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The effect of organic carbon on fixed nitrogen loss in the eastern tropical South Pacific and Arabian Sea oxygen deficient zones

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Abstract

The three major oxygen deficient zones (ODZs) of the world oceans (Eastern tropical North and South Pacific (ETNP and ETSP, respectively), and Arabian Sea (AS) host the vast majority of pelagic fixed nitrogen (N) loss and up to half of total marine N loss. An important control on the absolute and relative importance of the two main pathways of N removal (denitrification and anammox) is thought to be the input of organic matter. In this study, we investigated the response of N loss in the ETSP and AS ODZs to addition of organic matter in the form of glucose and naturally-derived dissolved and particulate organic matter (DOM and POM, respectively). Fresh POM was collected using sediment traps deployed above the ODZ and trap leachate was used as the DOM treatment. In the ETSP ODZ, the addition of glucose stimulated denitrification (1.6 fold increase after 5 d) but not anammox (14 fold decrease after 5 d). In the AS ODZ, only POM, not DOM, significantly increased rates of denitrification at the base of the oxycline (5.4 – 6.4 fold increase after 2 d), but not at the secondary nitrite maximum. This may be due to the more refractory nature of the DOM vs. POM (C:N ratio = 9.3 vs. 7.8, respectively) and a higher carbon consumption rate at the shallower depth. These results suggest that denitrification was generally limited by organic matter supply at the time of this study in both the ETSP and AS ODZs, although the lability of the organic matter is important. Interestingly, $^{15}N_2$ produced in ETSP and AS incubations were not binomially distributed relative to the reactants after the influence of anammox was taken into account, suggesting an alternative unknown production mechanism or pathway of N removal.

Introduction

The vast majority of pelagic fixed nitrogen (N) removal occurs in three major oxygen deficient zones (ODZs) of the world: the eastern tropical North and South Pacific (ETNP and ETSP, respectively), and the Arabian Sea (AS). Although they comprise less than 1% of the total volume of the ocean (Codispoti et al. 2001), the ODZs are responsible for at least a quarter of total marine N loss (Codispoti et al. 2001). Wind-driven upwelling stimulates high productivity in the overlying waters of the ODZs, which sinks and fuels substantial respiration at depth. Combined with poor ventilation of these regions, the result is a depletion of water column oxygen to the extent that it becomes thermodynamically favorable for microbes to utilize NO_3^-, IO_3^- (Farrenkopf and Luther 2002), oxidized metals (Luther et al. 1997; Moffett et al. 2007), and SO_4^{2-} (Canfield et al. 2010) as electron acceptors during organic matter oxidation. Within the open ocean ODZs, the majority of organic matter respired is ultimately coupled to the reduction of NO_3^-. Although the SO_4^{2-} concentration is orders of magnitude higher than the NO_3^- concentration, there is no accumulation of H_2S, implying reduced S is efficiently reoxidized, likely via direct or indirect coupling to the reduction of NO_3^- (Canfield et al. 2010). Numerous transformations of fixed N can occur; however, once converted to N_2O or N_2, N becomes biologically inaccessible except to N_2 fixing microbes.

The two major pathways of fixed N loss in the ODZs are denitrification and the anaerobic oxidation of ammonium (anammox). Denitrification is a heterotrophic process that proceeds via a stepwise process in which organic carbon oxidation is coupled to the sequential reduction of N-oxides to gaseous end products: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O\ (g) \rightarrow N_2\ (g)$. Anammox is an autotrophic process, gaining energy from the oxidation of NH_4^+ to N_2 using NO_2^-. Due to the importance of the 3 major ODZs to the balance of fixed N in the ocean, there has been

substantial interest in determining the contribution of these two processes to total pelagic N removal, especially given the potential of these regions to change with changing climate.

^{15}N-labeling experiments have suggested that anammox is responsible for the majority of N_2 production in the ETSP (Thamdrup et al. 2006; Hamersley et al. 2007; Kalvelage et al. 2013) and AS (Jensen et al. 2011) ODZs. In contrast, other studies in the ETSP (Dalsgaard et al. 2012) and the AS ODZs (Nicholls et al. 2007; Ward et al. 2009) identified heterotrophic denitrification to be the dominant N loss pathway. There is evidence that both denitrification and anammox are regulated by the availability of organic matter in the ODZs (Ward et al. 2008; Kalvelage et al. 2013), and this discrepancy in the relative contributions of denitrification and anammox to N removal in the ETSP and AS ODZs has been ascribed to the differing responses of anammox and denitrifying bacteria to organic matter availability (Thamdrup et al. 2006; Ward et al. 2009; Dalsgaard et al. 2012).

Seasonally changing wind patterns in all three ODZs give rise to temporally varying productivity, which, in turn, affect the timing and quantity of organic matter reaching the ODZs (Lee et al. 1998). If the flux of organic matter to the ODZs is linked to surface productivity, the degree to which carbon is available to microbes in either the ETSP or AS ODZ would also be both spatially and temporally heterogeneous. Denitrifiers have been found to be abundant and diverse, capable of rapid growth in response to episodic inputs of organic matter (Ward et al. 2008). Anammox bacteria grow more slowly (Van de Graaf et al. 1995) and may maintain a lower though more constant rate of N removal in the ODZs. Although anammox is itself an autotrophic process, the substrates anammox depends upon (i.e., NH_4^+ and NO_2^-) are produced by the oxidation of organic matter. NH_4^+ is generated at each step of the successive reduction of N-oxides during heterotrophic denitrification. Additionally, dissimilatory nitrate reduction to

ammonium (DNRA, Lam et al. 2009; Jensen et al. 2011) and sulfate reduction (Canfield et al. 2010), both heterotrophic processes, may supply anammox with NH_4^+. NO_2^- is formed via the reduction of NO_3^- during the oxidation of organic matter. NO_2^- might also be produced by NH_4^+ oxidation (Kalvelage et al. 2013), however this pathway depends on the availability of NH_4^+ and thus, organic matter oxidation.

The objectives of this work were to investigate the effect of organic matter quality and availability on the rates of N loss in the ETSP and AS ODZs. In the ETSP ODZ, we measured the rates and relative contributions of denitrification and anammox in incubations with and without the addition of a simple organic compound (glucose). In the AS ODZ, we used freshly collected dissolved organic matter (DOM) and sinking particulate organic matter (POM) from sediments traps deployed directly above the ODZ in order to assess the response of N_2 production to naturally derived organic matter. We chose these forms of organic matter because although bacteria most readily take up DOM, a significant source of DOM in the interior of the ocean is the degradation of POM. In addition to acting as a source of DOM, bacteria directly colonize POM and take advantage of living adjacent to both substrate and other bacteria performing complementary redox transformations (Karl et al. 1984)

Methods

Study site and sample collection

Incubation experiments were carried out at one station in the ETSP ODZ aboard the R/V *Knorr* (October – November 2005) and at two stations in the AS ODZ aboard the R/V *Roger Revelle* (September – October 2007) (Table 1). Samples were collected using a rosette of 10 or

30 L Niskin bottles equipped with dissolved oxygen (O_2) sensors calibrated by Winkler titrations, in addition to conductivity, temperature, and pressure sensors.

Sampling depths were chosen based on O_2 and nitrite (NO_2^-) concentrations: at the secondary NO_2^- maximum (SNM), and at the shallowest depth where O_2 was undetectable (base of the oxycline; in AS only). The base of the oxycline was chosen based on previous studies, which have reported the highest rates of N_2 production at the top of the ODZ, possibly due to the relatively high flux of POM compared to deeper in the ODZ. The SNM was also sampled as it is associated with the most oxygen depleted waters (Thamdrup et al. 2012) and has classically been regarded as a zone of active N loss (Codispoti and Christensen 1985).

Incubations were carried out in duplicate in acid-washed, large-volume, gas-tight trilaminate bags (Pollution Management Corporation) fitted with three-way stopcocks. Prior to sampling, bags were flushed at least three times with CO_2 to eliminate O_2 from any possible headspace and evacuated. Approximately 8 L of water were gravity-fed into each bag directly from a Niskin bottle, taking care to prevent any contact with the atmosphere. The headspace of each Niskin bottle was continuously flushed with CO_2 through the vent to prevent atmospheric O_2 contamination during sampling. Bags received additions of ^{15}N-labeled NO_3^- (in ETSP only), NO_2^- (in AS only) or NH_4^+ (in both, Table 2).

In the ETSP, dissolved organic matter (DOM) in the form of glucose was also added to $^{15}NO_3^-$ and $^{15}NH_4^+$ labeled incubations to a final concentration of 2 μmol C L^{-1}. In the AS experiments, $^{15}NO_2^-$ labeled incubations were amended with POM and DOM collected in situ from the AS ODZ (*see* below for details of collection and preparation) with and without $^{14}NH_4^+$. Tracers and amendments were added while filling each bag either by injecting directly into the Tygon tubing connecting the Niskin to the bag or through the three-way valve. POM was added

144 to the bags to a final concentration of 2.6 μmol C L^{-1} and 0.33 μmol N L^{-1} at Sta. 1 and 1.3 μmol
145 C L^{-1} and 0.17 μmol N L^{-1} at Sta. 2, determined by CHN elemental analyzer. At both Sta. 1 and
146 2, DOM was added to the bags to a final concentration of 0.24 μmol C L^{-1} and 0.026 μmol N L^{-1},
147 respectively, determined by total organic carbon and nitrogen analyzer (TOC-V/Total Nitrogen
148 M-1 Shimadzu).
149 For the AS experiments, POM was collected at a depth in the oxycline, approximately 20 and
150 50 m above the ODZ proper at Sta. 1 and 2, respectively, using a NetTrap (Petersen et al. 2005),
151 a large diameter (~2 m), free-floating sediment trap, based on the design of a closing plankton
152 net, capable of collecting large amounts of sinking POM (> 50 μm) in relatively short time
153 periods (24 – 36 h). Contents of the cod end were filtered through a glass fiber filter (0.7 μm
154 nominal pore size). Filtrate (trap leachate) was collected for use as the DOM amendment. The
155 material on the filter was resuspended into a smaller quantity of site water for use as the POM
156 amendment. Each organic matter addition was degassed before introduction to the bag by
157 applying a vacuum to cause continuous boiling for ~15 min.
158 Ambient and tracer concentrations of NO_3^-, NO_2^-, and NH_4^+ in all incubations were measured
159 by autoanalyzer using standard colorimetric techniques (Strickland and Parsons 1972).
160 Additionally, in the ETSP incubations, NO_3^- and NO_2^- were measured by autoanalyzer at
161 approximately 12 h intervals for the length of the incubation. Detection limits of NO_3^-, NO_2^-,
162 and NH_4^+ were 0.08, 0.01, and 0.07 μmol L^{-1}, respectively.
163
164 *^{15}N-labeled incubations*
165 Samples for the determination of $^{29}N_2$ and $^{30}N_2$ were taken following the method of Emerson
166 et al. (1999). Approximately 150 mL of water was drawn from the Niskin bottles (representing

167 initial N_2 gas and isotope concentrations) or from a bag into 300 mL $HgCl_2$-poisoned, pre-
168 evacuated glass flasks equipped with a 9 mm, gas-tight, single o-ring valves (Louwers-Hapert).
169 The flasks were returned to the University of Washington where they were weighed and
170 dissolved gases were equilibrated with the headspace of the flask at a constant temperature for 24
171 hours. The headspace gases were transferred to a stainless steel finger immersed in liquid He,
172 during which water and CO_2 were trapped cryogenically. Samples were analyzed on a Finnigan
173 Delta XL dual inlet isotope ratio mass spectrometer for mass ratios 29:28, 30:28, 28:40, relative
174 to an in-house gas standard with known gas and isotope ratios. Anammox and denitrification
175 rates were calculated from the production of ^{15}N-labeled N_2 following de Brabandere et al.
176 (2013).
177
178 *Statistical analyses*
179 The effects of treatments on the rates of N_2 production in the AS incubations were examined
180 using an analysis of variance (ANOVA). The factors affecting N_2 production that were
181 considered were location (Sta. 1 or 2), depth (base of oxycline or SNM), $^{14}NH_4^+$ (with or
182 without), and organic matter (control, POM, and DOM). A post hoc comparison using Tukey's
183 HSD test was used to evaluate differences in organic matter treatments. All statistical analyses
184 were performed using SPSS version 13.
185
186 ***Results***
187 *Hydrographic conditions*
188 At the ETSP study site, the ODZ (defined as the minimum value reached by the Seabird
189 oxygen sensor mounted to the sampling rosette) extended from approximately 75 to 400 m. The

mixed layer was shallow (< 20 m, defined as a change in σ_θ > 0.03 kg m^{-3} [de Boyer Montégut et al. 2004]), sea surface temperature (SST) was relatively low (15.3˚C), and [NO_3^-] and [PO_4^{3-}] were relatively high in surface waters (5.9 and 0.8 μmol L^{-1}, respectively), all of which are indicative of active upwelling. [NO_2^-] was detectable at the surface (0.2 μmol L^{-1}) and formed a broad secondary maximum within the ODZ (maximum 4.4 μmol L^{-1}). A distinct primary NO_2^- maximum (PNM) was not detected, probably due to the sampling distribution being too coarse to resolve the narrow peak. [NH_4^+] was as high as 0.3 μmol L^{-1} in the mixed layer, decreasing to below detection (< 0.05 μmol L^{-1}) in and below the ODZ.

The AS field campaign took place at the end of the high productivity southwest monsoon, although conditions were already transitioning to the more oligotrophic fall intermonsoon as indicated by relatively high SST (28.8˚C) and a shallow mixed layer (40 m) in which [NO_3^-] and [PO_4^{3-}] were undetectable. Hydrographic conditions were previously reported by Ward et al. (2009), which we will summarize here. A distinct PNM was present at the base of the mixed layer (maximum 0.9 and 2.8 μmol L^{-1}, at Sta. 1 and 2, respectively), underlain by a much larger SNM (maximum 5.7 and 7.7 μmol L^{-1}, at Sta. 1 and 2, respectively), which extended from approximately 100-150 to 400 m. At the more northern station sampled (Sta. 1), O_2 was undetectable from approximately 100 to 800 m. At the more southern station sampled (Sta. 2), the ODZ was slightly thinner, extending from 150 to 800 m. [NH_4^+] was as high as 0.6 μmol L^{-1} in the mixed layer, decreasing to below detection (< 0.05 μmol L^{-1}) in and below the ODZ.

N transformations and loss in ETSP ODZ incubations

At Sta. 20 in the ETSP, in the incubations with glucose, the initial concentration of 24 μmol L^{-1} NO_3^- was rapidly drawn down to < 1 μmol L^{-1} in less than 3 days, and undetectable by 5.5

213 days (Fig. 1a). There was some NO_2^- accumulation until 1.5 days, after which NO_2^- was quickly
214 consumed to undetectable levels by 4.5 days (Fig. 1b). This pattern of nutrient consumption was
215 in contrast to the incubation in which no organic carbon was added. In these incubations, NO_3^-
216 remained unchanged or even slightly increased until approximately 3 days, after which NO_3^-
217 steadily decreased to undetectable levels by 11.5 days. Nitrite decreased ~1 μmol L^{-1} for one day
218 before slowly increasing to maximum values by 9.5 – 10 days followed by rapid consumption to
219 undetectable levels by 11.5 days.
220 Samples for $^{15}N_2$ production were taken at 5.7 days (Fig. 2). In the control incubations (only
221 ^{15}N tracers added), the average denitrification rate over the length of the incubation was 2.6 nmol
222 N_2 h^{-1}, and the average anammox rate was 0.9 nmol N_2 h^{-1} over the course of the incubation. In
223 the incubations amended with glucose, the average denitrification rate was 4.2 nmol N_2 h^{-1}
224 during the length of the incubation and the average anammox rate was 0.06 nmol N_2 h^{-1} over the
225 course of the incubation.
226
227 *Sediment trap organic matter composition in the AS*
228 Arabian Sea DOM (trap leachates) had an atomic C:N ratio of 9.3. This ratio is higher than
229 that of the particulate trap flux, which had an average C:N ratio of 7.8.
230
231 *N loss in AS ODZ incubations*
232 The addition of $^{14}NH_4^+$ had no significant effect on $^{29}N_2$ or $^{30}N_2$ production in the AS
233 regardless of station, depth, or organic matter addition ($F_{1,21} = 0.486$, $p > 0.05$, data not shown).
234 Since the addition of $^{14}NH_4^+$ had no significant effect on $^{15}N_2$ production rates, all rates with and

235 without organic matter additions are presented as an average of the treatments with and without
236 $^{14}NH_4^+$.

237 In the AS incubations, samples for $^{15}N_2$ production were taken at approximately 2 days. $^{15}N_2$
238 production rates by anammox (in $^{15}NH_4^+$ amended incubations) and denitrification (in $^{15}NO_2^-$
239 amended incubations with no organic matter additions) were previously published in Ward et al.
240 (2009). We present them here as the 'control' to assess the effect of organic matter additions on
241 N_2 production rates. At Sta. 1, in the control incubations (no organic matter additions), the
242 average denitrification rates over the length of the incubations were 0.21 ± 0.14 and 0.15 ± 0.06
243 nmol N_2 h^{-1}, at the base of the oxycline and SNM, respectively (Fig. 3a). At Sta. 2, in the control
244 incubations, the average denitrification rates over the course of the incubations were 0.06 ± 0.02
245 and 0.05 ± 0.02 nmol N_2 h^{-1}, at the base of the oxycline and SNM, respectively (Fig. 3b).

246 The two organic carbon treatments, DOM and POM collected freshly from sediment traps,
247 had different effects on denitrification rates (determined from $^{30}N_2$ production) in the AS ODZ
248 (Fig. 3a,b). Tukey's post hoc test revealed that denitrification rates with and without DOM were
249 not significantly different ($p > 0.05$). The addition of POM from sediment traps significantly
250 increased denitrification rates at both stations relative to the control ($p < 0.05$) at the base of the
251 oxycline only ($F_{1,10} = 10.255$, $p < 0.01$), however the response of denitrification to the addition
252 of POM was not significantly different between the two stations ($F_{1,10} = 0.565$, $p > 0.05$).
253 Average denitrification rates in the POM treatment at the base of the oxycline was 6.4 times
254 larger than the control at Sta. 1 and 5.4 times larger at Sta. 2.

255 Parallel incubations with $^{15}NH_4^+$ only without any additions of organic matter were carried
256 out at each station and depth in the AS (Fig. 3a,b), which allowed the determination of the
257 control anammox rate. At Sta. 1, the average anammox rates during incubations were 0.005 ±

258 0.0004 and 0.007 ± 0.0009 nmol N_2 h^{-1}, at the base of the oxycline and SNM, respectively. At
259 Sta. 2, the average anammox rates during incubations were 0.009 ± 0.008 and 0.06 ± 0.05 nmol
260 N_2 h^{-1}, at the base of the oxycline and SNM, respectively.
261
262 ***Discussion***
263 *Effect of dissolved organic carbon (glucose) on N loss in the ETSP*
264 Concentrations of DOC in the Pacific Ocean range from as high as 70 μmol L^{-1} in the low
265 latitude surface ocean to < 40 μmol L^{-1} in the deep ocean (Hansell and Carlson 1998). The
266 amount of glucose added to incubations in this study (2 μmol C L^{-1}) was only a small fraction of
267 the DOC naturally present. However, this ambient DOC is thought to be very old (4000 – 6000
268 y B. P.), estimated from radiocarbon measurements (Druffel et al. 1992), indicating that the
269 majority of this DOC is refractory and is returned to the deep unrespired after a complete ocean
270 mixing cycle. Glucose ($C_6H_{12}O_6$) is a simple carbohydrate that is a readily utilizable energy
271 source by microorganisms that yields ATP via glycolysis, thus, even a relatively small amount of
272 glucose may fuel relatively high rates of bacterial respiration.
273 At Sta. 20 in the ETSP, both the control and glucose-amended incubations exhibited NO_3^-
274 consumption, and NO_2^- accumulation and consumption in a classic denitrifying sequence, as
275 observed in bacterial cultures, which is controlled by the enzyme kinetics of each step of
276 denitrification (Betlach and Tiedje 1981). The addition of glucose to these incubations
277 stimulated NO_3^- and NO_2^- reduction by supplying substrate to a carbon-limited system (Fig.
278 1a,b). The average denitrification rate measured by the production of $^{15}N_2$ was 1.6 times higher
279 in the incubations with the addition of glucose compared to without (Fig. 2). Conversely, the
280 average anammox rate was an order of magnitude lower in the glucose treatments compared to

the control. Although glucose does not contain organic N, which would be remineralized to NH_4^+ potentially for anammox, it is unlikely that anammox bacteria in these incubations were NH_4^+-limited due to the addition of 1 μmol L^{-1} $^{15}NH_4^+$ tracer.

These results support the findings of Dalsgaard et al. (2012) in the ETSP ODZ, who hypothesize a lack of close coupling between denitrification and anammox based on the observation that denitrification rates were high when anammox rates were low and vice versa. Dalsgaard et al. (2012) argue that the lack of close coupling may be due to the relative response times of the different microorganisms involved: denitrifiers are fast-growing and can respond quickly to episodic inputs of organic matter, whereas anammox bacteria are relatively slow-growing which limits the rate of their response to increased substrate; however, they subsequently maintain lower rates for longer periods of time.

Effect of NH_4^+ on denitrification in the AS

Previous researchers have also found no significant increase in the anammox rate (determined from $^{29}N_2$ production) in $^{15}NO_{2,3}^-$ labeled incubations with $^{14}NH_4^+$ compared to without (Kuypers et al. 2005; Thamdrup et al. 2006). This is somewhat unexpected since [NH_4^+] in both the Benguela upwelling and ETSP ODZs is frequently below detection suggesting anammox could be limited by [NH_4^+]. Thamdrup et al. (2006) speculated that no stimulation of anammox by the addition of NH_4^+ may reflect extremely efficient ammonium uptake by anammox bacteria, which would lead to reaching saturating concentrations at a relatively low [NH_4^+].

Effect of dissolved organic matter on denitrification in the AS

303 The absence of a significant response from the addition of DOM indicated that denitrifiers in
304 the AS ODZ were either restricted in their ability to respire the DOM added due to its possibly
305 refractory nature (Druffel et al. 1992), or did not receive sufficient additional DOM to cause a
306 response. Due to the significant increase in denitrification rates by the addition of POM (*see*
307 discussion in following section), it is unlikely that denitrifiers had no response to the addition of
308 DOM because they were not carbon-limited.
309 The C:N ratio of the DOM was high (9.3) compared to both the Redfield ratio (6.6) and the
310 POM collected concurrently in the sediment trap (7.8), suggesting the DOM was more
311 chemically degraded. Labile N-rich proteins and amino acids are preferentially remineralized
312 leaving behind the more refractory fraction of DOM (Walker and McCarthy 2012). Another
313 possibility is that not enough DOM was added to cause a response. Approximately an order of
314 magnitude less organic C and N were added to DOM amended incubations compared to POM
315 treatments. Although the aim was to add a comparable amount of organic matter to all POM and
316 DOM treatments, the exact composition and concentration of both the POM and DOM were
317 determined only upon return to a land-based laboratory and as such, was unknown at the time the
318 incubations were being carried out.
319
320 *Effect of particulate organic matter on denitrification in the AS*
321 Stimulation by the addition of only the particulate form of organic matter suggests that we
322 may have essentially inoculated the bag incubations with denitrifying microbes associated with
323 the POM, or provided ample labile organic carbon for respiration, or both. Previous work
324 analyzing the chemical composition of sinking POM captured in sediment traps has concluded
325 that sinking POM is composed of more labile organic matter relative to suspended POM or

DOM. A significant increase in denitrification rates was observed at the base of the oxycline only, and not at the SNM, at both stations suggesting that it was the POM itself, not particle associated denitrifiers that gave rise to increased denitrification rates. If the stimulation were due to new bacteria introduced with the POM, a comparable absolute increase in rates at both depths at each station would be expected. Additionally, although double the amount of POM was added to incubations at Sta. 1 relative to Sta. 2, the proportional increase in the average denitrification rates relative to the control at the base of the oxycline of both stations was similar (6.4 vs. 5.4 fold increase at Sta. 1 and 2, respectively) suggesting the original bacterial population in each incubation had comparable responses to the addition of POM. However, the absolute increase in denitrification rates at Sta. 1 was triple that of Sta. 2 (1.1 vs. 0.3 nmol L^{-1} h^{-1}, respectively), although double the POM was added at Sta. 1 relative to Sta. 2, further suggesting that the increased denitrification rates were due to a stimulation of the original bacterial population in the bags, not due to bacteria introduced with the POM.

The flux of organic matter is correlated to the rate of fixed N removal in the ETSP and AS ODZs (Jensen et al. 2011; Kalvelage et al. 2013) suggesting organic matter is an important control on this process. Further supporting this conclusion is the observation that rates of N_2 production generally decrease with increasing depth in both these regions (Thamdrup et al. 2006; Jensen et al. 2011; Dalsgaard et al. 2012), following the trend of decreasing organic matter flux with increasing depth (Martin et al. 1987). Additionally, carbon limitation of denitrification in the ETSP has been directly measured in incubations (Ward et al. 2008). In the present study, we hypothesize that the significant stimulation of denitrification at the base of the oxycline only, not the SNM, by the addition of POM is due to increased respiration leading to elevated organic carbon demand at the shallower depth.

The magnitude of the POM additions (2.6 and 1.3 μmol C L^{-1} at both depths at Sta. 1 and 2, respectively) can be evaluated in the context of ambient POC fluxes and remineralization rates in the AS. The Martin equation (Martin et al. 1987):

$$\text{POC flux at depth } z = \text{POC flux at } 100\text{m} \times (z/100)^{-b} \quad (1)$$

was used to estimate the POC flux to the depths sampled in this study. Temporal patterns of productivity and POC export in the AS are dominated by seasonal monsoonal cycles with the highest productivity and export during the Northeast and Southwest monsoons (Lee et al. 1998). A ^{234}Th-based average export from a comparable depth, location, and season as this study (100 m at an open ocean AS ODZ station during the SW monsoon) is 10.85 mmol C m^{-2} d^{-1} (Lee et al. 1998). An estimate of the attenuation coefficient (b) in Eq. 1 at the same open ocean AS ODZ station is 0.74 (Berelson 2001). In order to specify a depth interval over which POC is consumed, the 8 L bag incubation is assumed to be a cube with a height of 0.2 m. Using these values, the average in situ rates of POC consumption in these incubations would be 0.08, 0.04, and 0.02 μmol C L^{-1} d^{-1} at 100, 150, and 200 m. These calculated POC consumption rates are comparable to measured bacterial carbon demand associated with different forms of POC (Smith et al. 1992). Although the POC additions in this study exceeded the estimated POC consumption rate at all depths, it may be that the base of the oxycline harbors a more active and/or denser bacterial population relative to the deeper SNM due to a chronically larger POC flux and thus, were able to produce a larger response to the addition of fresh POC.

Amammox and the source of excess $^{29}N_2$ production in ETSP and AS incubations

During the 2005 R/V *Knorr* cruise to the ETSP, additional $^{15}NO_3^-$ amended bag incubations were carried out at other stations using the same method described here (data not shown). After

372 2 days, $^{29}N_2$ and $^{30}N_2$ produced by denitrification were binomially distributed after taking the
373 production due to anammox into account, such that the total $^{29}N_2$ produced was equal to the sum
374 of $^{29}N_2$ from anammox (determined from a parallel incubation with $^{15}NH_4^+$) and $^{29}N_2$ from
375 denitrification (predicted from $^{30}N_2$ assuming a binomial distribution of N_2 relative to the initial
376 fraction labeled of $^{15}NO_3^-$).

377 However, this was not true after 5.7 days in the ETSP incubations (Fig. 2) and in all of the AS
378 incubations after 2 days (Fig. 4a,b). In these incubations more $^{29}N_2$ was produced than could be
379 accounted for based on the fraction of the initial $NO_{2,3}^-$ that was labeled, after taking into account
380 the production due to anammox. We name this excess the residual $^{29}N_2$ (Δ_{resid}). It is defined as
381 the difference between the total measured $^{29}N_2$ production rate and the $^{29}N_2$ production rate
382 predicted from the sum of anammox and denitrification rates, assuming that the isotopic
383 composition of the products is binomially distributed relative to the reactants.

384 Nicholls et al. (2007) in the AS found the isotopic composition of $^{15}N_2O$ produced by
385 denitrification was binomially distributed relative to the starting pool of NO_2^-. However, similar
386 to the results in this study, $^{15}N_2$ production could not be predicted by the binomial distribution.
387 Trimmer and Purdy (2012) measured N_2 production that was not via canonical denitrification or
388 anammox, and hypothesized amine groups on allylthiourea (ATU) were directly oxidized to N_2
389 by NO_2^-. In incubations with $^{15}NO_2^-$ in the ETSP, de Brabandere et al. (2013) observed $^{15}N_2$
390 production that was non-binomially distributed after production by anammox had been taken into
391 account. These researchers speculated that production of $^{29}N_2$ via denitrification in the $^{15}NO_2^-$
392 incubations might be underestimated if denitrifiers reduced ambient NO_3^- directly to N_2
393 intracellularly, without allowing the NO_2^- to mix completely with the ambient NO_2^- pool ('nitrite
394 shunting').

Production of $^{29}N_2$ in excess of the predicted binomial distribution indicates that the ^{15}N-labeled fraction of the reactant pool is not well known. To further explore possible mechanisms responsible for the production of Δ_{resid}, we calculated the average ^{15}N-labeled fraction of the reactant pool that would have been required to produce the observed $^{29}N_2$ and $^{30}N_2$, with no Δ_{resid}, assuming the anammox rates measured in the control incubations remained constant across all treatments (this is reasonable considering the slow growth rate of anammox bacteria relative denitrifiers) and $^{15}N_2$ was binomially distributed relative to the reactants. In the ETSP incubations, the fraction of the NO_3^- pool that was ^{15}N-labeled was approximately 0.1. In order to produce the observed distributions of $^{29}N_2$ and $^{30}N_2$ at 5.7 d, the ^{15}N-labeled fraction of the reactant pool must have been much lower: 0.002. In the AS incubations, the fraction of the NO_2^- pool ^{15}N-labeled was between 0.4 – 0.7. For $\Delta_{resid} = 0$, the fraction of the reactant pool ^{15}N-labeled must have been 0.001 – 0.5. These results indicate that direct oxidation of organic N to N_2 is probably not the only mechanism producing the observed distributions of $^{15}N_2$, given that an organic ^{14}N pool up to 1000 times greater than the $^{15}NO_{2,3}^-$ concentration would be required. Thus in this study, 'nitrite shunting' (de Brabandere et al. 2013) is a reasonable mechanism to explain a significant portion of the Δ_{resid}. At 2 days in the ETSP incubations, $^{29}N_2$ and $^{30}N_2$ were binomially distributed, which is consistent with 'nitrite shunting' not affecting the predicted distribution of $^{29}N_2$ and $^{30}N_2$ since $^{15}NO_3^-$ was used as the tracer. At 5.7 d, Δ_{resid} becomes significant which may be due to changes in the fraction ^{15}N-labeled of the substrates, suggested by significant changes in the concentrations of NO_3^- and NO_2^-. Additionally, similar to denitrification rates, Δ_{resid} was significantly affected by only the addition of POM ($p < 0.005$); no significant effect was observed between stations, depths, or with the addition of NH_4^+. This response suggests a heterotrophic source for Δ_{resid}.

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Table 1

Station locations, sampling depths, and associated hydrographic characteristics. [O_2] measured by Seabird O_2 sensor, detection limit ~1 μmol L^{-1}. Detection limits of NO_3^-, NO_2^-, and NH_4^+ were 0.08, 0.01, and 0.07 μmol L^{-1}, respectively.

Station	Latitude	Longitude	Bottom depth (m)	Sampling depth (m)	Feature	[O_2] (μmol L^{-1})	[NO_3^-] (μmol L^{-1})	[NO_2^-] (μmol L^{-1})	[NH_4^+] (μmol L^{-1})
ETSP									
20	13.3°S	77°W	788	260	SNM	nd	24.3	4.4	0.1
AS									
1	19.4°N	66.7°E	3095	100	base of oxycline	nd	22.3	1.9	nd
				150	SNM	nd	15.1	5.7	0.06
2	15°N	64°E	3900	150	base of oxycline	nd	10.7	3.7	nd
				200	SNM	nd	7.6	7.7	nd

nd – not detected

Table 2
Summary of ^{15}N-labeled tracer additions and treatments.

Station	Feature	^{15}N-tracer[a]	Treatment
ETSP			
20	SNM	$^{15}NO_3^-$ (2)	–
		$^{15}NO_3^-$ (2)	glucose
		$^{15}NH_4^+$ (1)	–
		$^{15}NH_4^+$ (1)	glucose
AS			
1	SNM, base of oxycline	$^{15}NO_2^-$ (5)	–
		$^{15}NO_2^-$ (5)	$^{14}NH_4^+$
		$^{15}NO_2^-$ (5)	POM[b]
		$^{15}NO_2^-$ (5)	POM + $^{14}NH_4^+$ (5)
		$^{15}NO_2^-$ (5)	DOM[c]
		$^{15}NO_2^-$ (5)	POM + $^{14}NH_4^+$ (5)
		$^{15}NH_4^+$ (5)	$^{14}NO_2^-$
2	SNM, base of oxycline	$^{15}NO_2^-$ (5)	–
		$^{15}NO_2^-$ (5)	$^{14}NH_4^+$
		$^{15}NO_2^-$ (5)	POM
		$^{15}NO_2^-$ (5)	POM + $^{14}NH_4^+$ (5)
		$^{15}NO_2^-$ (5)	DOM
		$^{15}NO_2^-$ (5)	POM + $^{14}NH_4^+$ (5)
		$^{15}NH_4^+$ (5)	$^{14}NO_2^-$

[a]Target concentration of ^{15}N tracer and $^{14}NH_4^+$ in parentheses (μmol L^{-1}); [b]POM – particulate organic carbon; [c]DOM – dissolved organic carbon. Both organic matter treatments came from sediment traps deployed approximately 20 and 50 m above the ODZ proper at Sta. 1 and 2, respectively. *See* text for details of preparation.

523 ***Figure legends***

524

525 Figure 1: (A) [NO_3^-] and (B) [NO_2^-] in incubations with and without glucose added at Sta. 20 in
526 the ETSP ODZ. Vertical dashed line indicates when sample for $^{15}N_2$ was taken. Error bars
527 represent range of duplicates.

528

529 Figure 2: Denitrification, anammox, and Δ_{resid} rates in incubations with and without glucose
530 added at Sta. 20 in the ETSP ODZ.

531

532 Figure 3: Denitrification rates in control, DOM, and POM amended incubations at (A) Sta. 1 and
533 (B) Sta. 2 in the AS ODZ. Also shown are anammox rates measured in parallel incubations with
534 $^{15}NH_4^+$. Error bars are 1 standard deviation (SD). Anammox and denitrification (control only)
535 rates previously published in Ward et al. (2009).

536

537 Figure 4: Δ_{resid} in control, DOM, and POM amended incubations at (A) Sta. 1 and (B) Sta. 2 in
538 the AS ODZ. Δ_{resid} is the $^{29}N_2$ production rate in excess of the amount predicted from the $^{30}N_2$
539 production rate in $^{15}NO_2^- + {}^{14}NH_4^+$ incubations, assuming the N_2 isotopomers generated by
540 denitrification are binomially distributed. $^{29}N_2$ production from anammox was taken into
541 account in the calculation of Δ_{resid}. Error bars are 1 SD.

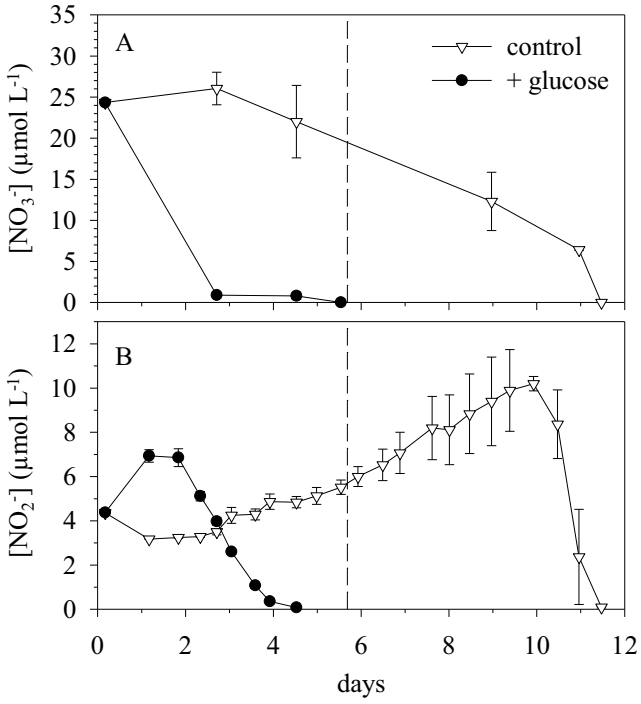
A
B
control
+ glucose
[NO3-] (µmol L-1)
[NO2-] (µmol L-1)
days
0
5
10
15
20
25
30
35
2
4
6
8
12

Figure 1

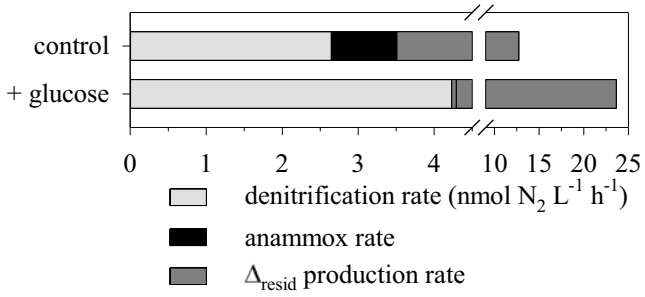
control
+ glucose
0
1
2
3
4
10
15
20
25
denitrification rate (nmol N_2 L^{-1} h^{-1})
anammox rate
Δ_{resid} production rate

Figure 2

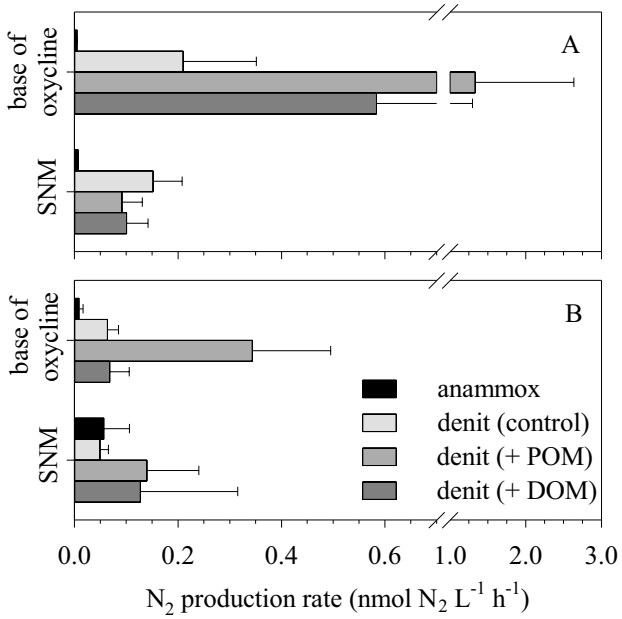
base of oxycline
SNM
A
B
anammox
denit (control)
denit (+ POM)
denit (+ DOM)
0.0
0.2
0.4
0.6
1.0
2.0
3.0
N2 production rate (nmol N2 L-1 h-1)

Figure 3

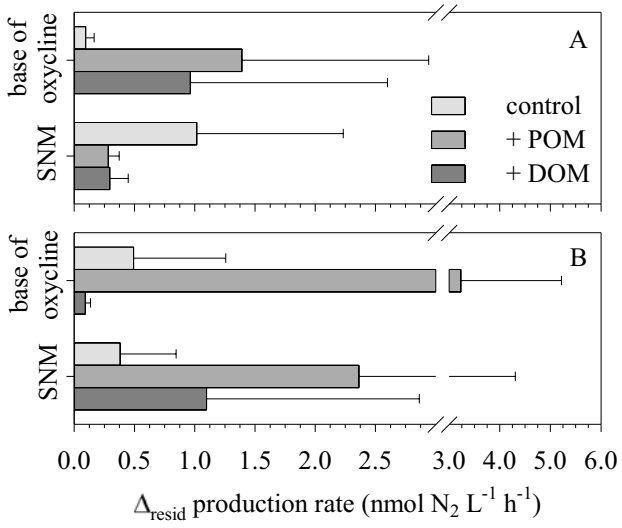
base of oxycline
SNM
A
control
+ POM
+ DOM
B
0.0 0.5 1.0 1.5 2.0 2.5 3.0 4.0 5.0 6.0
Δ_{resid} production rate (nmol N_2 L^{-1} h^{-1})

Figure 4