

1 **Denitrification exceeds Anammox as a nitrogen loss pathway in the Arabian**
2 **Sea Oxygen Minimum Zone**

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24 **ABSTRACT**

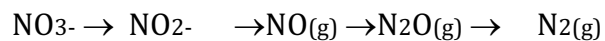
25 The oxygen minimum zones (OMZs) of the world oceans are sites of enhanced
26 anaerobic nitrogen transformations, such that the largest OMZ, the Arabian Sea, is
27 responsible for 30-50% of the oceanic water column fixed N loss. Measurements of
28 excess N₂ and nitrate deficits have been interpreted to indicate a large net loss of
29 fixed nitrogen through N₂ production in waters with very low oxygen
30 concentrations. Rates of N₂ production both by denitrification and anaerobic
31 ammonium oxidation (anammox) were investigated by measuring ¹⁵N-N₂
32 production from ¹⁵NH₄⁺, ¹⁵NO₂⁻, and ⁴⁶N₂O in incubations at 3-4 depths at each of
33 three stations in the central region of the Arabian Sea OMZ. In addition to
34 conventional measurements of anammox and denitrification rates from incubations
35 in 12 mL exetainers purged with He, we also investigated the effect of ammonium
36 and organic carbon additions on N₂ production rate. Denitrification was detected at
37 every depth at every station; the maximum rate (25.3±9.1 nmol l⁻¹ d⁻¹ from the
38 ¹⁵NO₂ tracer and 21.2±5.6 nmol l⁻¹ d⁻¹ from the ⁴⁶N₂O tracer) occurred at the depth
39 of the secondary nitrite maximum in the core of the OMZ. The ⁴⁶N₂O tracer was the
40 most direct method to measure the denitrification rate. Rates of denitrification
41 correlated strongly with water column nitrite concentration. Addition of organic
42 carbon or ammonium did not consistently stimulate denitrification rates.
43 Distribution of anammox was patchy; low rates of anammox production of ²⁹N₂ in
44 ¹⁵NH₄⁺ incubations were detected at only four of eleven depths. The highest rate of
45 anammox production (4.23±0.35 nmol l⁻¹ d⁻¹) occurred near the upper boundary of

46 the OMZ at one station. Overall, denitrification dominated N₂ production at this time
47 in the Arabian Sea OMZ.

48 **Key Words:** anammox; denitrification; Arabian Sea; oxygen minimum zone

49 **1. Introduction**

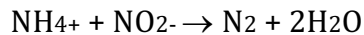
50 Available fixed nitrogen is the limiting nutrient that controls the growth of
51 phytoplankton in over half of the surface ocean (Tyrell 1999, Moore et al. 2004), and
52 denitrification is the main sink for oceanic fixed N (Codispoti 1995). The Arabian
53 Sea Oxygen Minimum Zone (OMZ) is responsible for up to 40 Tg yr⁻¹ (30-50%) of
54 that fixed nitrogen loss (Codispoti 2001, Naqvi 2008). Nitrogen lost as N₂O and N₂
55 has traditionally been attributed to the respiratory (also referred to as canonical)
56 denitrification process:



58 This pathway proceeds under very low conditions (less than 1-2% of saturation,
59 Codispoti 1995). Denitrification is performed by a diverse assemblage of microbes
60 (Zumft 1997), and water column denitrification has been largely attributed to
61 heterotrophic denitrifiers (Codispoti 2001). Due to the low sensitivity of previously
62 available isotope methods, direct rate measurements of N₂ production by
63 denitrification are rare. Instead, denitrification (which may have included
64 anammox) has often been calculated stoichiometrically from nitrate deficits (Naqvi
65 1987, Bange 2000, Codispoti 2001) or estimated from electron transport activity
66 (ETS) (Naqvi and Shailaja 1993).

67 Recently anaerobic ammonia oxidation (anammox) has been identified as an
68 important pathway for anaerobic N₂ production in the marine environment

69 (Thamdrup and Daalsgard 2002, Daalsgard et al. 2003). Anammox bacteria are a
70 deeply branching group of the *Planctomycetales* (Strous et al. 1999). In contrast to
71 the heterotrophic denitrifying bacteria, anammox bacteria are autotrophic and
72 produce N₂ by combination of NO₂⁻ with NH₄⁺ through a hydrazine intermediate
73 (Strous et al. 1998):



74
75 While heterotrophic denitrifiers obtain their nutrition from organic matter and their
76 respiratory substrate from the NO₃⁻ that is plentiful in the deep ocean, anammox
77 bacteria require ammonium and nitrite ions produced from the remineralization of
78 organic matter. Interestingly, anammox alone has been shown to be responsible for
79 fixed N loss in oxygen depleted water columns off the coast of Chile, Peru, and the
80 Benguela (Thamdrup et al. 2006, Hamersley et al. 2007, Kuypers et al. 2005). Just
81 how ammonium and nitrite are supplied to anammox bacteria in these
82 environments without denitrification is perplexing. It has been suggested episodic
83 denitrification may occur in these systems (Ward et al. 2009, Thamdrup et al. 2006,
84 Ward et al. 2008) or that alternative nitrogen cycling process, such as aerobic
85 nitrification or dissimilatory nitrate reduction to ammonium, may be important
86 (Lam et al. 2009). It appears that the Arabian Sea is different and those conditions
87 favor denitrification as the dominant N-loss pathway (Ward et al., 2009). The
88 temporal component of the relative importance of denitrification and anammox still
89 has not been addressed, however, due to the limited sampling reported thus far.

90 Both denitrifier and anammox bacterial N₂ production require suboxic
91 conditions, such as found in the water column in the three major OMZs in the world:

92 in the eastern tropical north Pacific (ETNP) off of the coast of Mexico, in the eastern
93 tropical south Pacific (ETSP) off the coasts of Peru and Chile, and in the Arabian Sea
94 (AS). The Arabian Sea contains the largest permanent oxygen minimum zone
95 (OMZ), 44% of the area of all three major OMZs together (Helly and Levin 2004).
96 The AS OMZ is the thickest of the three OMZs, as oxygen concentrations remain
97 below 10 μ M between 150 to 988 meters throughout this region (boundaries are
98 upper and lower quartiles, Helly & Levin 2004). As with the other OMZs, there is no
99 evidence of sulfide production and the AS OMZ is characterized by a high secondary
100 nitrite maximum (SNM) of 0.5-6 μ M (Naqvi 1994), suggesting that N-oxides are the
101 preferred electron acceptors in respiration. The size of the AS OMZ alone
102 emphasizes its importance in global marine fixed N loss.

103 The relative contributions of denitrification and anammox processes to fixed
104 N loss in the AS are still not clear, although recent ¹⁵N experiments and molecular
105 studies indicate that both types of organisms and processes are present. The
106 denitrifier nitrite reductase (*nirS*) gene and the anammox 16S genes have been
107 detected in the Arabian Sea OMZ (Woebken et al. 2008, Jayakumar et al. 2009).
108 Denitrification rates from ¹⁵N incubations have been measured in the Arabian Sea
109 by Devol et al. (2006) and Nicholls et al. (2007), and those results suggest that
110 denitrification alone cannot be the only loss term for fixed N in the Arabian Sea.
111 Nicholls et al. (2007) reported a few, very low anammox rates, but the anammox
112 ²⁹N₂ production was $\leq 10\%$ of the total.

113 Although Ward et al. (2008) found that denitrification was carbon limited in
114 both the ETNP and ETSP OMZs, it was not carbon limited in the AS OMZ during the

115 SW Monsoon. This is consistent with the report by Ducklow et al. (2001) which
116 described seasonally uniform DOC and low variability in surface heterotrophic
117 bacterial production throughout one year in the AS. We hypothesized that
118 conditions in the Arabian Sea might consistently favor denitrifying bacteria over
119 anammox bacteria.

120 The aim of this study was to determine the relative contribution of
121 denitrification and anammox to fixed N loss in the Arabian Sea by measuring rates of
122 N₂ production through ¹⁵N tracer incubations in exetainers. In addition to ¹⁵NO₂-
123 additions, we examined additions of ⁴⁶N₂O as an alternative treatment to measure
124 denitrification. We designed experiments to test if the N₂ production rate was
125 limited by carbon or ammonium supply. Since He sparging typically used in the
126 exetainer protocol might artificially enhance the rates of either anammox or
127 denitrification, we tested this potential artifact by including a set of experiments
128 that was not sparged with He. Here we report rate measurements for denitrification
129 and anammox, and the effect of the various treatments on those rates.

130 **2. METHODS**

131 **2.1 Sample Collection**

132 Experiments were conducted in the Arabian Sea during the late
133 monsoon/intermonsoon transition in September and October 2007 on the KNOX08
134 cruise leg of the R/V Roger Revelle. All three stations were in water depth >3000m.
135 Stations 1 (19°N 66°E) and 2 (15.15°N 64°E) were located within the region
136 historically documented as the permanent OMZ (Figure 1). Oxygen concentration
137 was less than 1 μM between 100 or 140m and 750m at Stations 1 and 2,

138 respectively. Station 3 (11°N 68°E) was located on the periphery of the permanent
139 OMZ (Figure 1), and the depleted oxygen layer was less than 200m thick at this
140 location. Samples were collected from 10 L Niskin bottles on a conductivity-
141 temperature-depth (CTD) rosette system. A Seabird O₂ sensor (calibrated to
142 shipboard Winkler assays, Carpenter 1965) was used to determine the depth and
143 location of the OMZ. Nitrite concentrations were measured on a Shimadzu UV-
144 Visible spectrometer following Strickland and Parsons (1972), and nitrate and
145 ammonium concentrations were measured on a segmented flow autoanalyzer
146 (SKALAR Analytical) onboard ship.

147 Samples were collected at three or four depths at the three stations
148 corresponding to specific features of the NO₂- depth profile: 1) within the oxycline at
149 the top of the OMZ (112-126m depth), 2) at the secondary nitrite maximum (SNM,
150 ~150-200m), 3) either above or below the SNM, and 4) toward the lower boundary
151 of the OMZ where the O₂ concentration was still <2 μM and nitrite was ≤0.5 μM
152 (Table 1).

153 **2.2 N tracer incubations**

154 Water was collected into the bottom of a 250 mL glass bottle by allowing it to
155 overflow at least three times before sealing it with a glass stopper. The Niskin
156 bottles were plumbed with N₂ to fill the headspace created during sample collection
157 to help prevent O₂ contamination. To measure denitrification and anammox rates,
158 the incubation protocol followed Dalsgaard et al. (2003). Briefly, all manipulations
159 were carried out in a glove bag that was flushed and filled three times with N₂ gas.
160 Positive pressure was maintained throughout the procedure. Water samples kept at

161 12°C for no longer than 3 h were transferred to an N₂ flushed glove bag, where the
162 ¹⁵N tracers were added and amended seawater was transferred to Exetainers
163 (Labco). Tracer additions for a final concentration of 3 μM of ¹⁵NO₂⁻ or ¹⁵NH₄⁺ were
164 added to each 250 mL bottle, and aliquots (8 mL) of sample water were pipetted
165 into 12 mL Exetainers. Tracers and additions for each incubation are listed in Table
166 2. The Exetainers were sealed with butyl rubber septa in screw caps, and then
167 sparged by bubbling the water using a canula with He for 5 minutes, with slight He-
168 overpressure, except for the no He-sparge treatment (which was incubated with no
169 headspace). ⁴⁶N₂O tracer was added after the He-sparge step. For the ¹⁵NO₂⁻ + no He
170 sparge treatment, the Exetainer vials were filled with 12 mL sample water with no
171 headspace. Three replicates of each sample were killed with 0.05 mL of 7M ZnCl₂ at
172 12-hour intervals between 0 and 48 hours.

173 **2.3 N Analyses and Calculations**

174 The amount of excess ¹⁵N-N₂ produced during the incubations was measured
175 directly in the incubation vials with a continuous flow isotope ratio mass
176 spectrometer (Europa Scientific 20-20), in-line with an automated gas preparation
177 unit (Europa Scientific, ANCA-G Plus). Denitrification was defined as binomially
178 distributed ²⁹N₂ and ³⁰N₂ production in the ¹⁵NO₂⁻ incubation and ³⁰N₂ in the ⁴⁶N₂O
179 incubation. Anammox was defined as ²⁹N₂ production in ¹⁵NH₄⁺ incubation and
180 calculated from ²⁹N₂ in the ¹⁵NO₂⁻ incubation (see equations below). The method of
181 Thamdrup and Dalsgaard (2002) was followed to determine the amount of ²⁹N₂ and
182 ³⁰N₂ in excess of natural abundance produced in the vials. NO₃⁻+NO₂⁻ was measured
183 using a NO_x analyzer (Teledyne) following vanadium reduction to nitric oxide (Cox

184 1980). The Strickland and Parsons (1972) method was used to measure NO₂-
185 spectrophotometrically.

186 At depths where statistically significant anammox production of N₂ was
187 detected in the ¹⁵NH₄⁺ incubations by linear regression (p<0.05), the amount of ¹⁵N-
188 N₂ produced by anammox (A_{total}) and denitrification (D_{total}) was calculated according
189 to the isotope pairing method of Thamdrup and Dalsgard (2002). The ¹⁵N-atom% of
190 NO₂ (F_N) in the incubations where ¹⁵NO₂⁻ was the tracer was determined as:

$$191 \quad F_N = \frac{(^{15}\text{N}_{\text{added}} - ^{15}\text{N}_{2\text{excess}})}{\text{total } [\text{NO}_2]^{-1}} \quad (1)$$

192 The total N₂ produced from respiratory denitrification (D_{total}) was calculated
193 using the measured amount of ³⁰N₂ excess and the F_N. The isotope pairing method
194 was used to calculate ²⁹N₂ from respiratory denitrification (D₂₉) and N₂ from
195 anammox (A_{total}) according to the following equations:

$$196 \quad D_{\text{total}} = P_{30} F_N^{-2} \quad (2)$$

$$197 \quad D_{29} = P_{30} 2(1 - F) F^{-1} \quad (3)$$

$$198 \quad A_{29} = P_{29} - D_{29} \quad (4)$$

$$199 \quad A_{\text{total}} = P_{29} F^{-1} - D_{\text{total}} 2 (1 - F_N) \quad (5)$$

200

201 For depths at which significant anammox production was not detected
202 during the ¹⁵NH₄⁺ incubations (p-value >0.05), the total denitrification rate was
203 calculated according to the binomial distribution calculations of Nielson (1992).
204 These equations derive the rate from both ³⁰N₂ (P₃₀) and ²⁹N₂ (P₂₉), and therefore
205 we found them to be more accurate when anammox was not a factor. N₂ production
206 values for both P₃₀ and P₂₉ were zeroed to the average of the T₀ timepoints. The rate

207 was determined from the regression line slopes of the production of $^{29}\text{N}_2$ (F29) and
208 $^{30}\text{N}_2$ (F30), using the following equation:

$$\begin{aligned} 209 \quad & \text{Total respiratory denitrification rate} = \\ 210 \quad & (F29 - 2 F30) - 1 (F29 + 2 F30) \quad (6) \end{aligned}$$

Statistical analyses were performed using the JMP software program (SAS).

211 The ^{15}N - N_2 production rates were calculated from the 36-hour (4 time points)
212 slope (wherever possible) of the standard least squares regression over the time
213 course of the incubations (from 0 to 36 hours). Vials with exponential production at
214 36 hours were considered outliers and not included when calculating the slope, so
215 the line was fit to the maximum number of data points that resulted in the most
216 linear (based on R^2) and significant ($p < 0.05$) regression line. Student t-tests were
217 performed to determine whether the slope was significantly different from zero
218 ($p < 0.05$). Error was calculated as the standard error of the slope. An F-test was
219 performed on the anammox production slopes to determine if rates between
220 treatments were significantly different. Error for denitrification rates calculated
221 according to Nielson (1992) was determined by propagation of error from the
222 slopes of F29 and F30. When comparing among denitrification rates, two rates were
223 considered significantly different if their standard errors did not overlap. All slopes
224 and errors are reported in supplementary Table 1.

225 **3. RESULTS**

226 Depth profiles of the distributions of DIN ($\text{NO}_3^- + \text{NO}_2^-$) and oxygen at all three
227 stations are shown (Figure 2). NH_4^+ concentrations ranged from undetectable to
228 $< 1.0 \text{ M}$ (data not shown). The thickness and intensity of the OMZ at Stations 1 and
229 2 were similar: oxygen concentrations declined to $1 \text{ } \mu\text{mol l}^{-1}$ between 100 and

230 150m and increased to $>1 \mu\text{mol l}^{-1}$ again by 750m depth. The OMZ at Station 3 was
231 less thick, with the oxygen concentration declining to $\leq 1 \mu\text{mol l}^{-1}$ by 110m and
232 increasing to $> 1 \mu\text{mol l}^{-1}$ by 250m. The maximum concentration of the secondary
233 nitrite maximum was also higher at Stations 1 ($5.0\mu\text{M}$ at 140m) and 2 ($6.5\mu\text{M}$ at
234 180-200m) than at Station 3 ($3.5\mu\text{M}$ at 150m).

235 Changes in the DIN concentration in incubations with $^{15}\text{NO}_2^-$ tracers were
236 usually not observed until after 24 or 36 hours (e.g., Station 1, Figure 3). All
237 incubations showed the same general patterns of DIN change over time: NO_2^-
238 production was positive, with the greatest increase between 36 and 48 hours.
239 Occasionally, most or all of the DIN was completely converted to NO_2^- in one of the
240 three replicate exetainer vials before 48 hours. For example, Stn 1 at 400m had a
241 nitrite concentration of $25.5\mu\text{M}$ by 48 hours (Figure 3). Similar to Thamdrup et al
242 (2006), when this occurred before 48 hours, the outlier vial was excluded from
243 further calculations.

244 **3.1 Denitrification**

245 Respiratory denitrification was detected at all eleven depths through
246 significant production of $^{30}\text{N}_2$ from additions of $^{46}\text{N}_2\text{O}$. At the seven depths where
247 anammox was not detected, respiratory denitrification in $^{15}\text{NO}_2^-$ additions followed
248 the pattern at Station 1 at 151m: significant production of $^{29}\text{N}_2$ between 0 and 36
249 hours, and exponential production of $^{29}\text{N}_2$ by 48 hours (and sometimes by 36 hours)
250 (Fig. 4). In the $^{46}\text{N}_2\text{O}$ additions, the pattern was similar except $^{30}\text{N}_2$ was produced
251 instead of $^{29}\text{N}_2$. Total N_2 production was calculated from excess $^{29}\text{N}_2$ and $^{30}\text{N}_2$
252 production over the initial 36-hr time course, and the 48-hr data were not included

253 in the rate calculations. Rates derived from each treatment are described in the
254 following sections.

255 **3.1.1 $^{15}\text{NO}_2$ tracer incubation**

256 The highest rates of total N_2 production from denitrification measured in
257 treatments that received only the $^{15}\text{NO}_2$ - tracer were observed at Station 1, ranging
258 from $1.27 \pm 0.27 \text{ nmol l}^{-1} \text{ day}^{-1}$ at 400m to $25.4 \pm 9.12 \text{ nmol l}^{-1} \text{ day}^{-1}$ at 151m (Figure
259 5a). The rates of total N_2 production observed at the shallowest depths at Station 2
260 (126m, 150m) and Station 3 (112m) were not significantly above zero (Figures 5b,
261 5c). The maximum denitrification rate at Station 2 of $3.08 \pm 1.33 \text{ nmol l}^{-1} \text{ day}^{-1}$
262 occurred at 200 m (Figure 5b), while at Station 3, the maximum rate (4.12 ± 1.53
263 $\text{nmol l}^{-1} \text{ day}^{-1}$) occurred 150m (Figure 5c).

264 **3.1.2 $^{46}\text{N}_2\text{O}$ tracer incubation**

265 Significant production of total N_2 from denitrification was measured in
266 incubations from all stations at all depths in the $^{46}\text{N}_2\text{O}$ tracer incubations (Figure 5).
267 The pattern of total N_2 production rates was very similar to that observed from the
268 $^{15}\text{NO}_2$ - tracer. The highest rates occurred at Station 1, ranging from $1.83 \pm 0.60 \text{ nmol}$
269 $\text{l}^{-1} \text{ day}^{-1}$ at 400m to $21.2 \pm 5.56 \text{ nmol l}^{-1} \text{ day}^{-1}$ at 151m. Low rates were detected at
270 the two shallowest depths at Station 2 (0.55 ± 0.14 and $0.93 \pm 0.54 \text{ nmol l}^{-1} \text{ day}^{-1}$) and
271 the shallowest depth at Station 3 (0.48 ± 0.15) $\text{nmol l}^{-1} \text{ day}^{-1}$). The highest rate at
272 Station 2 occurred at 400m ($2.99 \pm 0.52 \text{ nmol l}^{-1} \text{ day}^{-1}$) and at Station 3 at 151m
273 ($2.40 \pm 0.70 \text{ nmol l}^{-1} \text{ day}^{-1}$).

274 **3.1.3 $^{15}\text{NO}_2$ - $^{14}\text{NH}_4^+$ treatment**

275 In the $^{15}\text{NO}_2^-+^{14}\text{NH}_4^+$ tracer and addition treatment, the denitrification rates
276 observed at Station 1 were the highest overall, ranging from $1.24 \pm 0.43 \text{ nmol l}^{-1} \text{ day}^{-1}$
277 at 400m to $7.46 \pm 2.31 \text{ nmol l}^{-1} \text{ day}^{-1}$ at 200m (Figure 5). Total N_2 production was
278 not significantly greater than zero at the shallowest depths at Stations 2 and 3. The
279 greatest rate at Station 2 occurred at 200m ($9.47 \pm 3.33 \text{ nmol l}^{-1} \text{ day}^{-1}$) and at Station
280 3 at 400m ($0.17 \pm 0.03 \text{ nmol l}^{-1} \text{ day}^{-1}$). This treatment resulted in the highest rates at
281 Station 3 ($9.23 \pm 4.10 \text{ nmol l}^{-1} \text{ day}^{-1}$) of any treatment.

282 **3.1.4 $^{15}\text{NO}_2^-+\text{C}$ treatment**

283 The rates of total N_2 production following the $^{15}\text{NO}_2^-$ tracer incubations
284 which received an organic carbon addition (mixture of labile substrates, Table 2)
285 were not significantly different from rates with the $^{15}\text{NO}_2^-$ tracer alone (Station 1,
286 Figure 5). Again, the highest denitrification rates (average $5.19 \pm 2.51 \text{ nmol l}^{-1} \text{ day}^{-1}$)
287 were observed at Station 1, ranging from $1.13 \pm 0.27 \text{ nmol l}^{-1} \text{ day}^{-1}$ at 400m to
288 $9.94 \pm 1.94 \text{ nmol l}^{-1} \text{ day}^{-1}$ at 151m. The maximum rate at Station 2 occurred at 200 m
289 ($5.17 \pm 3.38 \text{ nmol l}^{-1} \text{ hr}^{-1}$) and at Station 3 the maximum was $4.50 \pm 1.86 \text{ nmol l}^{-1} \text{ day}^{-1}$
290 at 150m. The rate of total N_2 production was not significantly above zero at the
291 shallowest depths at Station 2 (126m) or Station 3 (112m). The highest
292 denitrification rate ($1.42 \pm 0.37 \text{ nmol l}^{-1} \text{ day}^{-1}$) observed at Station 2, 151m $^{15}\text{NO}_2^-+\text{C}$
293 treatment was significantly higher than the rate from the $^{15}\text{NO}_2^-$ tracer alone, but
294 was not significantly different than the N_2 production rate from the $^{46}\text{N}_2\text{O}$ addition.

295 **3.1.5 $^{15}\text{NO}_2^-$ tracer incubation with no He sparge**

296 The addition of $^{15}\text{NO}_2^-$ without sparging the exetainers with helium was
297 performed only at Stations 2 and 3. A significant ($p<0.05$), positive rate was

298 detected at only one depth: Station 3 at 112m with a rate of $1.08 \pm 0.36 \text{ nmol l}^{-1} \text{ day}^{-1}$
299 (Figure 5).

300 **3.1.6 ^{15}N -glycine+ $^{14}\text{NH}_4^+$ treatment**

301 The ^{15}N -glycine+ $^{15}\text{NH}_4^+$ addition did not result in the production of excess
302 $^{29}\text{N}_2$ or $^{30}\text{N}_2$ in any of the experiments (data not shown).

303 **3.2 Anammox**

304 Only four of the eleven depths sampled showed evidence of anammox
305 activity, based on significant $^{29}\text{N}_2$ -excess production from $^{15}\text{NH}_4^+$ tracer incubations.
306 Station 2, 151m was the only depth at which high anammox rates were detected.
307 These high anammox rates coincided with some of the lowest observed
308 denitrification rates. Significant ($p < 0.05$), linear $^{29}\text{N}_2$ production occurred at Station
309 2, 150m in both the $^{15}\text{NO}_2^-$ and $^{15}\text{NH}_4^+$ tracer incubations, but there was no
310 significant $^{30}\text{N}_2$ production before 48 hours in either the $^{15}\text{NO}_2^-$ incubation with no
311 He-sparge or the $^{46}\text{N}_2\text{O}$ incubation (Figure 6). As in the $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production
312 from denitrification, the values at 48 hours were not included in the rate
313 calculations.

314 Rates of total N_2 production by anammox in the $^{15}\text{NH}_4^+$ tracer incubation
315 ranged from $0.66 \pm 0.19 \text{ nmol l}^{-1} \text{ day}^{-1}$ at Station 1 at 200m up to $4.23 \pm 0.35 \text{ nmol l}^{-1}$
316 day^{-1} at Station 2 at 150m (Figure 7). The rates of N_2 production calculated from
317 the $^{15}\text{NO}_2^-$ tracer incubations were higher than those calculated from the $^{15}\text{NH}_4^+$
318 tracer incubations at all 4 depths; a maximum of $6.49 \pm 0.40 \text{ nmol l}^{-1} \text{ day}^{-1}$ was
319 observed at Station 2 at 151m. Anammox rates in incubations with additions of
320 $^{15}\text{NH}_4^+$ or carbon as well as $^{15}\text{NO}_2^-$ were not significantly different from the $^{15}\text{NO}_2^-$

321 addition alone ($F=0.01$, $p=0.987$), with the exception of the $^{15}\text{NO}_2\text{-+C}$ addition at
322 Station 2 at 151m ($F=6.08$, $p=0.005$). With the addition of carbon, the rate total N_2
323 production at this depth increased from 6.49 ± 0.40 to 9.23 ± 1.50 $\text{nmol l}^{-1} \text{ day}^{-1}$.

324 4. DISCUSSION

325 We found evidence for production of N_2 by both denitrification and anammox
326 in the Arabian Sea OMZ using the standard methods—measuring excess $^{30}\text{N}_2$ and
327 $^{29}\text{N}_2$ from $^{15}\text{NO}_2^-$ and $^{15}\text{NH}_4^+$ tracer incubations, respectively—as well as
328 denitrification as the production of $^{30}\text{N}_2$ from a $^{46}\text{N}_2\text{O}$ tracer. Conversion of $^{46}\text{N}_2\text{O}$ to
329 $^{30}\text{N}_2$ is strong evidence of respiratory denitrification, because N_2O is an intermediate
330 in denitrification but is not a known intermediate of anammox bacteria (Kartal et al.
331 2007). Denitrification rates of N_2 production were on average at least four-fold
332 greater than anammox rates at these three stations. We detected significant
333 anammox activity at four of eleven depths (two depths at Station 1 and two at
334 Station 2), and the rate was lower than that from denitrification at three of the four.
335 These findings are in contrast to those of Jensen (ASLO abstract 2009), who
336 reported evidence of neither denitrification nor anammox in the open ocean
337 Arabian Sea OMZ, using the same exetainer protocol.

338 Denitrification was detected at every station and depth sampled. The
339 average rate of N_2 production from denitrification at each depth was correlated with
340 the *in situ* nitrite concentration, with an R^2 value of 0.67 (Figure 8). The highest
341 denitrification rates at all three stations occurred at the depth of the SNM. High
342 nitrite concentrations have been used to infer high rates of denitrification, but this is
343 the first time that measured open-ocean rates have been shown to correlate

344 strongly with nitrite concentration. The mechanism underlying the relationship is
345 still unclear, however, because denitrification both produces and consumes NO_2^- .
346 Nicholls et al. (2007) speculated that partially uncoupled rates of the steps in the
347 sequential reduction of nitrate to N_2 might be responsible for the accumulation of
348 nitrite in the OMZ, as nitrite accumulated in their $^{15}\text{NO}_3$ incubations before
349 reduction to N_2O .

350 At depths where measured O_2 was $>2 \mu\text{M}$ ($12.9 \mu\text{M}$ and $43 \mu\text{M}$, Table 1),
351 denitrification rates were at their lowest, indicating that low oxygen is necessary to
352 support significant denitrification, as has been long recognized. The rates were
353 lowest at the shallowest depth at Stations 2 and 3, where O_2 was $43.1 \mu\text{M}$ and
354 $12.9 \mu\text{M}$, respectively (Table 1), putting those depths above the heart of the OMZ,
355 well into the oxycline. These results are in direct contrast with those of Farias et al.
356 (2009) which showed, using acetylene block and allyl-thiourea inhibitor
357 incubations, N_2O consumption through denitrification in the ETSP in waters with
358 $>40 \mu\text{M}$ O_2 .

359 Denitrification alone cannot explain all of the fixed-N loss in the AS. Devol et
360 al. (2006) reported 'extra' N_2 calculated from difference between the nitrate deficit
361 and $\text{N}_2:\text{Ar}$ ratios that could not be explained by denitrification, and predicted that
362 conversion of organic N to N_2 was also occurring. Nicholls et al. (2007) found that
363 the production of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ was not binomially distributed as it should be if
364 produced by denitrification alone, resulting in 'extra' $^{29}\text{N}_2$. As this could not be
365 accounted for by anammox, they suggested that either organic N or DNRA was
366 contributing to N_2 production. We did not detect any $^{15}\text{N}-\text{N}_2$ production in

367 incubations with ^{15}N -glycine, suggesting that conversion of primary amines is not a
368 factor. We also found no evidence for DNRA based on comparing ^{15}N - N_2 production
369 between $^{15}\text{NO}_2$ and $^{46}\text{N}_2\text{O}$ additions, with and without anammox activity. While the
370 $^{30}\text{N}_2$ produced by linked DNRA and anammox from a $^{15}\text{NO}_2$ tracer addition is not
371 distinguishable from denitrification, the $^{30}\text{N}_2$ produced from a $^{46}\text{N}_2\text{O}$ tracer can only
372 be from respiratory denitrification. The denitrification rates determined from the
373 $^{46}\text{N}_2\text{O}$ tracer were not significantly different from other treatments at the same
374 depth, which suggests that if DNRA were occurring, its contribution to N_2
375 production was below our detection limit. Additionally, at depths where 'extra' (or
376 non-binomially distributed) $^{29}\text{N}_2$ was observed, anammox activity was also detected
377 in the $^{15}\text{NH}_4^+$ incubations. Conversely, at depths at which no anammox activity was
378 detected from the $^{15}\text{NH}_4^+$ addition, we observed no 'extra' $^{29}\text{N}_2$. Therefore, we infer
379 that any non-binomially distributed N_2 we observed was from anammox, with no
380 significant contribution from DNRA.

381 Anammox activity appeared to be more patchy and unpredictable in regard
382 to depth distribution compared to denitrification. At Station 1, we found evidence
383 for anammox in the upper OMZ, well within the oxycline, as well as below the SNM,
384 while at Station 2, anammox was observed only at the SNM and 150m below the
385 SNM. Three of the four depths at which anammox was observed had lower
386 anammox rates on average than denitrification rates, and $^{29}\text{N}_2$ production from the
387 $^{15}\text{NH}_4^+$ addition was $\leq 10\%$ of the $^{30}\text{N}_2$ production from the $^{46}\text{N}_2\text{O}$ addition. The
388 fourth depth (Station 2, 151m), however, had the highest anammox rates of all, and
389 about ten times greater than the denitrification rate at that depth. This was also the

390 only depth at which the carbon addition stimulated the rate of denitrification or
391 anammox, and here it did both. It is possible that this particular sample from this
392 depth was carbon-limited, and that may be why anammox is favored over
393 denitrification in this sample only.

394 Overall, the average anammox N₂ production rates of 0.77 ± 0.11 nmol l⁻¹ d⁻¹
395 (¹⁵NH₄⁺ treatment) to 2.5 ± 0.75 nmol l⁻¹ d⁻¹ (¹⁵NO₂⁻ treatment) were lower than
396 anammox rates measured in the ETSP, where anammox but not denitrification was
397 detected. Thamdrup and Daalsgard (2006) reported anammox rates of 4.8 to 16.8
398 nmol l⁻¹ d⁻¹ off the coast of Chile. Similarly, Hamersley et al (2007) reported rates of
399 2 to 6 nmol l⁻¹ d⁻¹, and Rich (unpublished data) found rates of 5-15 nmol l⁻¹ d⁻¹ off
400 the coast of Peru.

401 The anammox rates of N₂ production calculated from the ¹⁵NO₂⁻ tracer
402 incubations were consistently higher than those calculated from the ¹⁵NH₄⁺ tracer.
403 We suggest that this is due to uncertainty in the ¹⁵N enrichment of the substrate
404 pool. The fraction of labeled ammonium was much higher (99%) than the fraction
405 of labeled nitrite, which ranged from 16-70% depending on the station and depth.
406 The largest error associated with the isotope pairing calculations (Thamdrup and
407 Daalsgard 2002) derives from the labeled fraction of the tracer pool (F_N) when F_N is
408 small. This was often the case with nitrite in our ¹⁵NO₂⁻ tracer incubations, because
409 we attempted to simulate *in situ* environmental conditions by using relatively small
410 tracer additions. The labeled fraction, F_N, ranged from 0.11 to 0.99, and in equation
411 2, this factor occurs as the reciprocal squared and may vary by up to a factor of 100
412 if the size of the total nitrite pool is greatly reduced (i.e. when it is converted to N₂).

413 Indeed, at Station 1, the F_N values ranged from 0.35 to 0.40 over the course of 36
414 hours and the anammox rates in the $^{15}\text{NO}_2^-$ were 7.3-8.7 times greater than the rates
415 in the $^{15}\text{NH}_4^+$ incubations, while at Station 2 at 151m the F_N was 0.74-0.84 and the
416 rate in the $^{15}\text{NO}_2^-$ incubations was only 1.5 times greater than in the $^{15}\text{NH}_4^+$
417 incubations. Thus, the effect of F_N is weighted much more strongly in the $^{15}\text{NO}_2^-$ -
418 tracer incubations than in the $^{15}\text{NH}_4^+$ tracer incubations, and small errors in
419 calculating the F_N were magnified. For this reason, we chose to use the equation of
420 Nielsen (1992) for calculating denitrification when anammox was not detected. For
421 future studies, using the $^{15}\text{NH}_4^+$ tracer to measure anammox rates and the $^{46}\text{N}_2\text{O}$
422 tracer to measure denitrification rates are the most direct and least ambiguous
423 methods.

424 There were no clear trends between rates observed in incubations with
425 additional treatments. There was no consistent effect from the addition of
426 ammonium or carbon. The highest rates of denitrification in the $^{15}\text{NO}_2^-+^{14}\text{NH}_4^+$
427 treatment occurred at Station 2, but those rates were not significantly different from
428 the $^{15}\text{NO}_2^-+\text{C}$ treatment. The highest denitrification rates at Station 3 occurred in
429 the $^{15}\text{NO}_2^-+^{14}\text{NH}_4^+$ treatment, but again were not significantly different from the
430 rates in the $^{15}\text{NO}_2^-$ or $^{15}\text{NO}_2^-+\text{C}$ experiments. Additionally, the ^{15}N -glycine addition
431 did not result in measurable excess $^{15}\text{N}-\text{N}_2$, suggesting that anammox bacteria were
432 not oxidizing NH_4^+ from amino acids. Thus, we conclude that neither carbon nor
433 nitrogen was limiting denitrifying and/or anammox bacteria in these samples. The
434 lack of carbon limitation on denitrification agrees with previous incubation

435 experiments and persistently high primary production in the AS (Ward et al. 2008,
436 Ducklow et al. 2001)

437 Aspects of the experimental design were devised to prevent or test suspected
438 methodological artifacts of the exetainer method. The high denitrification rates
439 (three orders of magnitude higher than those reported here) in the Arabian Sea
440 reported by Ward et al. (2008) were based on NO_3^- disappearance at 48 hours and
441 later, when large changes in DIN were observed. Thus the incubations in this study
442 were designed to target the early stage of the incubation, before DIN changes were
443 observed. To the extent possible, linear production rates were calculated from 0 to
444 36 hours.

445 Sparging with helium is another potential artifact that could cause an
446 artificially anoxic environment, thus enhancing both denitrification and anammox.
447 Rates of N_2 production, however, from both denitrification and anammox, were
448 lowest (often below the limit of detection) in the $^{15}\text{NO}_2^-$ treatment that was not
449 sparged with helium. These low rates probably resulted from existing N_2 , greatly
450 reducing the sensitivity of the method. Therefore, this study did not robustly test
451 the effect of sparging on measured rates. Because previous reports using the
452 sparged exetainer method had reported anammox and very little denitrification in
453 other low-oxygen environments (Thamdrup et al. 2006, Hamersley et al. 2007,
454 Kuypers et al. 2005), the no-sparge treatment was intended to investigate whether
455 sparging favored anammox. This concern was mooted by the results that
456 denitrification was dominant, thus making it unlikely that previous results were due
457 to experimental perturbation. Additionally, based on the sensitive O_2

458 measurements made by Revsbech et al. (2009), artificially low oxygen
459 concentrations in the exetainers do not appear to be a cause for concern, as the *in*
460 *situ* concentrations are already extremely low (<10 nmol O₂). Overall, we conclude
461 that the He purge does not favor anammox or denitrification over the other,
462 although it does enhance detection sensitivity.

463 The average denitrification rate (calculated from the ¹⁵NO₂⁻ and the ⁴⁶N₂O
464 tracer incubations at all 11 depths) was 8.54±2.78 nmol N l⁻¹ d⁻¹. This rate is very
465 similar to a previous estimate in the Arabian Sea of 9.1±1 nmol N l⁻¹ d⁻¹ (Devol et al.
466 2006), an average derived from sixteen ¹⁵N incubation experiments throughout the
467 year. These rates are also very similar to those measured in the ETNP by Farias et
468 al. (2009), who measured an average rate of N₂O consumption by denitrification of
469 14.71±3.53 nmol l⁻¹ d⁻¹ off the coasts of Peru and Chile, or Richards and Broenkow
470 (1971) who measured a nitrate reduction rate of 11.74 nmol l⁻¹ d⁻¹ in the oxygen-
471 deficient waters of Darwin Bay in the Galapagos. Our reported average
472 denitrification rate when extrapolated over the average area of the OMZ 1.5x10¹² m²
473 (assuming a constant 600 m thickness of the OMZ, following Devol et al. 2006, with
474 the area averaged from Mantoura et al. (1993) and Naqvi (1987)), results in an
475 average annual N loss from denitrification of 39±13 Tg N yr⁻¹. Previously reported
476 estimates of 13–34 Tg yr⁻¹ (Bange et al., 2000; Howell et al., 1997; Mantoura et al.,
477 1993; Naqvi, 1987; Naqvi and Shailaja, 1993; Yakushev and Neretin, 1997) are
478 equivalent to 9.5-44 Tg yr⁻¹ when normalized to the same area (Devol et al. 2006).
479 Most of those estimates were calculated from nitrate deficits or ETS activity. The
480 only other tracer measurements of denitrification from the Arabian Sea are from

481 Devol et al. (2006), who estimated a total loss of 41 ± 18 Tg N yr⁻¹, which is not
482 significantly different from the values reported here. The rates presented here and
483 those of Devol et al. (2006) would raise the AS contribution to global pelagic fixed N
484 from close to 1/3 to almost 1/2, suggesting an even greater role for the AS in the
485 global budget (Naqvi 2008).

486 Overall, denitrification appears to be the major pathway for fixed nitrogen
487 loss in the Arabian Sea. This may be correlated to a more consistent carbon supply,
488 in contrast to the carbon-limited ETSP and ETNP (Ducklow et al. 2001, Ward et al.
489 2008). Anammox appears to provide a secondary pathway for fixed N loss, although
490 its distribution is patchy and not consistently correlated with depth or other likely
491 environmental factors. In an environment with sufficient carbon supply, perhaps
492 the heterotrophic denitrifying bacteria are favored over autotrophic anammox
493 bacteria. An interesting avenue for further study would be to measure
494 denitrification and anammox rates during other times of the year, especially right
495 before monsoon season. Perhaps at times when the carbon supply is lower,
496 anammox bacteria might play a more significant role. Likewise, measuring
497 denitrification and anammox rates in the eastern Pacific during bloom times might
498 reveal a more substantial role for denitrifiers when carbon is not limiting.

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505

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636 Table 1. Geochemical information for each sample depth. Oxygen concentrations
 637 are from the CTD, and nitrite measurements are from the T₀ samples.
 638

Station	Bottom Depth (m)	Sample Depth (m)	Oxygen ($\mu\text{mol l}^{-1}$)	Nitrite conc. ($\mu\text{mol l}^{-1}$)
1	3095	120	0.9	4.92
19N 66E		151	1.0	5.37
		202	1.3	4.35
		400	1.4	1.02
2	3943	126	43.1	0.15
15.15N 64E		151	0.82	3.87
		200	0.79	5.96
		400	1.57	0.87
3	4439	112	12.9	0.13
11N 68E		151	0.63	3.62
		225	0.66	0.53

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644 Table 2. Tracers and additional treatments for 48-hour exetainer incubations.

Tracer	Concentration	Additional treatment	Concentration
¹⁵ NO ₂ ⁻	3 uM		
¹⁵ NO ₂ ⁻	3 uM	¹⁴ NH ₄ ⁺	3 uM
¹⁵ NO ₂ ⁻	3 uM	Carbon addition: Acetate, glucose, casamino acids	5 μ M, 5 μ M, 750 μ g/L
¹⁵ NO ₂ ⁻	3 uM	No He sparge	
¹⁵ NH ₄ ⁺	3 uM		
⁴⁶ N ₂ O	3 uM		
¹⁵ N glycine	3 uM	¹⁴ NH ₄ ⁺	3 uM

651 Figure captions:

652

653 Figure 1. Map of the Arabian Sea. Stations are marked with stars, and the dashed
654 line marks the $0.5\mu\text{M}$ nitrite threshold. Adapted from Jayakumar (2009).

655

656 Figure 2. Depth Profiles of 3 Stations in the Arabian Sea. Closed squares represent

657

658 diamonds oxygen concentration (μM).

659

660 Figure 3. Changes in DIN concentration over a 48 hour time course with $3\mu\text{M}$ $^{15}\text{NO}_2^-$ -
661 additions at Station 1. (A) Nitrite concentration, (B) Total DIN concentration.

662

663 Figure 4. Production of excess $^{29}\text{N}_2$ (black diamonds) and $^{30}\text{N}_2$ (open squares) over
664 48 hours time courses at Station 1, 150m following additions of (A) $3\mu\text{M}$ $^{15}\text{NO}_2^-$, (B)
665 $3\mu\text{M}$ $^{15}\text{NH}_4^+$ and (C) $3\mu\text{M}$ $^{46}\text{N}_2\text{O}$. Exponential production at 48 hours is shown in the
666 inset graphs.

667

668 Figure 5. Denitrification rates for all depths at all three stations. Starred rates are
669 significantly different than zero ($p < 0.05$).

670

671 Figure 6. Production of excess $^{29}\text{N}_2$ (black diamonds) and $^{30}\text{N}_2$ (open squares) over
672 48 hours time courses at Station 2, 151m following additions of (A) $3\mu\text{M}$ $^{15}\text{NO}_2^-$, (B)
673 $3\mu\text{M}$ $^{15}\text{NH}_4^+$ and (C) $3\mu\text{M}$ $^{46}\text{N}_2\text{O}$.

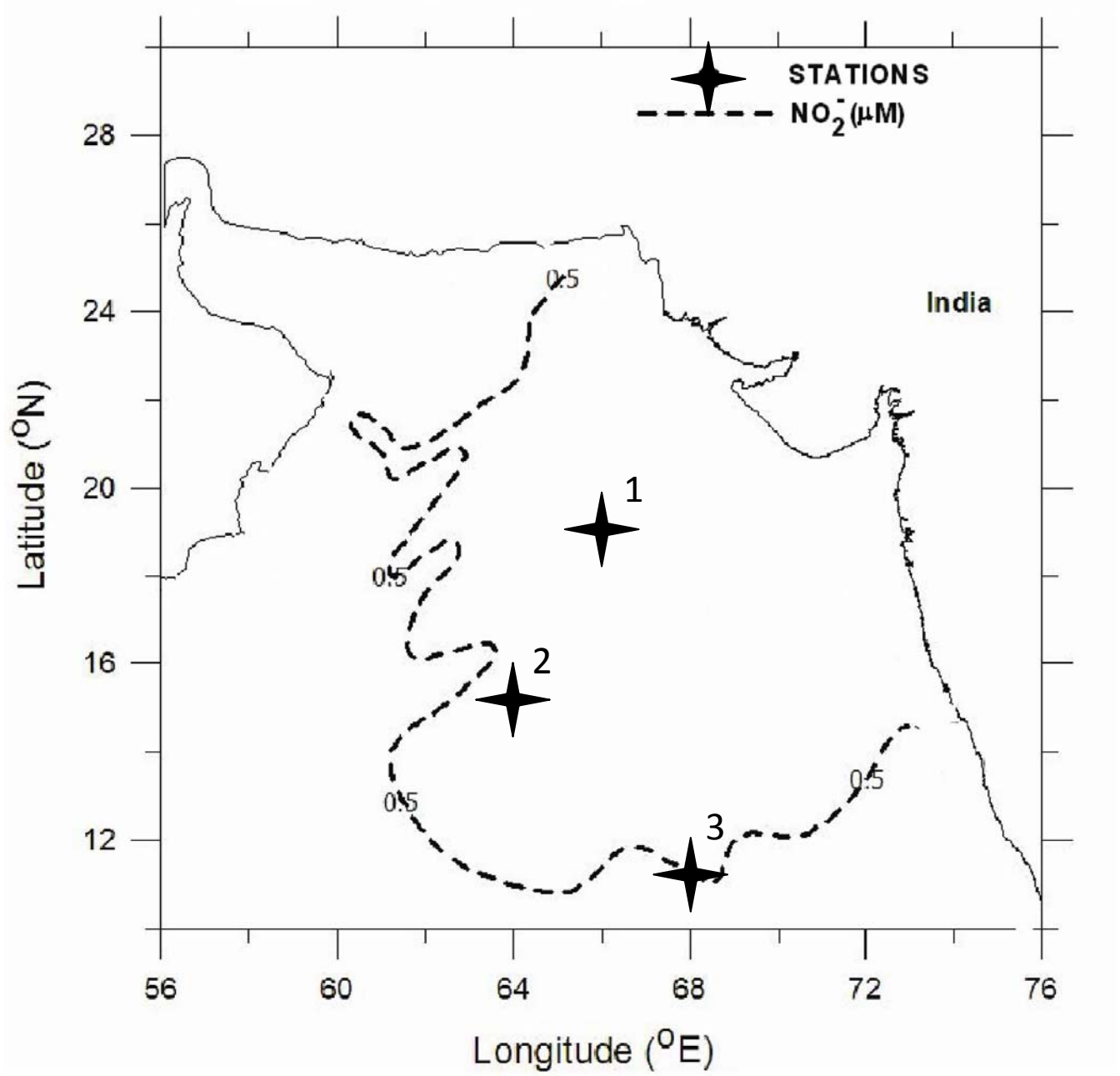
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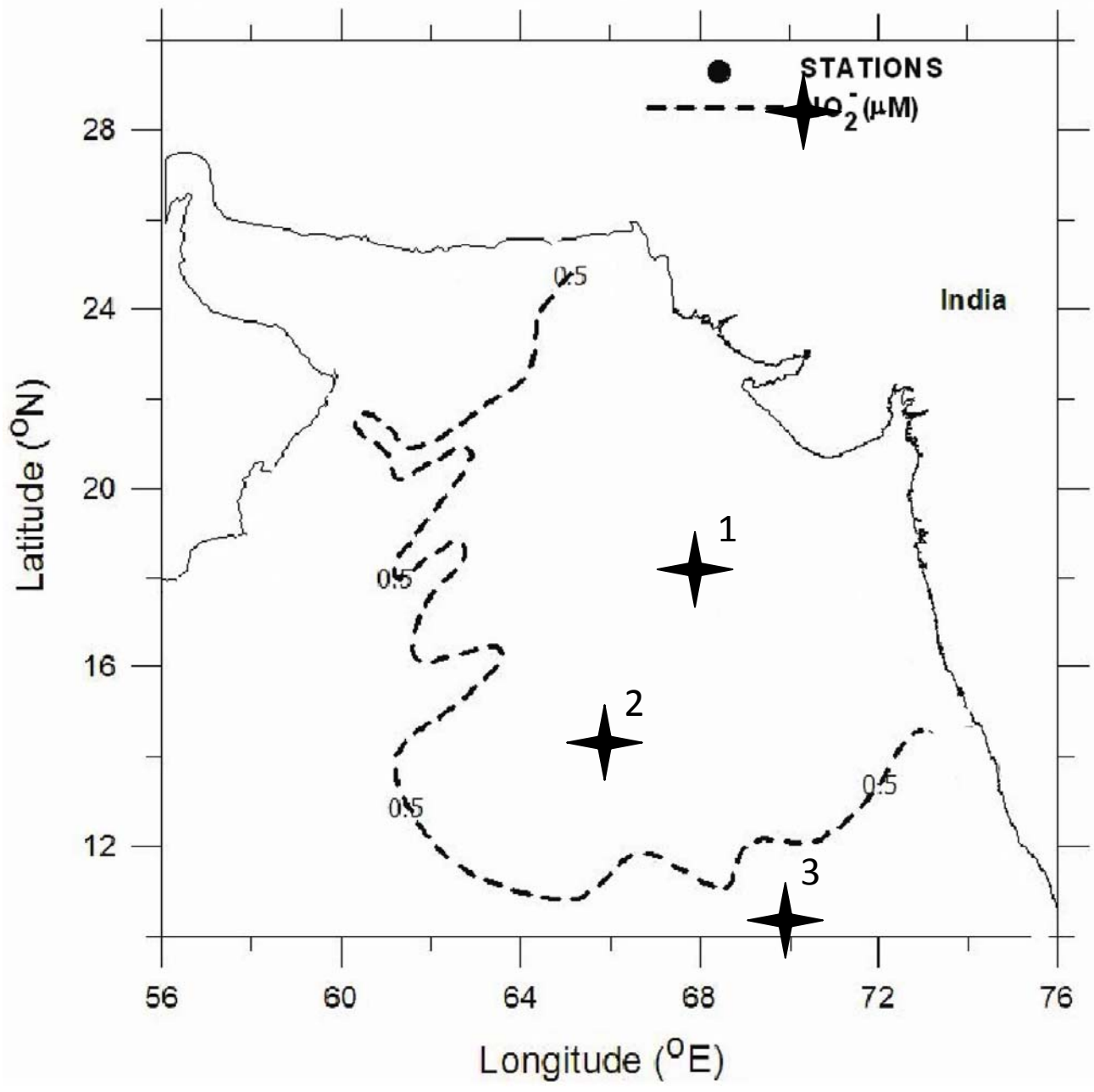
675 Figure 7. N_2 production rates from anammox for all treatments at all depths with
676 significant anammox activity. Starred rates are significantly different from zero
677 ($p < 0.05$).

678

679 Figure 8. Correlation between denitrification rate and ambient nitrite
680 concentration.

Figure 1





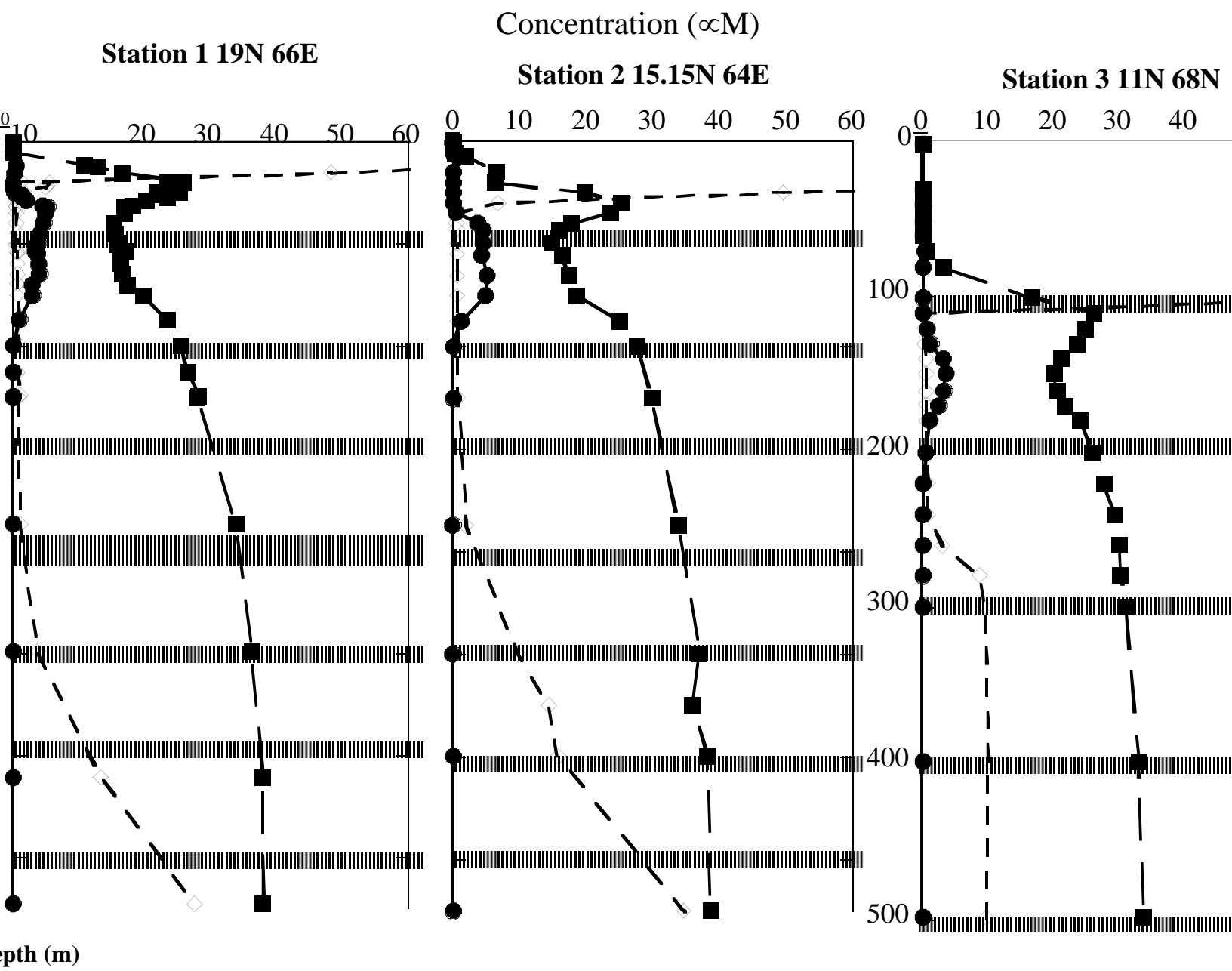


Figure 3

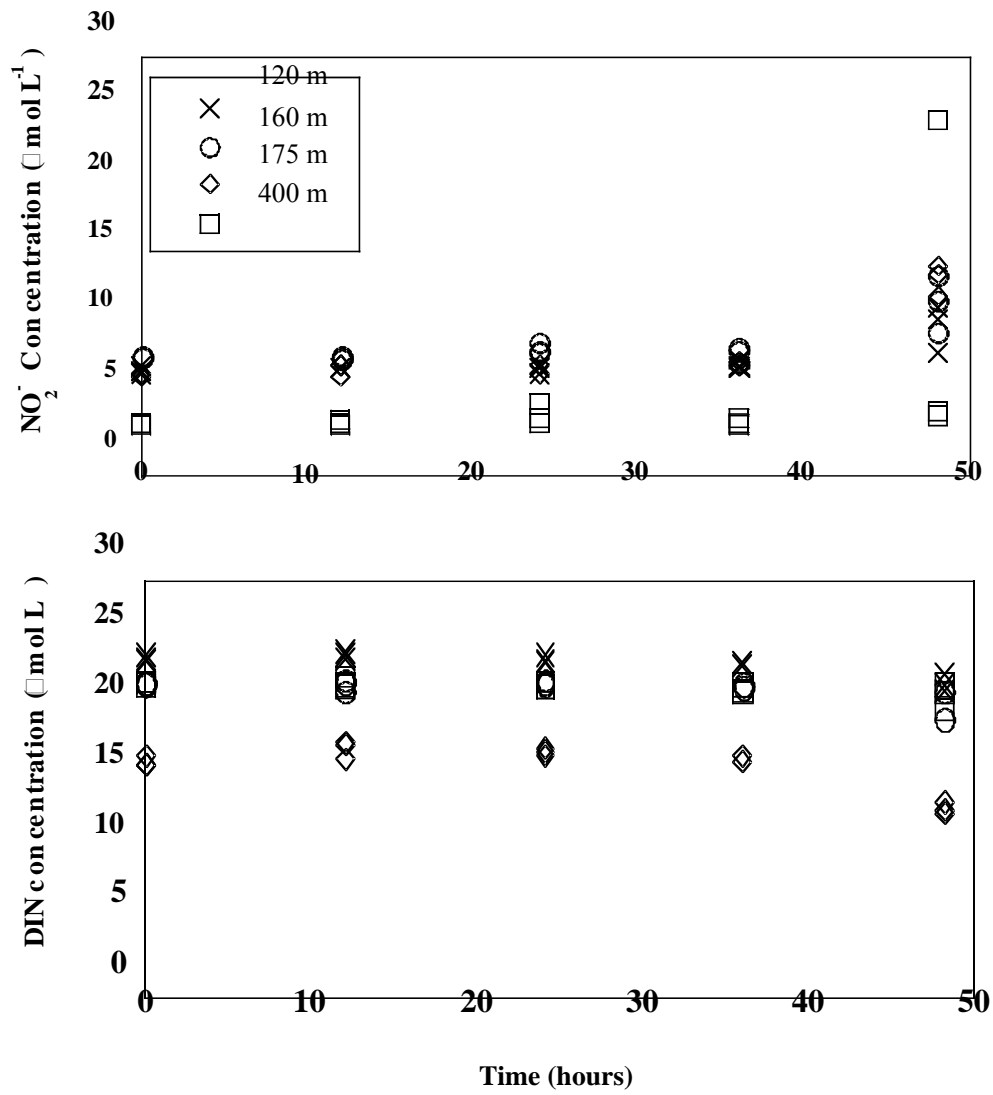


Figure 4

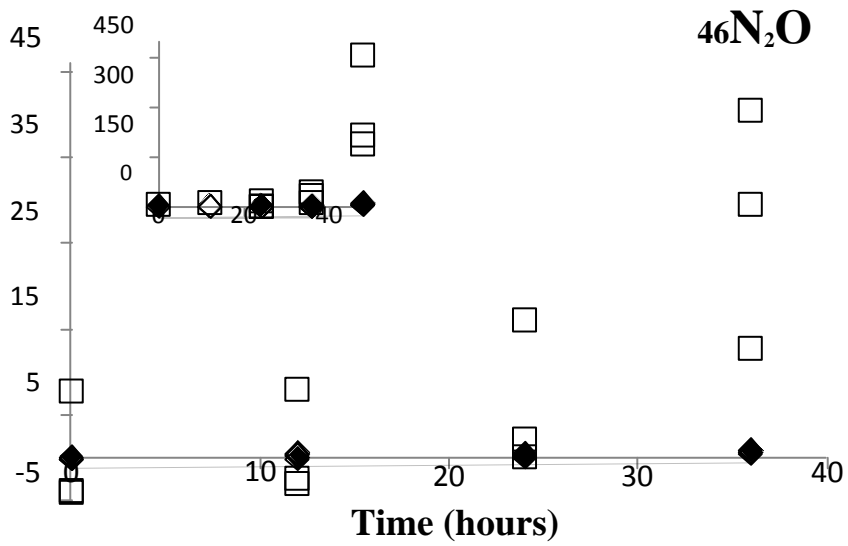
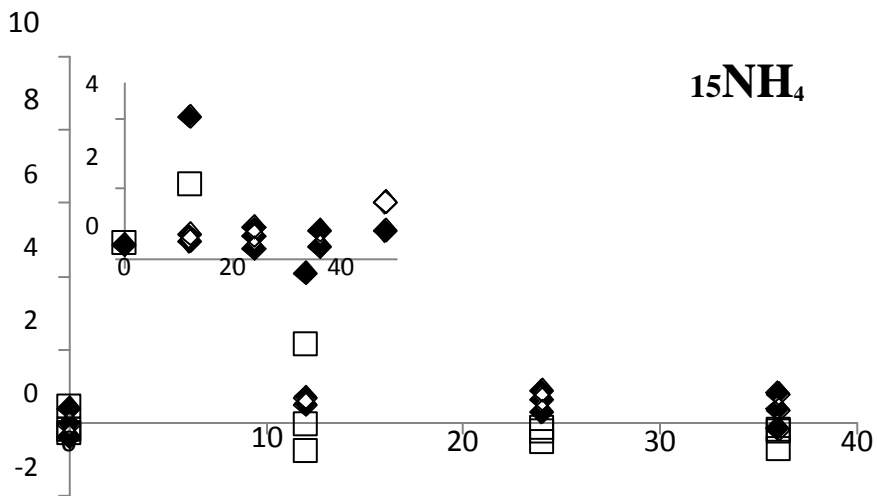
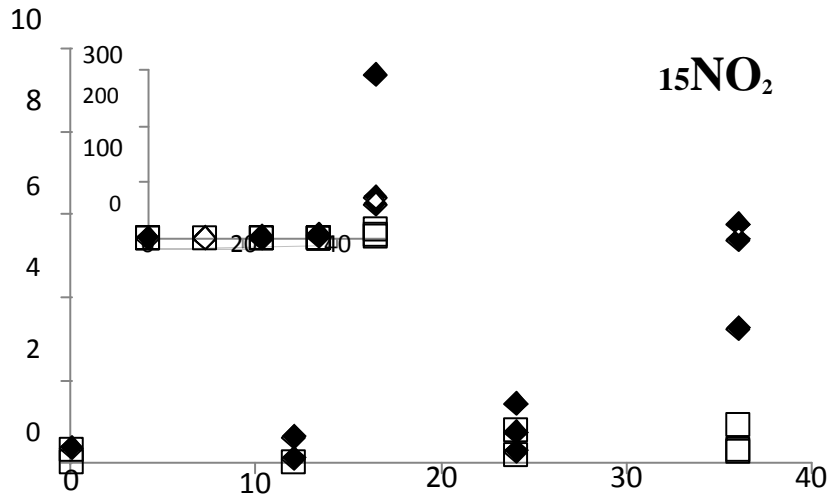
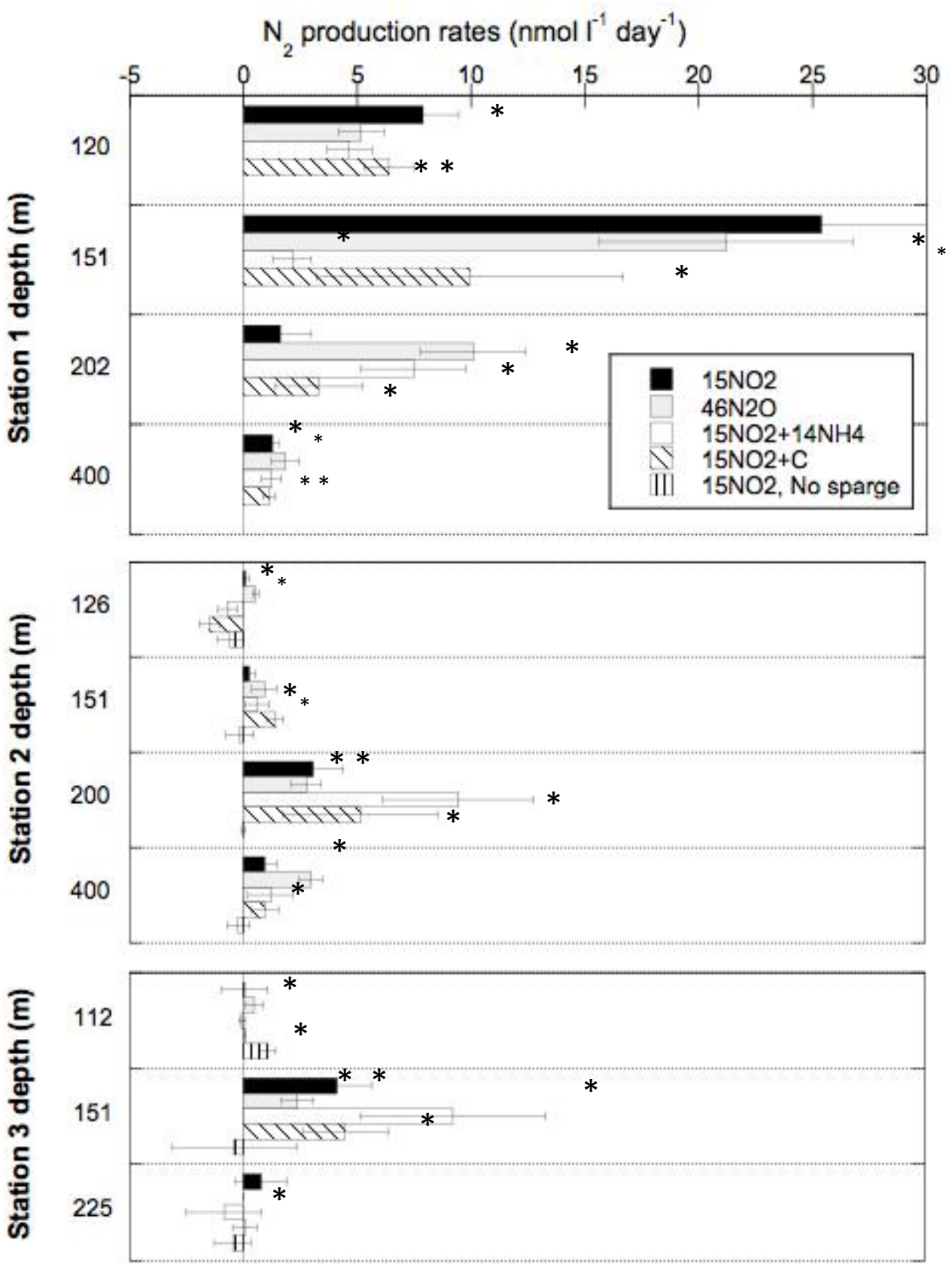
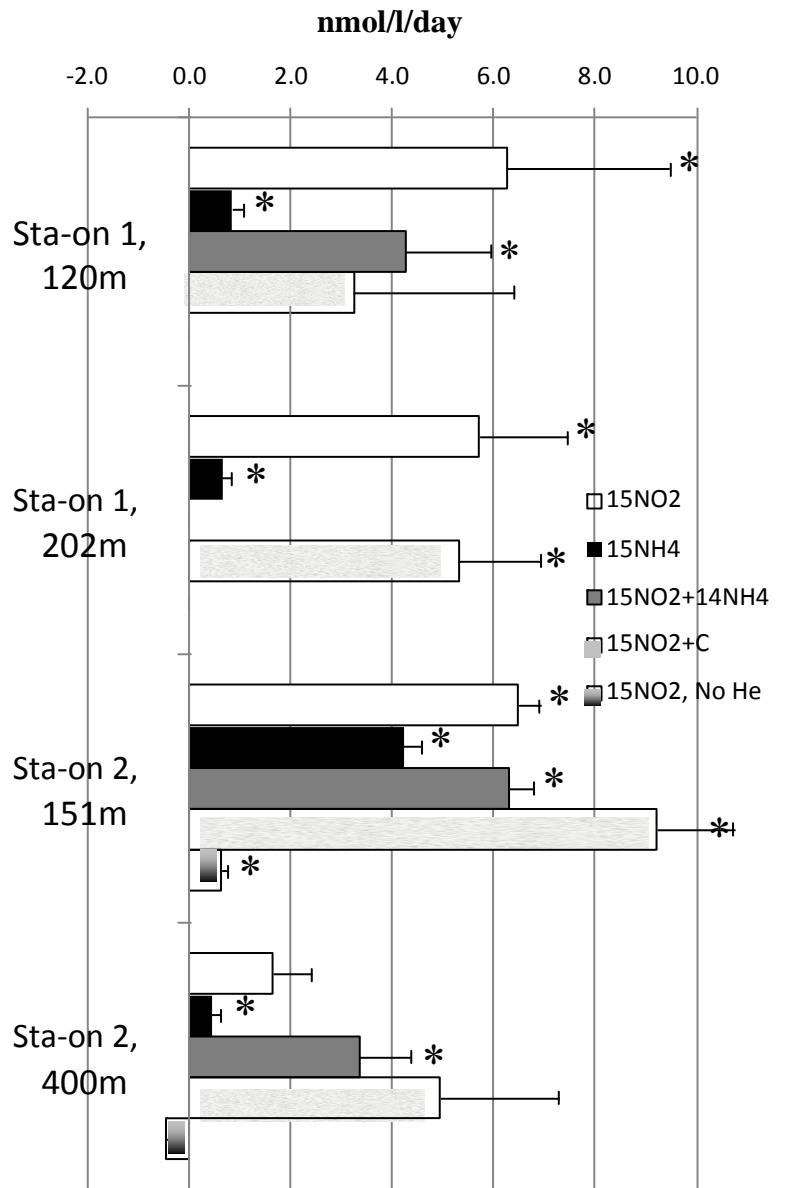


Figure 5



Anammox N₂ Production Rates



Nitrite conc vs. Denitrification Rate

