpha_{\text{k,NXR}} - 1) \cdot \ln(C) + \ln(^{15}\text{R}_{\text{NO}_2,0}/C_0^{(1/\alpha_{\text{k,NXR}} - 1)})$ , where  $^{15}\text{R}_{\text{NO}_2}$  is the  $^{15}\text{N}/^{14}\text{N}$  ratio of  $\text{NO}_2^-$   
248 (unitless) and  $^{15}\text{R}_{\text{NO}_2,0}$  is its initial value.  $C$  is the  $\text{NO}_2^-$  concentration ( $\mu\text{M}$ ) and  $C_0$  is its initial  
249 value.  $^{15}\alpha_{\text{k,NXR}}$  (unitless) is the ratio of the first order rate constants for  $^{14}\text{NO}_2^-$  and  $^{15}\text{NO}_2^-$   
250 oxidation, respectively ( $^{14}\text{k}_{\text{NXR}}/^{15}\text{k}_{\text{NXR}}$ ). Fractionation factors calculated in this way are the  
251 reciprocal of those discussed by Mariotti et al (MARIOTTI et al., 1981).

252 Dummy variables were used to obtain the average and standard deviation of  $^{15}\alpha_{\text{k,NXR}}$  for all  
253 experiments (SCOTT et al., 2004). This procedure utilizes the equation  $y = \beta x + \gamma_1 + \gamma_2 D_2 + \gamma_3$   
254  $D_3$ , where  $y = \ln(^{15}\text{R}_{\text{NO}_2})$ ,  $x = \ln(C)$ , and  $\beta = (1/^{15}\alpha_{\text{k,NXR}} - 1)$ . First, experimental data were  
255 combined from different flasks to obtain the y-intercept for experiment 1 ( $\gamma_1$ ) and the y-intercept  
256 offset from  $\gamma_1$  for experiments 2 ( $\gamma_2$ ) and 3 ( $\gamma_3$ ). Cell density did not appreciably change the  
257 observed kinetic isotope effect, so “A” and “B” flasks from each experiment were treated as  
258 replicates for this purpose. Next, the  $\beta$  value was determined from each x-y pair, and the  $^{15}\alpha_{\text{k,NXR}}$   
259 value was calculated from each  $\beta$  value. Finally, the average and standard deviation for  $^{15}\alpha_{\text{k,NXR}}$   
260 were calculated from the entire dataset.

261

## 262 **2.6 Ab Initio Calculations of Molecular Vibration Frequencies**

263 Molecular vibration frequencies for two isotopic forms (containing either  $^{15}\text{N}$  or  $^{14}\text{N}$ ) of  $\text{NO}_2^-$   
264 ,  $\text{HNO}_2$ , and  $\text{NO}_3^-$  were calculated using the Gaussian interface through the WebMO (Version  
265 8.0.011e) guest server at buchner.chem.hope.edu. The geometry of each molecule was first  
266 optimized in either 'water' or 'no solvent', depending on the desired phase of the vibration  
267 calculations. The optimized molecules were then used to calculate the molecular vibration  
268 frequencies using B3LYP density function theory with the 'routine' basis set (6-31G(d)) (ANBAR  
269 et al., 2005) in both 'no solvent' and in aqueous solution. The geometry optimization and  
270 vibration calculations were performed three times each, and the frequencies obtained varied by  
271 less than 0.01%.

272

273

## **3. RESULTS**

### 274 **3.1 Time Course Experiments**

275 In each independent experiment,  $\text{NO}_2^-$  was oxidized to  $\text{NO}_3^-$  by pure cultures of *N. mobilis*  
276 over a course of 2-6 days (Figure 1A, C, E).  $\text{NO}_2^-$  removal was nearly complete in each  
277 inoculated flask. Each experiment was conducted using a two-fold range of cell densities  
278 (approximately  $2\text{-}4 \times 10^7$  cells  $\text{mL}^{-1}$ ), depending on the volume of concentrated cell suspension  
279 added. The net rate of nitrite oxidation apparently increased with cell density (Figure 1A, C, E).  
280 For the most part, mass balance was maintained between  $\text{NO}_2^-$  and  $\text{NO}_3^-$  pools as  $\text{NO}_2^-$  was  
281 consumed. Concentrations of  $\text{NO}_2^- + \text{NO}_3^-$  in experimental flasks showed up to 10-20% variation  
282 at some time points, but as discussed below, there was no change in  $\delta^{15}\text{N}_{\text{NO}_2 + \text{NO}_3}$  corresponding  
283 to these apparent changes in  $[\text{NO}_2^- + \text{NO}_3^-]$ . Control flasks also showed similar variability in  
284  $[\text{NO}_2^- + \text{NO}_3^-]$ , suggesting that the variability was most likely not biological in nature. At this

285 time, the most likely explanation is error in concentration measurement at particular time points  
286 in the time course.

287 In every case, the nitrite became progressively depleted in  $^{15}\text{N}$ , decreasing from initial  $\delta^{15}\text{N}$   
288 values of +0.7‰ to less than -50‰ as it was oxidized to  $\text{NO}_3^-$  (Figure 1B, D, F). In contrast,  
289 uninoculated control flasks showed no change  $\delta^{15}\text{N}_{\text{NO}_2}$  values over the time course. Control  
290 flasks also showed no change in the  $\delta^{18}\text{O}$  value of  $\text{NO}_2^- + \text{NO}_3^-$  (data not shown), a measurement  
291 that is very sensitive to changes in the fractions of nitrate and nitrite in the mixture (CASCIO  
292 al., 2007). Finally, within the standard error of the measurement ( $\sim 0.3\%$ )  $\delta^{15}\text{N}_{\text{NO}_2 + \text{NO}_3}$  did not  
293 change over the time course in any flask, whether nitrite oxidation occurred (experimental flasks)  
294 or not (control flasks) (Figure 1 B, D, F). The consistency of  $\delta^{15}\text{N}_{\text{NO}_2 + \text{NO}_3}$  over the time course is  
295 a strong constraint on mass balance between  $\text{NO}_2^-$  and  $\text{NO}_3^-$  because any potential loss of N from  
296 the  $\text{NO}_2^-$  and  $\text{NO}_3^-$  pools would have needed to possess a constant  $\delta^{15}\text{N}$  value of +1‰, even as  
297  $\delta^{15}\text{N}_{\text{NO}_2}$  decreased from +1‰ to -52‰. Isotopically, it would be difficult to account for loss of  
298  $\text{NO}_2^-$  to other pools of N that were not measured (cell biomass,  $\text{NO}$ ,  $\text{NH}_4^+$ ).

299 Mass balance allows the calculation of the  $\delta^{15}\text{N}$  of accumulated  $\text{NO}_3^-$  over the time course  
300 (CASCIO and MCILVIN, 2007). As expected from a closed-system isotope mass balance,  
301  $\delta^{15}\text{N}_{\text{NO}_3}$  was highest initially, then decreased over the time course to a value within error of the  
302 initial  $\delta^{15}\text{N}_{\text{NO}_2}$  (not shown). The maintenance of mass (Figure 1A, C, E) and isotope (Figure 1B,  
303 D, F) balance between nitrate and nitrite in experimental flasks and the lack of nitrite oxidation  
304 to nitrate in control flasks points towards biological nitrite oxidation as the process responsible  
305 for  $\text{NO}_2^-$  consumption in these experiments.

306

### 307 3.2 Rayleigh Fractionation

308 Analysis of the observed trends in  $\delta^{15}\text{N}_{\text{NO}_2}$  using a closed system Rayleigh model yields  
309 estimates for  $^{15}\alpha_{\text{k,NXR}}$  of  $0.9873 \pm 0.0010$ ,  $0.9867 \pm 0.0018$ , and  $0.9882 \pm 0.0005$  for the three  
310 experiments, respectively (Figure 2). Averaging over all experiments yields an estimate of  
311  $0.9872 \pm 0.0015$  for  $^{15}\alpha_{\text{k,NXR}}$ . In all three experiments, the kinetic fractionation factor appears to  
312 be inverse. In addition, the three experiments were all very similar to each other, with individual  
313 estimates for  $^{15}\alpha_{\text{k,NXR}}$  within two standard deviations of the overall  $^{15}\alpha_{\text{k,NXR}}$ .

314 As observed in Figure 2, the fractionation factor appeared to increase slightly (become less  
315 inverse) after approximately 80% of the nitrite has been oxidized, as indicated by the deviation  
316 from the  $^{15}\alpha_{\text{k,NXR}} = 0.9872$  line at  $\ln([\text{NO}_2^-])$  values below 2 (corresponding to  $[\text{NO}_2^-]$  less than  
317  $\sim 7 \mu\text{M}$ ) (Figure 2). Both experiments 1 and 2 showed this change of slope. Inclusion of these  
318 points in the analysis may be one reason for the slight variation between  $^{15}\alpha_{\text{k,NXR}}$  in experiments  
319 1, 2, and 3. The apparent change in  $^{15}\alpha_{\text{k,NXR}}$  at later time points in the experiment may result  
320 from departures of the system from the basic assumption of a unidirectional closed system  
321 inherent in the Rayleigh distillation equation. Many systems show a tendency towards smaller  
322 isotope effects at high extents of substrate consumption as a result of diffusive limitation or  
323 active transport of substrate (FARQUHAR et al., 1982; GRANGER et al., 2008; GRANGER et al.,  
324 2006; NEEDOBA et al., 2004; O'LEARY, 1981). Given that the *N. mobilis* experiments were  
325 conducted with whole cells, these factors may play a role in the expressed isotope effect, which I  
326 will return to below. However, both diffusion of  $\text{NO}_2^-$  and uptake by the cell are likely to have  
327 small, normal kinetic isotope effects (GRANGER et al., 2008; WASER et al., 1998b), and are likely  
328 to be of secondary importance in the current analysis.

329

#### 4. DISCUSSION

330  
331 Given that true inverse kinetic isotope effects are rare, two alternative explanations were  
332 considered for the observed results. First, one must consider whether nitrite with low  $\delta^{15}\text{N}$  could  
333 have been produced in the experiments over time, thus mimicking an inverse kinetic isotope  
334 effect. The only mechanism possible for nitrite production in *N. mobilis* is through reduction of  
335 nitrate by reversible reaction of the nitrite oxidoreductase enzyme. The membrane-bound nitrite  
336 oxidoreductase enzymes isolated from *Nitrobacter agilis* and *Nitrobacter hamburgensis* have  
337 been shown to be reversible under low pH (TANAKA et al., 1983) and reducing conditions  
338 (MEINCKE et al., 1992; SUNDERMEYER-KLINGER et al., 1984). Although oxygen concentrations  
339 are not expected to have been limiting in the current *N. mobilis* experiments, enzyme-level  
340 reversibility is one mechanism that could produce an apparent inverse kinetic isotope effect, if  
341 the reverse reaction (nitrate reduction) is more highly fractionating than the forward reaction  
342 (nitrite oxidation). Therefore, the possibility for enzymatic back-reaction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  was  
343 carefully examined in section 4.1.2.

344 The second scenario under which a normal enzyme-level kinetic fractionation factor may  
345 appear to be inverse involves equilibrium between the true substrate for the reaction and a larger  
346 pool of non-reactive substrate. This is termed a “pre-equilibrium” scenario because the  
347 equilibrium reaction would occur prior to the enzymatic reaction, rather than at the enzyme level.  
348 For example, a pH-dependent pre-equilibrium between  $\text{H}_2\text{S}$  and  $\text{HS}^-$  was used to explain the  
349 small inverse kinetic isotope effect observed in anaerobic sulfide oxidation (FRY et al., 1984).  
350 The pH-dependent isotope exchange between  $\text{NO}_2^-$  and  $\text{HNO}_2$  is examined here in section 4.1.1.  
351 Rejecting these two scenarios, in which the apparent inverse fractionation factor would be driven  
352 by equilibrium or reversibility, it is shown that transition state theory supports the observation of



353 an inverse kinetic fractionation factor for nitrite oxidoreductase at the enzyme level (Section  
354 4.1.3).

355

## 356 **4.1 Potential Explanations for the Inverse Isotope Effect**

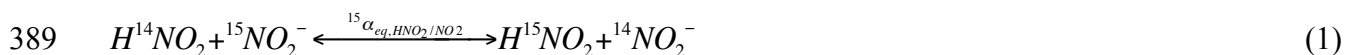
### 357 *4.1.1 Pre-equilibrium*

358 Apparent inverse fractionation factors have been observed in systems where a fractionating  
359 equilibrium reaction precedes a unidirectional enzymatic reaction. An example of this is in  
360 anaerobic oxidation of sulfide to elemental sulfur ( $S^0$ ) by the purple photosynthetic bacterium  
361 *Chromatium vinosum* (FRY et al., 1984). An inverse isotope effect was observed, whereby the  
362 sulfide undergoing oxidation became depleted  $^{34}S$  over time. The  $S^0$  produced by this reaction  
363 was initially enriched in  $^{34}S$ , but then also became depleted over time, approaching the  $\delta^{34}S$  of  
364 the initial substrate. However, in the sulfide oxidation reaction, the apparent inverse kinetic  
365 isotope effect was explained by co-occurring equilibrium and kinetic isotope effects, rather than  
366 a true inverse kinetic isotope effect (FRY et al., 1984). The equilibrium isotope effect between  
367 hydrogen sulfide ( $H_2S$ ) and bisulfide ( $HS^-$ ) caused the substrate for the reaction,  $H_2S$ , to be  
368 enriched in  $^{34}S$  by approximately 6‰ relative to the  $HS^-$  (FRY et al., 1984). Therefore, when  $H_2S$   
369 reacted with a small *normal* kinetic isotope effect the overall process produced  $S^0$  that was  
370 enriched in  $^{34}S$  relative to the total reduced sulfur pool, yielding an apparently inverse kinetic  
371 isotope effect.

372 In the case of nitrite oxidation to nitrate, it is often assumed that the substrate for the reaction  
373 is  $NO_2^-$ , although few experiments have been specifically designed to distinguish between  $HNO_2$   
374 and  $NO_2^-$  as substrates (BOON and LAUDELOUT, 1962). Given this uncertainty, the possibility that  
375  $HNO_2$  could have a significantly different  $\delta^{15}N$  from the total N(III) pool ( $NO_2^- + HNO_2$ ) must

376 be considered. Nitrous acid (HNO<sub>2</sub>) has a pKa of 2.8 (RIORDAN et al., 2005), and at pH 8 more  
 377 than 99.999% of the total N(III) occurs in the form of NO<sub>2</sub><sup>-</sup>, and less than 0.001% of the N(III)  
 378 occurs in the form of HNO<sub>2</sub>. In order for the pH-dependent pre-equilibrium to explain the  
 379 observed inverse kinetic isotope effect, 1) the minor pool (in this case, HNO<sub>2</sub>) must be the  
 380 substrate for the reaction, 2) HNO<sub>2</sub> must be enriched in <sup>15</sup>N relative to NO<sub>2</sub><sup>-</sup>, and 3) the  
 381 magnitude of the equilibrium isotope effect must be large enough to overcome a normal enzyme-  
 382 level kinetic isotope effect for the oxidation to nitrate. The scenarios that would allow the  
 383 observed inverse isotope effect to be consistent with a normal enzyme-level kinetic isotope  
 384 effect, were evaluated through *ab initio* calculation of the equilibrium isotope effect for N  
 385 isotope exchange between HNO<sub>2</sub> and NO<sub>2</sub><sup>-</sup>, in combination with six different Rayleigh model  
 386 simulations (see below for additional details). .

387 The nitrogen isotope exchange between HNO<sub>2</sub> and NO<sub>2</sub><sup>-</sup> is represented by equation (1):



390  
 391 where <sup>15</sup>α<sub>eq,HNO<sub>2</sub>/NO<sub>2</sub> is the fractionation factor for this isotope exchange equilibrium. As  
 392 discussed earlier, <sup>15</sup>α<sub>eq,HNO<sub>2</sub>/NO<sub>2</sub> defines the ratio of isotope ratios of HNO<sub>2</sub> and NO<sub>2</sub><sup>-</sup> at  
 393 equilibrium (<sup>15</sup>R<sub>HNO<sub>2</sub></sub>/<sup>15</sup>R<sub>NO<sub>2</sub></sub>)<sub>eq</sub>. This equilibrium fractionation factor can be calculated (with  
 394 some approximations that are suitable for isotopes other than hydrogen) using the reduced  
 395 partition functions (Q) if the vibration frequencies of the <sup>14</sup>N and <sup>15</sup>N-containing molecules of  
 396 both species are known (UREY, 1947). Equation 2 illustrates how <sup>15</sup>α<sub>eq,HNO<sub>2</sub>/NO<sub>2</sub> is related to the  
 397 reduced partition function ratios for the two species.</sub></sub></sub>

398

399 
$${}^{15}\alpha_{eq,HNO_2/NO_2} = \frac{{}^{15}Q_{HNO_2}/{}^{14}Q_{HNO_2}}{{}^{15}Q_{NO_2}/{}^{14}Q_{NO_2}} \quad (2)$$

400  
 401 Equation 3 shows how the reduced partition function ratio for either HNO<sub>2</sub> or NO<sub>2</sub><sup>-</sup> is calculated  
 402 from the normal mode molecular vibration frequencies of <sup>15</sup>N and <sup>14</sup>N-containing molecules.

403  
 404 
$$\frac{Q_{15}}{Q_{14}} = \frac{\sigma_{14}}{\sigma_{15}} \prod_i \frac{u_{15i}}{u_{14i}} \cdot \frac{e^{-u_{15i}/2}}{e^{-u_{14i}/2}} \cdot \frac{1 - e^{-u_{14i}}}{1 - e^{-u_{15i}}} \quad (3)$$

405  
 406 In equation 3, Q<sub>14</sub> and Q<sub>15</sub> are the reduced partition function ratios relative to the separated  
 407 atoms, for <sup>14</sup>N and <sup>15</sup>N-containing molecules, respectively. σ<sub>14</sub> and σ<sub>15</sub> are the symmetry  
 408 numbers for the two isotopically substituted species. In addition,  $u_i = hc\omega_i/kT$ , where ω<sub>i</sub>  
 409 corresponds to each of the i<sup>th</sup> normal frequencies (bent polyatomic molecules with N atoms have  
 410 3N-6 normal frequencies), *h* is Plank's constant, *k* is Boltzman's constant, *c* is the speed of light,  
 411 and *T* is the absolute temperature in K (here, *T* = 298 K). Therefore, in order to obtain a first-  
 412 order estimate for <sup>15</sup>α<sub>eq,HNO<sub>2</sub>/NO<sub>2</sub>, we need only the molecular vibration frequencies for H<sup>14</sup>NO<sub>2</sub>,  
 413 H<sup>15</sup>NO<sub>2</sub>, <sup>14</sup>NO<sub>2</sub><sup>-</sup>, and <sup>15</sup>NO<sub>2</sub><sup>-</sup>. For additional discussion of the assumptions and simplifications  
 414 involved in using equations 2 and 3, please see Bigeleisen and Mayer (1947) and Urey (1947).</sub>

415 A collection of observed (BEGUN and FLETCHER, 1960; DEELEY and MILLS, 1985) and  
 416 calculated (*this study*) vibration frequencies for NO<sub>2</sub><sup>-</sup> and HNO<sub>2</sub> are given in Table 2. Aqueous  
 417 vibration frequencies were available for NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> (BEGUN and FLETCHER, 1960), but not  
 418 for HNO<sub>2</sub>. For HNO<sub>2</sub> the only available vibration frequencies were in gas phase (DEELEY and  
 419 MILLS, 1985). Given that the molecular vibrations could be quite different in aqueous vs. gas  
 420 phase (RICHT et al., 1977), vibrational frequencies were needed for HNO<sub>2</sub> in the aqueous phase.

421 To check the accuracy of the calculation, the aqueous phase vibration frequencies for  $\text{NO}_2^-$  and  
422 gas phase vibration frequencies for  $\text{HNO}_2$  were also calculated to compare with observed  
423 frequencies (Table 2). Finally, to test the sensitivity to solvation the vibration frequencies for  
424  $\text{NO}_2^-$  were also calculated for the ‘no solvent’ case.

425 The calculated frequencies for  $\text{NO}_2^-$  and  $\text{HNO}_2$  were similar to observed frequencies, with  
426 differences ranging from -6.8 to +1.5% for  $^{14}\text{N}$ -containing species and -6.7 to +1.7% for  $^{15}\text{N}$ -  
427 containing species (Table 2). The frequency shifts upon isotopic substitution were also similar.  
428 On average, a 1.13% vibrational frequency shift upon substitution with  $^{15}\text{N}$  was obtained in the  
429 calculated frequencies, compared with a 1.16% vibrational shift in the observed frequencies.  
430 Finally, the normal modes that appeared insensitive to isotopic substitution in the modeled  
431 frequencies also showed little isotopic shift in the observed frequencies. The comparison  
432 between modeled and observed frequencies gives some confidence in the calculated results,  
433 although both observed and calculated frequencies could be inaccurate for different reasons. The  
434 differences between calculated aqueous and gas phase frequencies were less than 2.5% in all  
435 cases.

436 The modeled frequencies were used to calculate the equilibrium fractionation factor for  
437 nitrogen exchange between  $\text{NO}_2^-$  and  $\text{HNO}_2$  using equations 2 and 3. Little difference was  
438 obtained for  $^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2}$  in aqueous (0.9975) versus gas (0.9972) phase (Table 3). In addition,  
439 use of the observed frequencies (from mixed phases) yields an  $^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2}$  value of 0.9970,  
440 which is very similar to the  $^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2}$  values obtained from the *ab initio* frequencies. In all  
441 cases, the calculated  $^{15}\epsilon_{\text{eq,HNO}_2/\text{NO}_2}$  values were small at room temperature (-3 to -2.5‰), and  
442  $\text{HNO}_2$  was predicted to be depleted in  $^{15}\text{N}$  relative to  $\text{NO}_2^-$ . Given these results for  $\text{HNO}_2/\text{NO}_2^-$   
443 equilibration, it is anticipated that  $\text{HNO}_2$  would have been depleted in  $^{15}\text{N}$  relative to  $\text{NO}_2^-$  by 2.5

444 to 3.0‰ in the *N. mobilis* experiments (room temperature, pH 7.8 - 8.8). In contrast to the  
445 H<sub>2</sub>S/HS<sup>-</sup> equilibrium where protonation and deprotonation directly affect the bonding  
446 environment of the sulfur atom (a primary isotope effect), protonation and deprotonation of NO<sub>2</sub><sup>-</sup>  
447 does not occur at the central N atom. Therefore, it is not surprising that protonation and  
448 deprotonation of the NO<sub>2</sub><sup>-</sup>/HNO<sub>2</sub> couple should exhibit a small isotope effect.

449 Given the small equilibrium isotope effect between HNO<sub>2</sub> and NO<sub>2</sub><sup>-</sup>, it can be shown that  
450 reaction of either NO<sub>2</sub><sup>-</sup> or HNO<sub>2</sub> with a normal fractionation factor cannot explain an apparent  
451 inverse kinetic fractionation factor of the magnitude that we observe. This is illustrated in detail  
452 through Rayleigh simulations using various estimates for the kinetic (normal vs. inverse) and  
453 equilibrium isotope effects, and using either HNO<sub>2</sub> or NO<sub>2</sub><sup>-</sup> as substrate for the reaction  
454 (*Electronic Annex 1*). The pre-equilibrium between HNO<sub>2</sub> and NO<sub>2</sub><sup>-</sup> can therefore be rejected as  
455 a mechanism contributing to the apparent inverse kinetic fractionation factor.

456

#### 457 *4.1.2. Enzyme-Level Reversibility*

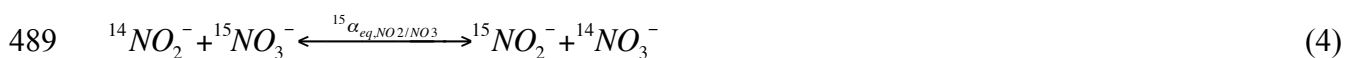
458 Enzyme reversibility could lead to an apparent inverse isotope effect, even if the forward and  
459 reverse reactions are normal, if 1) the reverse reaction has a larger isotope effect than the forward  
460 reaction, and 2) there is sufficient mass flux in the reverse reaction to lead to isotopic depletion  
461 of the primary substrate pool. The flux required to obtain an apparent inverse isotope effect will  
462 vary inversely with the magnitude of the isotope effect for the reverse reaction, in this case  
463 nitrate reduction to nitrite.

464 Nitrite oxidoreductase is known to be reversible (SUNDERMEYER-KLINGER et al., 1984;  
465 TANAKA et al., 1983). For example, some studies have shown growth of *Nitrobacter* under  
466 anaerobic conditions using nitrate as a terminal electron acceptor (AHLERS et al., 1990; BOCK et

467 al., 1988; FREITAG and BOCK, 1990; FREITAG et al., 1987), and other studies have shown that  
 468 *Nitrobacter* can assimilate nitrate-N (KUMAR and NICHOLAS, 1982). Both processes require a  
 469 mechanism for reducing nitrate to nitrite; however, nitrate reductase has not been found in any of  
 470 the nitrite oxidizer genomes studied thus far, including *Nitrococcus mobilis* (STARKENBURG et  
 471 al., 2008). Instead, it is believed that nitrite oxidoreductase acts as the nitrate reductase under  
 472 certain growth conditions. This theory is based on the biochemical similarity (YAMANAKA, 1996;  
 473 YAMANAKA and FUKUMORI, 1988) and evolutionarily relatedness of the genes for nitrite  
 474 oxidoreductase to the *narGH* family of dissimilatory nitrate reductases (STARKENBURG et al.,  
 475 2008), as well as observed reversibility of partially purified nitrite oxidoreductase from  
 476 *Nitrobacter* species (TANAKA et al., 1983; YAMANAKA and FUKUMORI, 1988). The biochemistry  
 477 of nitrite oxidoreductase has not been studied in *Nitrococcus* species directly, but the nitrite  
 478 oxidizing systems appear to be similar between the two genera (BARTOSCH et al., 1999; SPIECK  
 479 and BOCK, 2001), and some reversibility of this enzyme might be expected under some  
 480 circumstances.

481 Here the role of enzyme reversibility in the observed inverse kinetic isotope effect for nitrite  
 482 oxidation was evaluated. Two aspects of this question are discussed: first, whether equilibrium  
 483 calculations for the nitrite/nitrate redox system support a normal kinetic isotope effect for the  
 484 forward reaction (nitrite oxidation to nitrate) and second, whether significant back reaction  
 485 (nitrate reduction to nitrite) can be detected during experiments 1-3. To address the first  
 486 component, the equilibrium fractionation factor for isotope exchange between nitrate and nitrite  
 487 (equation 4) was first computed.

488



490

491 The equilibrium fractionation factor for the isotope exchange reaction ( $^{15}\alpha_{\text{eq},\text{NO}_2/\text{NO}_3}$ ) can be  
492 calculated using the same principles as applied above to calculate the isotope effect for nitrogen  
493 isotope exchange between  $\text{NO}_2^-$  and  $\text{HNO}_2$  (equations 2 and 3). Table 2 contains the relevant  
494 molecular vibration frequencies for  $\text{NO}_2^-$  and  $\text{NO}_3^-$  for use in equation 3. Using the calculated  
495 molecular vibrations for  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in aqueous solution, it is estimated that  $^{15}\alpha_{\text{eq},\text{NO}_2/\text{NO}_3}$  is  
496 approximately 0.945 in aqueous solution (Table 3). Furthermore, using experimentally-derived  
497 molecular vibrations for aqueous  $\text{NO}_2^-$  and  $\text{NO}_3^-$  ions yields  $^{15}\alpha_{\text{eq},\text{NO}_2/\text{NO}_3}$  values of 0.939 (BEGUN  
498 and FLETCHER, 1960) or 0.922 (SPINDEL, 1954), which compare favorably to the results  
499 presented in Table 3.

500 The equilibrium fractionation factor for this isotope exchange reaction can be represented by  
501 the ratio of kinetic fractionation factors for forward and reverse reactions (O'LEARY, 1981). In  
502 this case, the forward and reverse reactions are nitrite oxidation and nitrate reduction,  
503 respectively. The kinetic fractionation factor for nitrite oxidation ( $^{15}\alpha_{k,\text{NXR}}$ ) can therefore be  
504 estimated from the computed equilibrium fractionation factor ( $^{15}\alpha_{\text{eq},\text{NO}_2/\text{NO}_3}$ ) and the kinetic  
505 fractionation factor for nitrate reduction ( $^{15}\alpha_{k,\text{NAR}}$ ):

506

$$507 \quad ^{15}\alpha_{\text{eq},\text{NO}_2/\text{NO}_3} = \left[ \frac{^{15}\text{NO}_2^- / ^{14}\text{NO}_2^-}{^{15}\text{NO}_3^- / ^{14}\text{NO}_3^-} \right]_{\text{eq}} = \frac{^{15}\alpha_{k,\text{NXR}}}{^{15}\alpha_{k,\text{NAR}}} \quad (5)$$

508

509 Each estimate for  $^{15}\alpha_{\text{eq},\text{NO}_2/\text{NO}_3}$  was less than unity (Table 3), suggesting that  $^{15}\alpha_{k,\text{NXR}}$  is lower  
510 than  $^{15}\alpha_{k,\text{NAR}}$  and that  $^{15}\text{N}$  accumulates preferentially in  $\text{NO}_3^-$  at equilibrium. Assuming that the  
511 fractionation factor for nitrate reduction by the enzyme nitrite oxidoreductase (operating in

512 reverse) is between 1.020 and 1.030, as has been observed for nitrate reductases (Table 1;  
513 (BARFORD et al., 1999; DELWICHE and STEYN, 1970; GRANGER et al., 2006), the value of  
514  $^{15}\alpha_{k,NXR}$  estimated from equation 5 would vary between 0.964 and 0.973. Because of  
515 simplifications made in calculating the equilibrium isotope effects, they should be seen as  
516 approximations. However, rather than indicating a role for the reverse reaction in driving an  
517 artificial inverse kinetic isotope effect, these calculations suggest that the isotope effect for nitrite  
518 oxidation may actually be inverse. In order for the calculated  $^{15}\alpha_{eq,NO2/NO3}$  values to be consistent  
519 with a normal  $^{15}\alpha_{k,NXR}$ ,  $^{15}\alpha_{k,NAR}$  would have to be greater than 1.058, which is unlikely though  
520 not impossible (TCHERKEZ and FARQUHAR, 2006).

521 It is interesting that the observed  $^{15}\alpha_{k,NXR}$  value of 0.9872 is actually closer to 1 ('more  
522 normal') than expected from the results of these calculations. This may reflect partial rate  
523 determination by steps prior to nitrite oxidation to nitrate, such as transport into and out of the  
524 cell, diffusion to the enzyme active site, or fractionation associated with enzyme/substrate  
525 association and dissociation. The expression of enzyme-level isotope effects in the external  
526 medium does indeed require exchange of substrates and products between the interior and  
527 exterior of the cell (FARQUHAR et al., 1982; GRANGER et al., 2008; NEEDOBA et al., 2004;  
528 O'LEARY, 1981). If diffusion or uptake of nitrite is partially rate-limiting, these processes could  
529 invalidate the use of a simple unidirectional Rayleigh model to fit the *N. mobilis* data. However,  
530 in this case, the observed kinetic isotope effect should provide a minimum estimate of the inverse  
531 kinetic isotope effect because the processes of diffusion and transport would be expected to  
532 occur with normal isotope effects. There are a series of assumptions that should be tested by  
533 experiment, such as the isotope effect for nitrate reduction by nitrite oxidoreductase and the  
534 equilibrium fractionation between nitrite and nitrate. However, current calculations suggest that



535 in addition to an inverse kinetic fractionation in the oxidation reaction, any reversibility would  
536 tend to further lower the observed kinetic isotope effect.

537 The data from experiments 1-3 were also compared with a simple reaction reversibility  
538 model (*Electronic Annex 2*) to determine whether enzyme reversibility was detectable in the  
539 culture conditions employed here. Figure 3 shows the simulated  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  values for  
540 four scenarios with different rates of nitrate reduction, relative to nitrite oxidation. This ratio of  
541 forward (nitrite oxidation) and reverse (nitrate reduction) reactions is represented by the  
542 parameter  $x = {}^{14}k_{\text{NXR}}/{}^{14}k_{\text{NAR}}$ , which is set to values of 0, 0.2, 0.5, and 1.0 in the different  
543 simulations. This formulation assumes both nitrite oxidation and nitrate reduction are first order  
544 with respect to their substrates.

545 As the amount of reaction reversibility ( $x$ ) increases from 0 to 1, significant differences are  
546 seen in the relationship of  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  to the apparent extent of reaction (as determined  
547 from  $[\text{NO}_2^-]$ ) (Figure 3). With any significant amount of nitrate reduction, the forward reaction  
548 fails to go to completion and there is always some  $\text{NO}_2^-$  that remains in the simulation (Figure  
549 EA2.1). This is also indicated by  $f$  values failing to reach 0 (Figure 3). Nitrate reduction also  
550 leads to enhanced separation of  $^{15}\text{N}$  between  $\text{NO}_2^-$  and  $\text{NO}_3^-$  (Figure 3), and at equilibrium, the  
551 remaining  $\text{NO}_2^-$  would be expected to have a  $\delta^{15}\text{N}$  value that is approximately 50‰ lower than  
552  $\delta^{15}\text{N}_{\text{NO}_3}$ . Furthermore, at values of  $x$  greater than 0.2, the  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  values no longer  
553 appear to follow a closed-system “Rayleigh” model. Although  $\delta^{15}\text{N}_{\text{NO}_2}$  decreases in all  
554 simulations, the behavior of  $\delta^{15}\text{N}_{\text{NO}_3}$  changes dramatically. Rather than decreasing towards the  
555 initial  $\delta^{15}\text{N}_{\text{NO}_2}$  value,  $\delta^{15}\text{N}_{\text{NO}_3}$  gradually increases to its equilibrium  $\delta^{15}\text{N}$  value. This value is  
556 based on the concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  that remain in the system at equilibrium and the  
557 assumed value of  ${}^{15}\alpha_{\text{eq,NO}_2/\text{NO}_3}$ .

558 Comparison of these results to the *N. mobilis* data from experiments 1-3 suggests that little  
559 reversibility occurred (Figure 3). The  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  observations fall between the  $x = 0$   
560 and  $x = 0.2$ , consistent with little or no reversibility in the experiments described here.  
561 Furthermore, nitrite concentrations decreased to less than 1% of their initial value in all  
562 experiments, suggesting that there was at most 1% reaction reversibility ( $x = 0.01$ ). At this time,  
563 both the current observations and calculations are consistent with little reversibility of nitrite  
564 oxidoreductase under near-neutral pH conditions (HOLLOCHER, 1984; STRAAT and NASON, 1965;  
565 TANAKA et al., 1983; YAMANAKA and FUKUMORI, 1988).

566

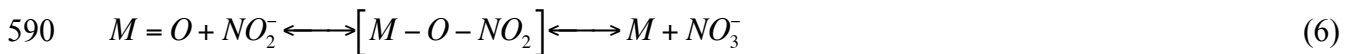
#### 567 *4.1.3. Enzyme-Level Inverse Isotope Effect*

568 Evaluations of both the pre-equilibrium and the enzyme reversibility scenarios imply that the  
569 kinetic fractionation factor for nitrite oxidation is likely to be inverse at the enzyme level.  
570 Enzyme-level inverse kinetic isotope effects have rarely, if ever, been demonstrated  
571 unambiguously in biochemical systems, making the results of the current study both surprising  
572 and novel. Several studies have reported apparent inverse kinetic isotope effects but these were  
573 shown to result from either pH-dependent pre-equilibrium (FRY et al., 1984), reaction branching  
574 (FRY et al., 1985; MCCREADY et al., 1975), or reaction reversibility (WEISS et al., 1988). In one  
575 case, the mechanism resulting in the observed inverse kinetic isotope effect was attributed to  
576 association and dissociation kinetics of the enzyme and substrate (GAWLITA et al., 1995), while  
577 in another case the mechanism was never identified (MCCREADY et al., 1975). However, the  
578 current results suggest that an inverse isotope effect is plausible, and even predictable, for the  
579 simple N-O bond forming reaction involved in nitrite oxidation. This hypothesis is examined

580 further using transition state theory applied to the current understanding of the mechanism for  
581 nitrite oxidation.

582 The reaction mechanism for nitrite oxidation has been studied using oxygen isotope transfer  
583 between H<sub>2</sub>O, NO<sub>2</sub><sup>-</sup>, and NO<sub>3</sub><sup>-</sup> species, and the currently accepted reaction mechanism involves a  
584 relatively simple oxygen (O) atom addition to NO<sub>2</sub><sup>-</sup> (Equation 6). Previous studies have  
585 suggested that the reaction proceeds by initial oxygenation of the enzyme's metal cofactor (M)  
586 from either NO<sub>3</sub><sup>-</sup> or H<sub>2</sub>O to form an oxo complex in the +2 oxidation state (M=O). This step is  
587 followed by NO<sub>2</sub><sup>-</sup> binding to the enzyme-bound O, reduction of the metal cofactor to the +0 state,  
588 and dissociation of the produced NO<sub>3</sub><sup>-</sup> (DISPIRITO and HOOPER, 1986; FRIEDMAN et al., 1986).

589



591

592 Using this reaction mechanism as a model, one can predict the isotope effect for nitrite  
593 oxidation using transition state theory (TST), which has often been used to describe the kinetics  
594 and isotopic fractionation of chemical and biochemical reactions (CLELAND, 1987; NORTHROP,  
595 1981; O'LEARY, 1977; THORNTON and THORNTON, 1978). Key assumptions in TST are that the  
596 transition state activated complex is in thermal equilibrium with the reactants and that the rate of  
597 reaction depends on the transmission coefficient and the concentration of molecules in the  
598 activated complex (BIGEISEN, 1949; BIGEISEN, 1952; BUDDENBAUM and SHINER, 1977).

599 With these assumptions, the kinetic fractionation factor can be estimated with knowledge of the  
600 vibrational frequencies for the substrate and transition state, using equation 7 (BIGEISEN, 1949;  
601 BIGEISEN and WOLFSBERG, 1958; HUSKEY, 1991):.

602

$$\alpha_k = k_1/k_2 = (v'_{1L}/v'_{2L}) \left[ 1 + \sum_i^{3n-6} G(u_i)\Delta u_i - \sum_i^{3n'-6} G(u'_i)\Delta u'_i \right] \quad (7)$$

604

605 In equation 7,  $u_i$  is defined as in equation 2,  $G(u_i) = [1/2 - (1/u_i) + 1/(e^{u_i} - 1)]$ , and primed

606 variables refer to properties of the transition state ([M-O-NO<sub>2</sub>] in equation 6). The term  $v'_{1L}/v'_{2L}$

607 is the ratio of imaginary frequencies in the transition state along the reaction coordinate for light

608 and heavy molecules, respectively; this term is always greater than 1 (FRY, 1970; SHEPPARD et

609 al., 1954) and can be determined from the reduced masses of the isotopic atoms involved in the

610 bond being formed or broken (SHEPPARD et al., 1954). The term in square brackets, which is

611 based on the zero point energy difference between the substrate and transition state for the light

612 and heavy molecules (BIGELEISEN, 1952), can be greater than or less than 1, depending on the

613 change in the bonding environment at the isotopically substituted atom between substrate and

614 activated complex (SHEPPARD et al., 1954). If N is more strongly bonded in the substrates than in

615 the transition state (a typical bond-breaking reaction), the sum of  $G(u_i)\Delta u_i$  for the substrates will

616 be greater than the sum of  $G(u'_i)\Delta u'_i$  in the transition state and the term in square brackets (eq. 7)

617 will be greater than 1, leading to a normal kinetic isotope effect. This is the case for most

618 nitrogen-oxide reduction reactions (nitrate reduction, nitrite reduction, nitrous oxide reduction),

619 which involve N-O bond breaking (BARFORD et al., 1999; BRYAN et al., 1983; GRANGER et al.,

620 2006). If, however, N is more strongly bonded in the transition state than in the substrates (a

621 bond forming reaction), the sum of  $G(u_i)\Delta u_i$  in the reactants will be less than the sum of

622  $G(u'_i)\Delta u'_i$  in the transition state, and the term in the square brackets will be less than one. This

623 can lead to an inverse kinetic isotope effect if the product of this term and the imaginary

624 frequency term ( $v'_{1L}/v'_{2L}$ ) leads to a ratio of rate constants ( $k_1/k_2$ ) that is less than one. If,

625 however, the product of the imaginary frequency term and the zero point energy term is greater  
626 than 1, a normal kinetic isotope effect would be expected, even for a bond-forming reaction.

627 Here, the kinetic isotope effect for nitrite oxidation was estimated from equation 7 assuming  
628 that the transition state contains the newly formed N-O bond (FRIEDMAN et al., 1986) and that  
629 the geometry and bonding environment of the N atom in this complex are similar to nitrate  
630 (FRIEDMAN et al., 1986; SHEPPARD et al., 1954). Assuming N-O bonding in the transition state is  
631 similar to that of the product (nitrate) may overestimate the bonding difference between the  
632 substrate and the transition state. This will be considered in the discussion below. The value of  
633 the imaginary frequency term was estimated to be 1.0182, from the square root of the ratio of  
634 reduced masses of the bond forming atoms,  $^{15}\text{N}-^{16}\text{O}$  and  $^{14}\text{N}-^{16}\text{O}$  (SHEPPARD et al., 1954). The  
635 zero point energy terms were estimated from the molecular vibration frequencies of both  
636 isotopically substituted forms of  $\text{NO}_2^-$  (assumed substrate) and  $\text{NO}_3^-$  (assumed geometry for N in  
637 the activated complex). Using both experimental and *ab initio* frequencies for  $\text{NO}_2^-$  and  $\text{NO}_3^-$   
638 (Table 2), the ZPE term was estimated to range from 0.933 to 0.941, depending on whether  
639 observed or calculated frequencies in aqueous or gas phase are used. Multiplying by the  
640 imaginary frequency term, the inherent kinetic isotope effect for nitrite oxidation to nitrate would  
641 therefore be predicted from TST (eqn. 7) to be on the order of 0.950-0.958. This  $^{15}\alpha_{\text{k,NXR}}$  value  
642 estimated from TST is lower (more inverse) than was observed experimentally (Figure 2). It is  
643 also similar, but a bit lower than the  $^{15}\alpha_{\text{k,NXR}}$  value predicted from equilibrium calculations  
644 (0.964-0.973; section 4.1.2.). However both of these estimates are consistent with the expectation  
645 of an inverse kinetic isotope effect for nitrite oxidation.

646 There are many reasons for the exact values of measured and calculated kinetic isotope  
647 effects to differ. Uncertainties involved in estimating  $^{15}\alpha_{\text{k,NXR}}$  from the equilibrium exchange

648 reaction for  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were discussed above (section 4.1.2). Application of transition state  
649 theory also involves inherent assumptions about the enzyme-bound transition state structure and  
650 vibrational frequencies, as well as the potential energy surfaces for isotopically substituted  
651 molecules (NORTHROP, 1981; THORNTON and THORNTON, 1978). Assuming that the transition  
652 state bonding and vibration frequencies are similar to  $\text{NO}_3^-$  is likely to exaggerate the difference  
653 in molecular properties between  $\text{NO}_2^-$  and the transition state. If the transition state properties are  
654 more intermediate between  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , the fractionation factor may indeed be closer to 1  
655 (less inverse). Because of this uncertainty, it may not be surprising that the observed  
656 fractionation factor is less inverse than the theoretical value calculated from TST.

657 Even if the estimates of  $^{15}\alpha_{k,\text{NXR}}$  based on TST provide an accurate estimate for the inherent  
658 kinetic isotope effect for nitrite oxidation, it may differ from the isotope effect observed  
659 experimentally because of the influence of cellular processes and enzymatic steps prior to the  
660 oxidation of nitrite (NORTHROP, 1981; O'LEARY, 1981; TCHERKEZ and FARQUHAR, 2006).  
661 Cellular processes that could cause some deviation of the observed isotope effect relative to the  
662 predicted (inherent) value cannot currently be ruled out (transport, diffusion, or leakage of  $\text{NO}_2^-$   
663 from the cell). Indeed, for the enzyme-level kinetic isotope effect to be expressed, some leakage  
664 of  $\text{NO}_2^-$  from the cell must occur. Given that diffusion and transport processes are expected to  
665 have normal kinetic isotope effects, these processes could cause some “renormalization” of the  
666 observed kinetic isotope effect relative to the theoretical values.

667 Two of the common causes for apparent inverse isotope fractionation have now been  
668 explored: pre-equilibrium (Section 4.1.1.) and enzyme reversibility (Section 4.1.2.), neither of  
669 which appear to be the underlying mechanism for inverse kinetic isotope fractionation during  
670 nitrite oxidation. Indeed, evaluation of the equilibrium isotope effect for  $^{15}\text{N}$  exchange between

671  $\text{NO}_2^-$  and  $\text{NO}_3^-$  suggested that the fractionation factor for nitrite oxidation is likely to be inverse.  
672 Evaluation of the fractionation factor using transition state theory provided an independent  
673 confirmation of the inverse enzyme-level inverse isotope effect for nitrite oxidoreductase. While  
674 there are uncertainties in each of these calculations, the fact that a new N-O bond is being formed  
675 without concurrent bond breakage in  $\text{NO}_2^-$  is a unique aspect of the nitrite oxidation reaction.  
676 Overall, the reaction results in increased bonding to the central N atom, and the observed inverse  
677 isotope effect suggests that the enzyme-bound transition state contains the new N-O bond.

678

#### 679 **4.2. Implications for Nitrogen Isotope Biogeochemistry**

680 An enzyme-level inverse kinetic isotope effect is unusual in biochemistry. The observation  
681 here of a large inverse kinetic isotope effect in the oxidation of nitrite by *N. mobilis* is supported  
682 qualitatively by theory, although further work is required to understand whether other cellular  
683 processes might mask an even higher inverse kinetic isotope effect. Understanding the cellular  
684 controls on the isotope effect for nitrite oxidation will allow us to better predict how the isotope  
685 effect for this process is expressed in the environment and how it should be parameterized in  
686 ocean biogeochemical models. At this point, it is important to recognize that this process occurs  
687 with a fractionation factor that is inverse (less than 1), which challenges the commonly held  
688 assumption that all biochemical processes fractionate against the heavier isotope.

689 An inverse isotope effect for nitrite oxidation may help explain the large  $\Delta\delta^{15}\text{N}$  ( $= \delta^{15}\text{N}_{\text{NO}_3} -$   
690  $\delta^{15}\text{N}_{\text{NO}_2}$ ) values observed in the eastern tropical north Pacific (ETNP), mentioned above. In a  
691 previous study, nitrite oxidation was considered as a mechanism for altering the  $\delta^{15}\text{N}_{\text{NO}_3}$  and  
692  $\delta^{18}\text{O}_{\text{NO}_3}$  profiles, particularly on the upper edge of the suboxic zone, where calculations  
693 suggested that nitrite and oxygen may be consumed concurrently (CASCIOTTI and MCILVIN,

694 2007). However, adequate explanations for the low  $\delta^{15}\text{N}_{\text{NO}_2}$  values and high  $\Delta\delta^{15}\text{N}$  values were  
 695 not found, holding to constraints of a 25‰ isotope effect for nitrate reduction ( $^{15}\epsilon_{\text{k,NAR}} =$   
 696  $(^{15}\alpha_{\text{k,NAR}}-1)*1000$ ) and either a ‘normal’ or non-fractionating nitrite consumption process. For  
 697 example, nitrite reduction with a normal isotope effect ( $^{15}\epsilon_{\text{k,NIR}}$ ) of +15‰ (BRYAN et al., 1983;  
 698 CASCIOTTI, 2002; CASCIOTTI et al., 2002) would tend to cause isotopic enrichment in the  $\text{NO}_2^-$   
 699 pool, counteracting the isotopic depletion of the nitrite pool generated by nitrate reduction. At  
 700 steady state, that is for nitrite concentrations and  $\delta^{15}\text{N}_{\text{NO}_2}$  values not changing (or changing  
 701 slowly relative to the production and consumption fluxes), the  $\Delta\delta^{15}\text{N}$  value would tend towards  
 702  $^{15}\epsilon_{\text{k,NAR}} - ^{15}\epsilon_{\text{k,NIR}}$ , or 25‰ – 15‰ = 10‰. The observed  $\Delta\delta^{15}\text{N}$  values of 28-35‰ are therefore  
 703 difficult to explain with nitrite reduction as the sole sink for nitrite. Non steady-state conditions  
 704 between nitrite production and consumption processes may contribute to  $\Delta\delta^{15}\text{N}$  values greater  
 705 than 25‰, but are unlikely to explain the low  $\delta^{15}\text{N}_{\text{NO}_2}$  values.

706 If nitrite oxidation by marine NOB routinely occurs with an inverse kinetic isotope effect, as  
 707 observed here for *N. mobilis*, then nitrite oxidation would tend to lower  $\delta^{15}\text{N}_{\text{NO}_2}$  values and  
 708 enhance the  $^{15}\text{N}$  depletion of  $\text{NO}_2^-$  achieved by nitrate reduction. A simple steady-state box  
 709 model including the fluxes of nitrite reduction ( $F_{\text{NIR}}$ ), nitrite oxidation ( $F_{\text{NXR}}$ ), and nitrate  
 710 reduction ( $F_{\text{NAR}} = F_{\text{NIR}} + F_{\text{NXR}}$ ), was used to estimate the dependence of  $\Delta\delta^{15}\text{N}$  on  $F_{\text{NXR}}/F_{\text{NAR}}$ .

711

$$712 \Delta\delta^{15}\text{N} = ^{15}\epsilon_{\text{k,NAR}} - ^{15}\epsilon_{\text{k,NXR}} * F_{\text{NXR}}/F_{\text{NAR}} - ^{15}\epsilon_{\text{k,NIR}} * F_{\text{NIR}}/F_{\text{NAR}} \quad (8)$$

713

714 Equation 8 predicts that  $\Delta\delta^{15}\text{N}$  values should increase as nitrite oxidation becomes a greater  
 715 fraction of the nitrite consumption flux. Figure 4 shows the steady state  $\delta^{15}\text{N}_{\text{NO}_2}$ ,  $\delta^{15}\text{N}_{\text{NO}_3}$ , and  
 716  $\Delta\delta^{15}\text{N}$  values obtained from this exercise, as well as the relationship between  $\Delta\delta^{15}\text{N}$  and



717  $F_{\text{NXR}}/F_{\text{NAR}}$  (inset). For these calculations,  $^{15}\epsilon_{\text{k,NAR}}$  was assumed to be +25‰,  $^{15}\epsilon_{\text{k,NXR}} = -13\text{‰}$ ,  
718 and  $^{15}\epsilon_{\text{k,NIR}} = +15\text{‰}$ . Data from the ETNP are overlaid on  $\Delta\delta^{15}\text{N}$  contours in Figure 4,  
719 illustrating the high  $\Delta\delta^{15}\text{N}$  values and low  $\delta^{15}\text{N}_{\text{NO}_2^-}$  values observed in the ETNP suboxic zone  
720 (CASCIOTTI and MCILVIN, 2007). The data from the ETNP oxygen minimum zone suggest that  
721 nitrite oxidation may be an important sink for  $\text{NO}_2^-$  in this region ( $\Delta\delta^{15}\text{N} > 28\text{‰}$ ,  $F_{\text{NXR}}/F_{\text{NAR}}$   
722  $> 0.7$ ) (Figure 4). This is consistent with previous interpretations based on  $\Delta(15,18)$  (CASCIOTTI  
723 and MCILVIN, 2007).

724 More generally, the opposing isotope effects for nitrite oxidation and reduction processes  
725 suggest that  $\Delta\delta^{15}\text{N}$  might be used to distinguish between oxidative and reductive fates for nitrite.  
726 Where reductive fates for nitrite dominate over oxidative fates ( $F_{\text{NIR}} < 0.5 * F_{\text{NAR}}$ ),  $\Delta\delta^{15}\text{N}$  would  
727 be expected to be less than the isotope effect for nitrate reduction to nitrite in a given setting  
728 (approximately 25‰ in suboxic zones, or 5‰ in the euphotic zone) (Equation 8). Conversely,  
729 where the consumption of nitrite through oxidation exceeds the flux of nitrite consumed by  
730 reductive processes ( $F_{\text{NXR}} > 0.5 * F_{\text{NAR}}$ ),  $\Delta\delta^{15}\text{N}$  would be expected to be greater than the isotope  
731 effect for nitrate reduction (Equation 8). These isotope effects are most likely to be observed in  
732 or near ocean suboxic zones with high concentrations of  $\text{NO}_2^-$ . However, the isotope effect for  
733 nitrite oxidation may also be expressed in the euphotic zone or in the primary nitrite maximum at  
734 the base of the euphotic zone, where competing fates for nitrite may lead to branching between  
735 nitrite assimilation into particulate nitrogen and nitrite oxidation to nitrate.

736

### 737 **4.3. Future Work**

738 It will be important to expand these observations beyond *N. mobilis* to examine the generality  
739 of these results among nitrite oxidizing microorganisms and to obtain comparable data on  $\delta^{18}\text{O}$

740 fractionation by nitrite-oxidizing bacteria. There are 4 known genera of nitrite oxidizing bacteria,  
741 which differ from each other in many ways: *Nitrococcus*, *Nitrobacter*, *Nitrospira*, and  
742 *Nitrospina*, and it will be important to determine whether the inverse isotope effect is universal  
743 among different species and growth conditions.

744 Examination of the  $\delta^{18}\text{O}$  fractionation factor during nitrite oxidation holds promise for  
745 independently verifying the conclusions from the current study based on  $\delta^{15}\text{N}$ . For example,  
746 these additional measurements have the power to independently verify the reversibility of the  
747 reaction, the structure of the transition state, and the reaction mechanism. At the same time,  
748 interpreting the  $\delta^{18}\text{O}$  data is more complex because of the analytical challenges posed by abiotic  
749 equilibration of oxygen atoms between nitrite and water (CASCIOTTI et al., 2007), as well as the  
750 potential for biochemical exchange of nitrite with water catalyzed by the bacteria themselves  
751 (DISPIRITO and HOOPER, 1986; FRIEDMAN et al., 1986; HOLLOCHER, 1984). Further work is  
752 required to fully resolve the effects of oxygen isotope equilibration from the interpretation of the  
753 kinetic isotope effect for oxygen isotopes, since in some situations equilibration will act in the  
754 same direction to lower  $\delta^{18}\text{O}_{\text{NO}_2}$ , and in other cases it will have counteracting effects on  $\delta^{18}\text{O}_{\text{NO}_2}$ .

755 It is also important to examine the kinetic isotope effects with isolated nitrite oxidoreductase  
756 enzyme to resolve the contribution of complicating factors, such as  $\text{NO}_2^-$  transport and diffusion,  
757 to the observed enzyme-level kinetic isotope effect. Nitrite oxidoreductase remains active in cell  
758 extracts (TANAKA et al., 1983; YAMANAKA and FUKUMORI, 1988) and can be partially purified  
759 for use in these experiments.

760

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767

768

## 768 TABLES

769 TABLE 1: Nitrogen isotope effects for microbial processes in pure culture

Process	Reaction	$^{15}\alpha_k$ = $^{14}k/^{15}k$	$^{15}\epsilon_k$ (‰) = $(^{15}\alpha_k - 1) \times 10^3$	References
Nitrate reduction (Denitrification)	$\text{NO}_3^- \rightarrow \text{NO}_2^-$	1.013 to 1.030	+13‰ to +30‰	BARFORD et al., 1999; DELWICHE and STEYN, 1970; GRANGER et al., 2006 BRYAN et al., 1983
Nitrite reduction (Denitrification)	$\text{NO}_2^- \rightarrow \text{NO}$	1.005 to 1.025	+5‰ to +25‰	
Nitrous oxide reduction (Denitrification)	$\text{N}_2\text{O} \rightarrow \text{N}_2$	1.004 to 1.013	+4‰ to +13‰	BARFORD et al., 1999; OSTROM et al., 2007
Nitrate reduction (Nitrate assimilation)	$\text{NO}_3^- \rightarrow \text{NO}_2^-$	1.005 to 1.010	+5‰ to +10‰	GRANGER et al., 2004; NEEDOBA and HARRISON, 2004; WASER et al., 1998a DELWICHE and STEYN, 1970; HOERING and FORD, 1960; MEADOR et al., 2007
Nitrogen fixation	$\text{N}_2 \rightarrow \text{N}_{\text{org}}$	0.998 to 1.002	-2‰ to +2‰	
Ammonium assimilation	$\text{NH}_4^+ \rightarrow \text{N}_{\text{org}}$	1.014-1.027	+14‰ to +27‰	HOCH et al., 1992; WASER et al., 1998a
Ammonia oxidation* (Nitrification)	$\text{NH}_4^+ \rightarrow \text{NO}_2^-$	1.014 to 1.038	+14‰ to +38‰	CASCIOTTI et al., 2003; MARIOTTI et al., 1981; YOSHIDA, 1988; DELWICHE AND STEYN, 1970
Nitrite oxidation (Nitrification)	$\text{NO}_2^- \rightarrow \text{NO}_3^-$	0.9872	-12.8‰	

770 \*  $^{15}\alpha_k$  for ammonia oxidation is reported relative to  $\text{NH}_4^+$ , which is not the substrate for ammonia oxidation. Instead,771 the observed  $^{15}\alpha_k$  relative to  $\text{NH}_4^+$  should be corrected for  $\text{NH}_4^+/\text{NH}_3$  equilibrium fractionation, dividing the772 observed  $^{15}\alpha_k$  by a correction factor of 1.0183 at pH 8 (WEISS et al., 1988), which yields isotope effects of 0.996 to773 1.0194 for ammonia oxidation relative to  $\text{NH}_3$ .

774

774 **TABLE 2:** Molecular vibration frequencies used to calculate isotope exchange equilibria.

	<sup>1</sup> Aqueous phase <i>ab initio</i> calculations $\omega_i$ (cm <sup>-1</sup> )		<sup>2</sup> Gas phase <i>ab initio</i> calculations $\omega_i$ (cm <sup>-1</sup> )		<sup>3</sup> Aqueous and <sup>4</sup> gas phase observations $\omega_i$ (cm <sup>-1</sup> )	
	<sup>14</sup> N	<sup>15</sup> N	<sup>14</sup> N	<sup>15</sup> N	<sup>14</sup> N	<sup>15</sup> N
NO <sub>2</sub> <sup>-</sup>	795.770	790.733	792.684	787.911	808	805
	1316.199	1288.788	1338.523	1310.613	1232	1208
	1386.788	1362.958	1377.186	1353.122	1326	1305
	This study		This study		<sup>3</sup> BEGUN and FLETCHER, 1960	
NO <sub>3</sub> <sup>-</sup>	708.514	706.678	706.396	704.737	716.8	714.8
	708.800	706.953	706.518	704.865	716.8	714.8
	829.003	807.405	837.870	816.030	830.9	809.0
	1089.308	1089.308	1077.496	1077.496	1049.2	1049.2
	1438.454	1404.619	1471.120	1436.134	1375.6	1343.7
	1438.681	1404.820	1471.151	1436.177	1375.6	1343.7
This study		This study		<sup>3</sup> BEGUN and FLETCHER, 1960		
HNO <sub>2</sub> (trans)	596.345	595.140	600.998	599.845	543.880	542.90
	671.375	668.799	630.216	627.954	595.620	593.20
	906.346	886.079	863.359	844.223	790.118	773.62
	1308.670	1308.112	1325.358	1324.390	1263.183	1262.45
	1731.762	1700.779	1794.972	1762.652	1699.800	1669.60
	3210.523	3210.478	3698.573	3698.576	3590.711	N/D
This study		This study		<sup>4</sup> DEELEY and MILLS, 1985		

775

776 <sup>1</sup>Calculated in the WebMO interface using Gaussian, B3LYP, 'routine', in aqueous solution.

777 <sup>2</sup>Calculated in the WebMO interface using Gaussian, B3LYP, 'routine', in gas phase.

778 <sup>3</sup>Observed in aqueous solution (BEGUN and FLETCHER, 1960).

779 <sup>4</sup>Observed in gas phase as trans-HONO (DEELEY and MILLS, 1985).

780

780 **TABLE 3:** Calculated isotope exchange equilibria

	Medium	$^{15}\alpha_{\text{eq}}^1$
HNO <sub>2</sub> /NO <sub>2</sub> <sup>-</sup>	aqueous	0.9975
	gas phase	0.9972
NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup>	aqueous	0.9454
	gas phase	0.9418

781

782 <sup>1</sup>Calculated from the ratios of Q<sub>2</sub>/Q<sub>1</sub> using equations 2 and 3 at T = 298 K and *ab initio* frequencies from Table 2.

783

784

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FIGURE 1: Time course results from *N. mobilis* experiments 1, 2, and 3.

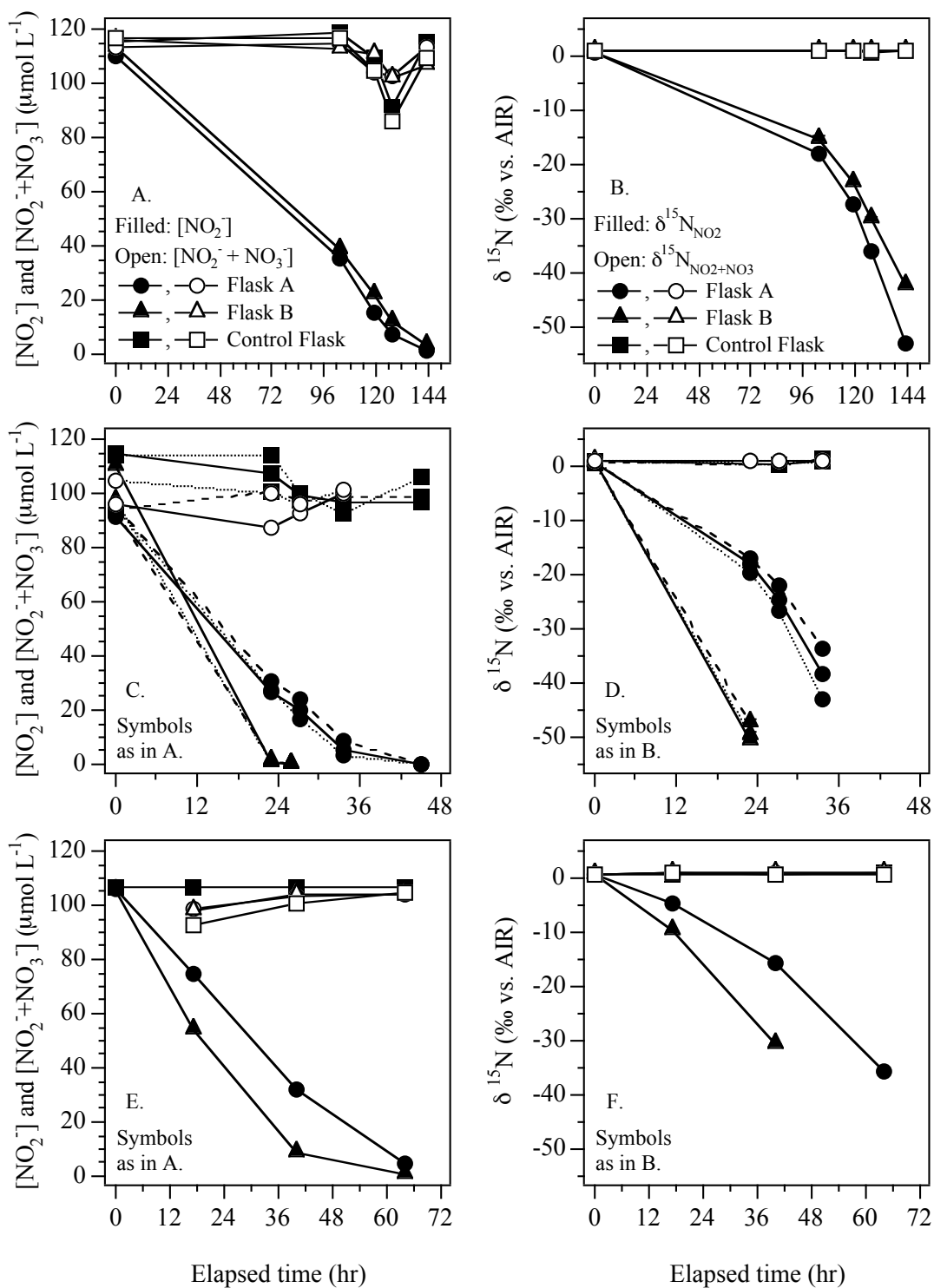


Figure 1.  $[\text{NO}_2^-]$  (filled symbols) and  $[\text{NO}_2^- + \text{NO}_3^-]$  (open symbols) are shown for time course experiments 1 (panel A), 2 (panel C), and 3 (Panel E). Please note the use of different x-axes for experiments 1, 2, and 3.  $\delta^{15}\text{N}_{\text{NO}_2}$  (filled symbols) and  $\delta^{15}\text{N}_{\text{NO}_2+\text{NO}_3}$  (open symbols) are shown for the same experiments in panels B, D, and F, respectively. In every panel circles represent data from flask A, triangles represent data from flask B, and squares denote data from the control flask. In experiment 2 (panels C and D), there were triplicate A, B, and control flasks, denoted by unique line dashing (solid, dotted, dashed) for replicate flasks. Error bars for  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_2+\text{NO}_3}$  are smaller than the symbols.

FIGURE 2: Rayleigh plot of nitrite  $\delta^{15}\text{N}$  from *N. mobilis* experiments 1, 2, and 3.

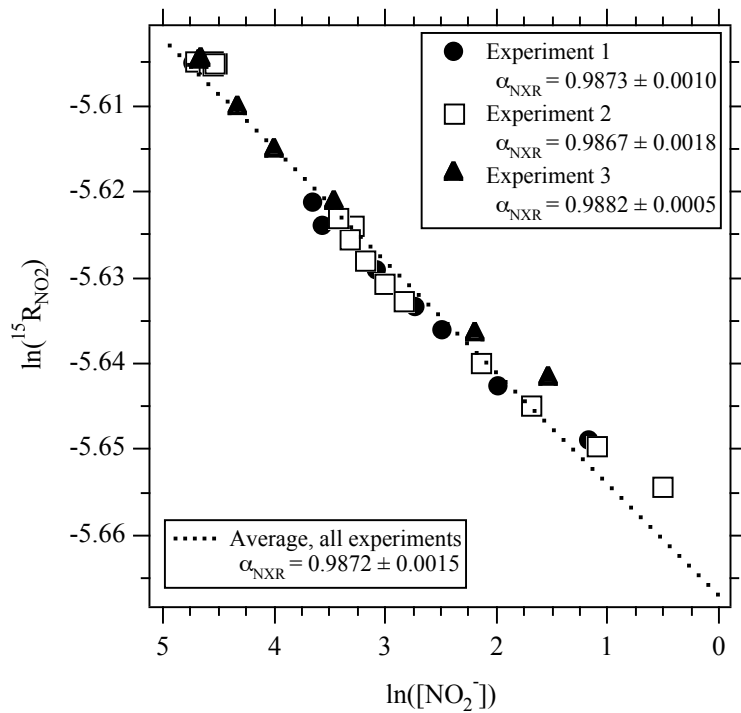


Figure 2. Rayleigh plot for nitrite oxidation, using  $\ln(^{15}\text{R}_{\text{NO}_2})$  vs.  $\ln([\text{NO}_2^-])$ , for experiments 1 (filled circles), 2 (open squares), and 3 (filled triangles). The fractionation factor for nitrite oxidation ( $^{15}\alpha_{\text{k,NXR}}$ ) estimated using dummy variables and averaging over all three experiments was  $0.9872 \pm 0.0015$ . For reference, the fractionation line  $^{15}\alpha_{\text{k,NXR}} = 0.9872$  is shown (dotted line). Error bars are smaller than the data points.

**FIGURE 3:** Modeled effect of enzyme reversibility on expression of KIE

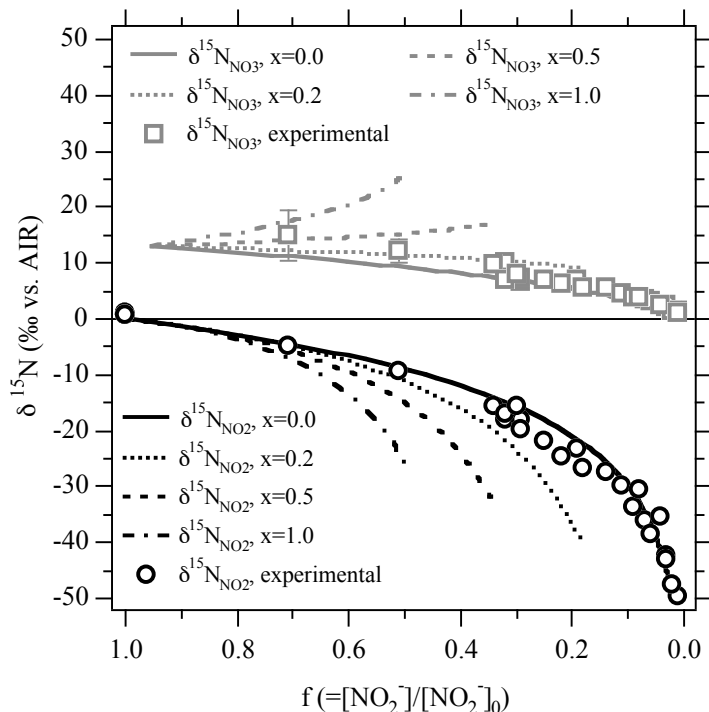


Figure 3. Model simulations for enzyme-level reversibility of nitrite oxidoreductase. Simulated  $\delta^{15}\text{N}_{\text{NO}_2}$  (black lines) and  $\delta^{15}\text{N}_{\text{NO}_3}$  (grey lines) are plotted versus  $f(=[\text{NO}_2^-]/[\text{NO}_2^-]_0)$  for varying amounts of back-reaction ( $x = {}^{14}k_{\text{NAR}}/{}^{14}k_{\text{NXR}}$ ; see *Electronic Supplement 2* for model details). Line dashing denotes simulations with a specified amount of back-reaction:  $x = 0.0$  (solid lines),  $x = 0.2$  (dotted lines),  $x = 0.5$  (dashed lines), and  $x = 1.0$  (dot-dashed lines). Overlain in each panel are experimental observations for  $\delta^{15}\text{N}_{\text{NO}_2}$  (open black circles) and  $\delta^{15}\text{N}_{\text{NO}_3}$  (open grey squares) from experiments 1, 2, and 3 (see text). Error bars for  $\delta^{15}\text{N}_{\text{NO}_2}$  measurements are smaller than the symbols. Experimental data are consistent with very low ( $0.0 \leq x \leq 0.2$ ) amounts of back-reaction.



**FIGURE 4:** Expression of Inverse KIE in  $\Delta\delta^{15}\text{N}$

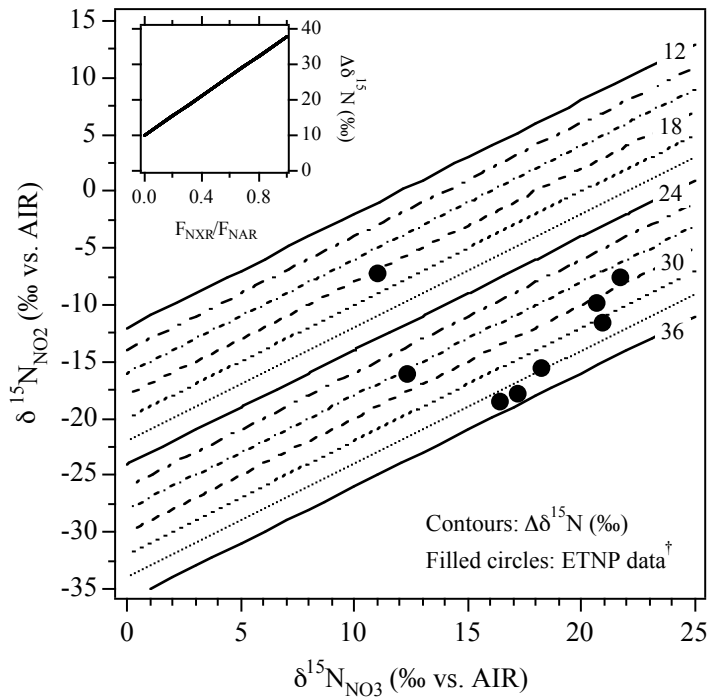


Figure 4. Contour plot of simulated suboxic zone  $\delta^{15}\text{N}_{\text{NO}_2}$  (% vs. AIR),  $\delta^{15}\text{N}_{\text{NO}_3}$  (% vs. AIR), and  $\Delta\delta^{15}\text{N}$  ( $= \delta^{15}\text{N}_{\text{NO}_3} - \delta^{15}\text{N}_{\text{NO}_2}$ ) for varying  $F_{\text{NXR}}/F_{\text{NAR}}$ . Assumptions for the simulation include:  $^{15}\epsilon_{\text{k,NAR}} = +25\%$ ,  $^{15}\epsilon_{\text{k,NXR}} = -12.8\%$ , and  $^{15}\epsilon_{\text{k,NIR}} = +15\%$ , with nitrite concentration and  $\delta^{15}\text{N}_{\text{NO}_2}$  held in steady state (see text/supplement for details). Contouring denotes 2‰ increments of  $\Delta\delta^{15}\text{N}$  (‰) with a range of 12-36‰. Overlaid on the contour plot are observations from the Eastern Tropical North Pacific (ETNP) (black circles; <sup>†</sup>Casciotti and McIlvin, 2007). Inset plot shows the dependence of  $\Delta\delta^{15}\text{N}$  (‰) on  $F_{\text{NXR}}/F_{\text{NAR}}$  in the simulation. ETNP data show  $\Delta\delta^{15}\text{N}$  values of 18-36‰, corresponding to simulated  $F_{\text{NXR}}/F_{\text{NAR}}$  values of 0.3-0.9.

## CASCIOTTI (W5860) ELECTRONIC ANNEX 1

### EA.1. Simulations for Pre-equilibrium Scenarios

In order to illustrate the role of a pre-equilibrium between  $\text{HNO}_2$  and  $\text{NO}_2^-$  on the expressed kinetic isotope effect for nitrite oxidation, six different closed-system simulations were conducted with different assumptions for  $^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2}$ ,  $^{15}\alpha_{\text{k,NXR}}$ , and enzyme substrate (Table EA1.1).

**Table EA1.1. Adjustable parameters for pre-equilibrium simulations.**

Simulation	Substrate	$^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2}$	$^{15}\alpha_{\text{k,NXR}}$
S1	$\text{HNO}_2$	1.0280	1.0150
S2	$\text{NO}_2^-$	1.0280	1.0150
S3	$\text{HNO}_2$	0.9972	1.0150
S4	$\text{NO}_2^-$	0.9972	1.0150
S5	$\text{HNO}_2$	0.9972	0.9872
S6	$\text{NO}_2^-$	0.9972	0.9872

In each simulation, the reaction was taken to be first order in substrate concentration. It was also assumed that isotope exchange equilibration between  $\text{HNO}_2$  and  $\text{NO}_2^-$  was rapid relative to the rate of oxidation, and that the oxidation reaction itself was irreversible. For simulations 1, 3, and 5 where  $\text{HNO}_2$  was taken to be the substrate for oxidation, the following finite-difference equations were used:

$$[^{14}\text{N}_T] = [^{14}\text{NO}_3^-] + [^{14}\text{NO}_2^-] + [\text{H}^{14}\text{NO}_2] \quad (\text{EA1.1})$$

$$[^{14}\text{NO}_3^-]_t = [^{14}\text{NO}_3^-]_{t-1} + {}^{14}k_{\text{NXR}} \cdot [\text{H}^{14}\text{NO}_2]_{t-1} \cdot dt \quad (\text{EA1.2})$$

$$[^{14}\text{NO}_2^-]_t = ([^{14}\text{N}_T]_t - [^{14}\text{NO}_3^-]_t) / (1 + 10^{\text{pKa-pH}}) \quad (\text{EA1.3})$$

$$[\text{H}^{14}\text{NO}_2]_t = ([^{14}\text{N}_T]_t - [^{14}\text{NO}_3^-]_t) \cdot (10^{\text{pKa-pH}}) / (1 + 10^{\text{pKa-pH}}) \quad (\text{EA1.4})$$

$$[^{15}\text{N}_T] = [^{15}\text{NO}_3^-] + [^{15}\text{NO}_2^-] + [\text{H}^{15}\text{NO}_2] = \text{constant} \quad (\text{EA1.5})$$

$$[^{15}\text{NO}_3^-]_t = [^{15}\text{NO}_3^-]_{t-1} + ({}^{14}k_{\text{NXR}} / {}^{15}a_{\text{k,NXR}}) \cdot [\text{H}^{15}\text{NO}_2]_{t-1} \cdot dt \quad (\text{EA1.6})$$

$$[^{15}\text{NO}_2^-]_t = ([^{15}\text{N}_T]_t - [^{15}\text{NO}_3^-]_t) / (1 + {}^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2} \cdot [\text{H}^{14}\text{NO}_2]_t / [^{14}\text{NO}_2^-]_t) \quad (\text{EA1.7})$$

$$[\text{H}^{15}\text{NO}_2]_t = {}^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2} \cdot [\text{H}^{14}\text{NO}_2]_t \cdot [^{15}\text{NO}_2^-]_t / [^{14}\text{NO}_2^-]_t \quad (\text{EA1.8})$$

For simulations 2, 4, and 6 where  $\text{NO}_2^-$  was taken to be the substrate for oxidation, the following finite-difference equations were used:

$$[^{14}\text{NO}_3^-]_t = [^{14}\text{NO}_3^-]_{t-1} + {}^{14}k_{\text{NXR}} \cdot [^{14}\text{NO}_2^-]_{t-1} \cdot dt \quad (\text{EA1.9})$$

$$[^{15}\text{NO}_3^-]_t = [^{15}\text{NO}_3^-]_{t-1} + ({}^{14}k_{\text{NXR}} / {}^{15}a_{k,\text{NXR}}) \cdot [^{15}\text{NO}_2^-]_{t-1} \cdot dt \quad (\text{EA1.10})$$

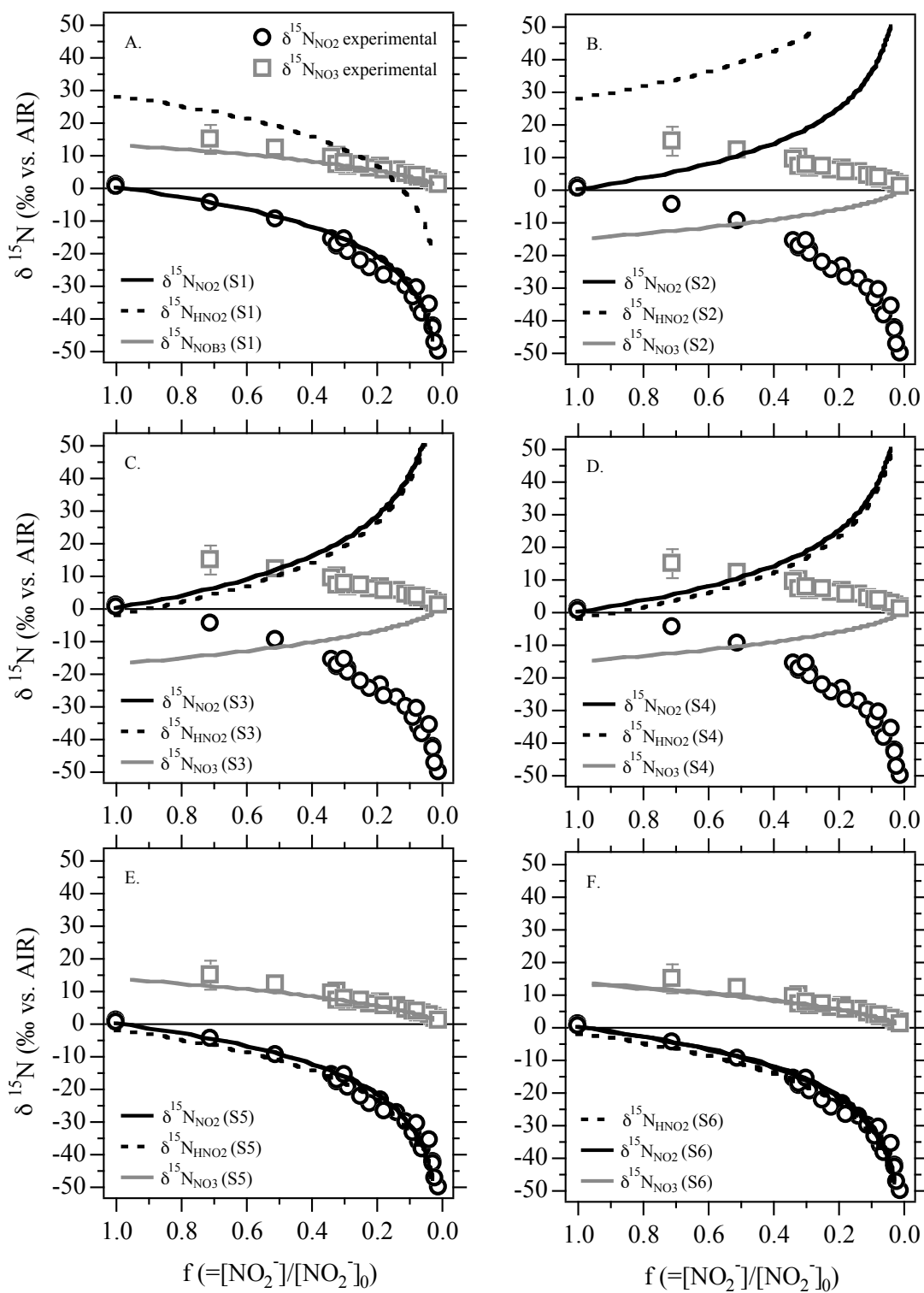
Equations for  $[^{14}\text{N}_T]$ ,  $[^{15}\text{N}_T]$ ,  $[^{14}\text{NO}_2^-]_t$ ,  $[^{15}\text{NO}_2^-]_t$ ,  $[\text{H}^{14}\text{NO}_2]_t$ , and  $[\text{H}^{15}\text{NO}_2]_t$  were the same as in simulations 1, 3, and 5 (above).

The simulated  $\delta^{15}\text{N}$  of  $\text{NO}_2^-$ ,  $\text{HNO}_2$ , and  $\text{NO}_3^-$  are plotted versus simulated  $f(=[\text{NO}_2^-] / [\text{NO}_2^-]_0)$  in Figure EA1.1. The  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  data collected from experiments 1-3 are overlaid on the simulated  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  in each panel. Simulations 1, 2, 3, and 4 were all conducted with a normal  ${}^{15}\alpha_{k,\text{NXR}}$  acting on either  $\text{HNO}_2$  (S1, Figure EA1.1A; S3, Figure EA1.1C) or  $\text{NO}_2^-$  (S2, Figure EA1.1B; S4, Figure EA1.1D) as the substrate. Of these first four simulations, only S1 fit the observed data. These results illustrate that in order for the observed  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  data to be explained using a normal kinetic isotope effect,  $\text{HNO}_2$  (the minor pool) must be the substrate for the reaction, it must be enriched in  $^{15}\text{N}$  relative to the bulk pool, and the equilibrium isotope effect must be about 13‰ higher than the kinetic isotope effect (in this case  ${}^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2} = 1.0280$  and  ${}^{15}\alpha_{k,\text{NXR}} = 1.0150$ ; Table EA1.1). For example, S1 appears to explain the observations with  $\text{HNO}_2$  as the substrate, whereas S2 does not fit the observations with identical fractionation factors, but with the enzyme acting on  $\text{NO}_2^-$  as the substrate rather than  $\text{HNO}_2$ . However, in order to fit the observations in S1 using a normal kinetic isotope effect,  ${}^{15}\alpha_{\text{eq,HNO}_2/\text{NO}_2}$  was set to 1.028. As discussed in the main text, however, our best estimate for the

equilibrium fractionation factor is estimated to be small (0.9975-0.9978), and HNO<sub>2</sub> is expected to be depleted in <sup>15</sup>N relative to NO<sub>2</sub><sup>-</sup>. Therefore, S1 and S2 can be eliminated.

Constraining <sup>15</sup>α<sub>eq,HNO2/NO2</sub> to 0.9975 in S3-S6 illustrates that an inverse kinetic isotope effect for either HNO<sub>2</sub> (S5, Figure EA1.1E) or NO<sub>2</sub><sup>-</sup> (S6, Figure EA1.1F) oxidation is required to explain the observations. A normal kinetic isotope effect for either HNO<sub>2</sub> (S3, Figure EA1.1C) or NO<sub>2</sub><sup>-</sup> (S4; Figure EA1.1D) cannot explain the observations.

FIGURE EA1.1: Simulated effects of  $\text{HNO}_2/\text{NO}_2^-$  pre-equilibrium on expression of  $^{15}\alpha_{k,\text{NXR}}$ .



**FIGURE EA1.1 LEGEND.** Simulated effects of  $\text{HNO}_2/\text{NO}_2^-$  pre-equilibrium on expression of  $^{15}\alpha_{\text{k,NXR}}$ . The different simulations were carried out using equations EA1.1-EA1.10 and the adjustable parameters (substrates, kinetic and equilibrium isotope effects) given in Table EA1.1. Simulated  $\delta^{15}\text{N}_{\text{NO}_2}$  (solid black line),  $\delta^{15}\text{N}_{\text{HNO}_2}$  (dashed black line), and  $\delta^{15}\text{N}_{\text{NO}_3}$  (solid grey line) are plotted versus simulated  $f(=[\text{NO}_2^-]/[\text{NO}_2^-]_0)$ , with pH held constant at 8.2. Overlaid in each panel are experimental observations for  $\delta^{15}\text{N}_{\text{NO}_2}$  (open black circles) and  $\delta^{15}\text{N}_{\text{NO}_3}$  (open grey squares) vs.  $f$  from experiments 1, 2, and 3 (see main text). Error bars for  $\delta^{15}\text{N}_{\text{NO}_3}$  are shown, and for  $\delta^{15}\text{N}_{\text{NO}_2}$  are smaller than the symbols.

## CASCIOTTI (W5860) ELECTRONIC ANNEX 2

### EA2. Model Formulation for Enzyme Reversibility

Reaction reversibility would be expected to affect the time course of  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  values and their relationship to  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$ . As noted in the main text, increased reaction reversibility would be expected to lead to the expression of lower (more inverse)  $^{15}\alpha_{\text{k,NXR}}$  values. Therefore, it is important to know whether nitrate reduction is occurring simultaneously with nitrite oxidation in order to best interpret the results of the current study. To first order, reversibility can be evaluated in the nitrite oxidation/nitrate reduction system through mass and isotope balance calculations. Here, a simple finite difference model was used to examine the effects of reaction reversibility on observed  $[\text{NO}_2^-]$ ,  $[\text{NO}_3^-]$ ,  $\delta^{15}\text{N}_{\text{NO}_2}$ , and  $\delta^{15}\text{N}_{\text{NO}_3}$ . The results from the model are then compared with the experimental results to evaluate evidence for reaction reversibility in the current experiments.

The following equations comprise the model formulation:

$$[^{14}\text{NO}_2^-]_t = [^{14}\text{NO}_2^-]_{t-1} + ^{14}\text{k}_{\text{NAR}} \cdot [^{14}\text{NO}_3^-]_{t-1} \cdot dt - ^{14}\text{k}_{\text{NXR}} \cdot [^{14}\text{NO}_2^-]_{t-1} \cdot dt \quad (\text{EA2.1})$$

$$[^{14}\text{NO}_3^-]_t = [^{14}\text{NO}_3^-]_{t-1} - ^{14}\text{k}_{\text{NAR}} \cdot [^{14}\text{NO}_3^-]_{t-1} \cdot dt + ^{14}\text{k}_{\text{NXR}} \cdot [^{14}\text{NO}_2^-]_{t-1} \cdot dt \quad (\text{EA2.2})$$

$$[^{15}\text{NO}_2^-]_t = [^{15}\text{NO}_2^-]_{t-1} + ^{15}\text{k}_{\text{NAR}} \cdot [^{15}\text{NO}_3^-]_{t-1} \cdot dt - ^{15}\text{k}_{\text{NXR}} \cdot [^{15}\text{NO}_2^-]_{t-1} \cdot dt \quad (\text{EA2.3})$$

$$[^{15}\text{NO}_3^-]_t = [^{15}\text{NO}_3^-]_{t-1} - ^{15}\text{k}_{\text{NAR}} \cdot [^{15}\text{NO}_3^-]_{t-1} \cdot dt + ^{15}\text{k}_{\text{NXR}} \cdot [^{15}\text{NO}_2^-]_{t-1} \cdot dt \quad (\text{EA2.4})$$

$$^{15}\alpha_{\text{k,NXR}} = ^{14}\text{k}_{\text{NXR}} / ^{15}\text{k}_{\text{NXR}} \quad (\text{EA2.5})$$

$$^{15}\alpha_{\text{k,NAR}} = ^{14}\text{k}_{\text{NAR}} / ^{15}\text{k}_{\text{NAR}} \quad (\text{EA2.6})$$

$$^{14}\text{k}_{\text{NAR}} = x \cdot ^{14}\text{k}_{\text{NXR}} \quad (\text{EA2.7})$$

$$^{15}\text{k}_{\text{NAR}} = x \cdot ^{15}\text{k}_{\text{NXR}} \cdot ^{15}\alpha_{\text{k,NXR}} / ^{15}\alpha_{\text{k,NAR}} \quad (\text{EA2.8})$$

$$\delta^{15}\text{N}_{\text{NO}_2} (\%) = (([^{15}\text{NO}_2^-]_t / [^{14}\text{NO}_2^-]_t \div ^{15}\text{R}_{\text{AIR}}) - 1) \cdot 1000 \quad (\text{EA2.9})$$

$$\delta^{15}\text{N}_{\text{NO}_3} (\%) = (([^{15}\text{NO}_3^-]_t / [^{14}\text{NO}_3^-]_t \div ^{15}\text{R}_{\text{AIR}}) - 1) \cdot 1000 \quad (\text{EA2.10})$$

In these equations, the molar concentrations (moles L<sup>-1</sup>) of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> containing <sup>14</sup>N and <sup>15</sup>N are given in brackets, t represents time, dt represents the time step for integration, k's represent first order rate constants (with the dimension t<sup>-1</sup>) for nitrite oxidation (subscript 'NXR') and nitrate reduction (subscript 'NAR') for <sup>14</sup>N-containing molecules (superscript '14') and <sup>15</sup>N-containing molecules (superscript '15'). The factor 'x' (dimensionless) represents the ratio of reverse and forward rate constants for <sup>14</sup>N-containing molecules. <sup>15</sup>α<sub>k,NXR</sub> is taken to be 0.983 and <sup>15</sup>α<sub>k,NAR</sub> is taken to be 1.040 so that <sup>15</sup>α<sub>eq,NOx</sub> (= <sup>15</sup>α<sub>k,NXR</sub> / <sup>15</sup>α<sub>k,NAR</sub>) is 0.945, as discussed in the main text. Initial conditions were taken to be [<sup>14</sup>NO<sub>2</sub><sup>-</sup>]<sub>0</sub> = 99.6337 x 10<sup>-6</sup> moles L<sup>-1</sup>, [<sup>15</sup>NO<sub>2</sub><sup>-</sup>]<sub>0</sub> = 0.3663 x 10<sup>-6</sup> moles L<sup>-1</sup> and [<sup>14</sup>NO<sub>3</sub><sup>-</sup>]<sub>0</sub> = [<sup>15</sup>NO<sub>3</sub><sup>-</sup>]<sub>0</sub> = 0 x 10<sup>-6</sup> moles L<sup>-1</sup>.

Four time course 'experiments' were run in MS Excel over 70 time steps (with dt = 1), for x equal to 0, 0.2, 0.5 or 1 (Figure EA2.1). In each case with non-zero reversibility (x>0), initial rates of net nitrite oxidation decreased over time, as the reaction came to equilibrium and the mass fluxes through the forward and reverse reaction became balanced (Figure EA2.1A). Increasing reversibility also lowers the initial net rate of nitrite oxidation and the apparent extent of nitrite oxidation, with correspondingly less nitrate accumulation. The only scenario in which nitrite is completely consumed is with no back reaction (x=0).

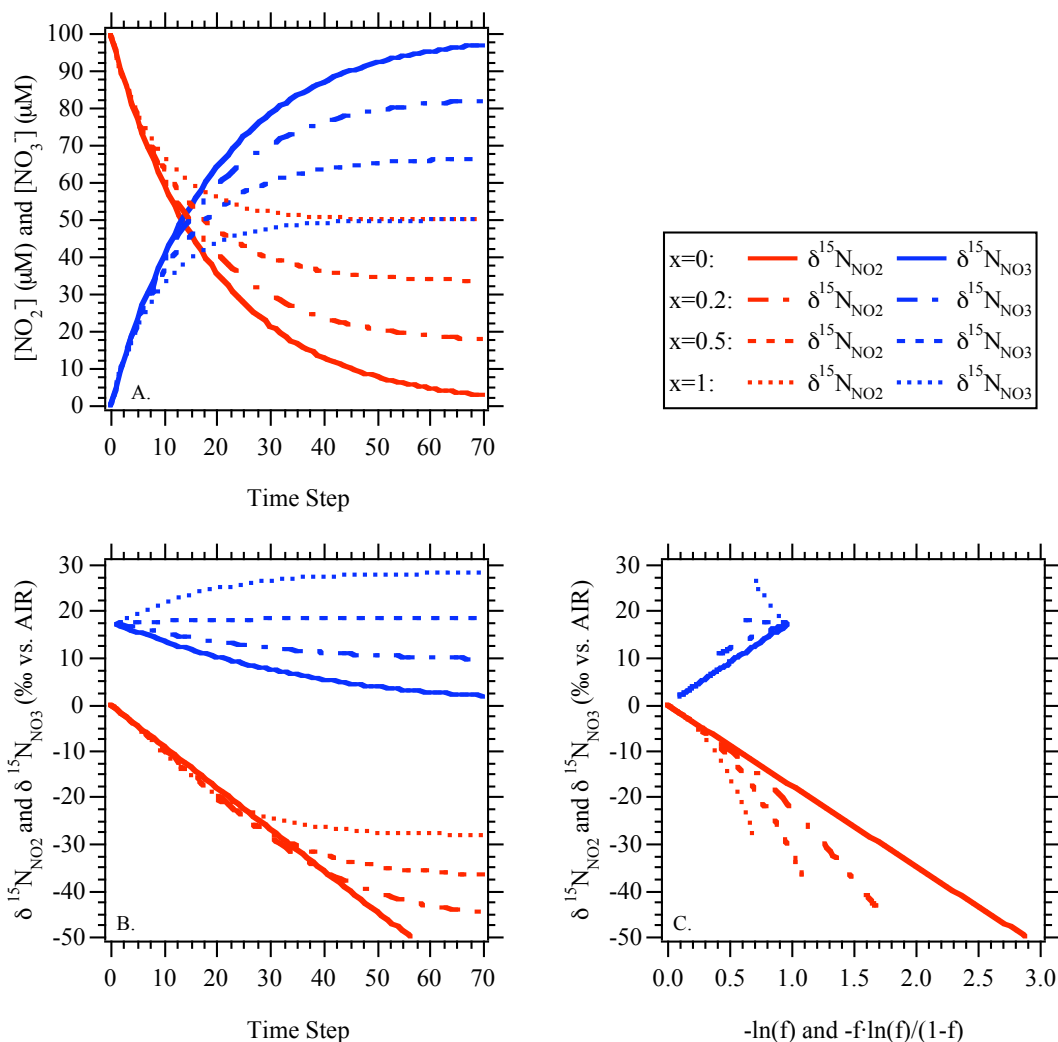
In each model run, δ<sup>15</sup>N<sub>NO2</sub> decreased over time as expected with the inverse isotope effect (Figure EA2.1B). Increasing amounts of nitrate reduction caused δ<sup>15</sup>N<sub>NO2</sub> to decrease more quickly for a given amount of nitrite oxidation. From the perspective of δ<sup>15</sup>N<sub>NO2</sub>, increasing reversibility lead to an increasingly inverse apparent isotope effect (Figure EA2.1C), and the extent of back reaction was difficult to diagnose from δ<sup>15</sup>N<sub>NO2</sub> alone. However, reaction reversibility also led to unique trends in δ<sup>15</sup>N<sub>NO3</sub> over time that should allow us to distinguish between a large irreversible isotope effect and a contribution from enzyme reversibility.



At low values of  $x$ ,  $\delta^{15}\text{N}_{\text{NO}_3}$  decreased over time as it was dominated by the inverse isotope effect for nitrite oxidation (Figure EA2.1B,  $x = 0$ ,  $x = 0.2$ ). As the value of  $x$  was increased to 0.5,  $\delta^{15}\text{N}_{\text{NO}_3}$  remained relatively constant over the time course (Figure EA2.1B), and in the most extreme case ( $x=1$ ),  $\delta^{15}\text{N}_{\text{NO}_3}$  actually increased over time as it approached equilibrium with  $\text{NO}_2^-$  (Figure EA2.1B). When  $x$  was greater than 0.5, the isotope effect for nitrite oxidation based on  $\delta^{15}\text{N}_{\text{NO}_3}$  switched from inverse to apparently normal (Figure EA2.1C) while  $\delta^{15}\text{N}_{\text{NO}_2}$  fractionation continued to become increasingly inverse. The differential behavior of  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  under different levels of reaction reversibility present a diagnostic pattern for reversibility in the  $\delta^{15}\text{N}_{\text{NO}_2}/\delta^{15}\text{N}_{\text{NO}_3}$  system, with apparent isotope effects based on  $\delta^{15}\text{N}_{\text{NO}_2}$  and  $\delta^{15}\text{N}_{\text{NO}_3}$  diverging as the amount of back reaction increases (Figure EA2.1C).

In experiments with *Nitrococcus mobilis*, discussed in the main text, nitrite was completely oxidized to nitrate over the time course experiments (Figure 1). Both nitrite and nitrate become depleted in  $^{15}\text{N}$  over time (Figure 1), with  $\delta^{15}\text{N}_{\text{NO}_3}$  approaching the initial  $\delta^{15}\text{N}_{\text{NO}_2}$  after all of the nitrite had been oxidized to nitrate. The Rayleigh plots were also consistent with a mass balance between  $\text{NO}_2^-$  and  $\text{NO}_3^-$  and a nearly irreversible reaction of  $\text{NO}_2^-$  to  $\text{NO}_3^-$  under our experimental conditions (Figure 2). Therefore, both the mass and isotope balance observed in *N. mobilis* experiments suggest that reaction reversibility was insignificant under the conditions employed.

**FIGURE EA2.1:** Reversibility of Nitrite Oxidoreductase



**FIGURE EA2.1.** Results from nitrite oxidoreductase reversibility model. (A) Modeled nitrite (red) and nitrate (blue) concentrations versus time for  $x$  ( $=^{14}k_{NAR}/^{14}k_{NXR}$ ) values of 0, 0.2, 0.5, and 1.0. (B) Modeled  $\delta^{15}N_{NO_2}$  (red) and  $\delta^{15}N_{NO_3}$  (blue) versus time for the same values of  $x$ . (C) Modeled  $\delta^{15}N_{NO_2}$  (red) and  $\delta^{15}N_{NO_3}$  (blue) versus  $-\ln(f)$  and  $-f \cdot \ln(f)/(1-f)$ , respectively. In each panel,  $x = 0$  is indicated by the solid line,  $x = 0.2$  by the dot-dashed line,  $x = 0.5$  by the dashed line, and  $x = 1$  by the dotted line.