SHORT COMMUNICATION

Evidence for the direct oxidation of organic nitrogen to N₂ gas in the Arabian Sea

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We performed a suite of ^{15}N incubations ($^{15}NO_2^-$, $^{15}NO_3^-$ and $^{15}NH_4^+$) with and without the organic-nitrogen (N) compound allylthiourea (ATU), in the suboxic waters of the Arabian Sea. Production of $^{29}N_2$ in control (-ATU) incubations with either $^{15}NH_4^+ + {}^{14}NO_2^-$, or their analogues, $^{15}NO_2^- + {}^{14}NH_4^+$, though small, confirmed the presence of anammox. In contrast, when we added ATU, along with $^{15}NO_2^-$ and $^{14}NH_4^+$, there was a much greater production of $^{29}N_2$, with 92% of the ^{15}N-label being recovered as $^{29}N_2$ on average. Such stimulated production of $^{29}N_2$ could not be due to anammox, as the addition of ATU, along with $^{15}NH_4^+ + {}^{14}NO_2^-$, only produced $^{29}N_2$ equivalent to that in the controls. The ratios of $^{29}N_2$ to $^{30}N_2$ produced also precluded stimulation of denitrification. We present this as evidence for a hitherto uncharacterised metabolism potentially capable of oxidising organic-N (e.g. NH_2 groups) directly to N_2 gas at the expense of NO_2^-.

Much of the surface water across the ocean is near air-saturation with respect to oxygen. In parts of the tropics, however, such as the eastern Pacific (North and South), Arabian Sea and Bay of Bengal, a core of permanently and severely oxygen-deplete ($1 \leqslant O_2 < 20 \mu M$) water (extending over $>300 m$ on average) can be found between 160 and 1080 m depth (Paulmier and Ruiz-Pino, 2009). Although the cores of these oxygen-minimum zones occupy only $\sim 0.8\%$ of the total ocean volume, they are responsible for a large amount of the N_2 produced (i.e., loss of fixed nitrogen (N)) on Earth (Seitzinger *et al.*, 2006; Codispoti, 2007). Given that the balance between N fixation and its removal through N_2 production is key to carbon assimilation by primary production and CO_2 modulation in the atmosphere (Falkowski, 1997), the oxygen-minimum zones are vital to the functioning of the biosphere. Evidence supports both denitrification and anammox as being responsible for this N_2 production, though discrepancies in the N-budget indicate that this two-path model is incomplete (Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2005; Thamdrup *et al.*, 2006; Ward *et al.*, 2009).

Primarily we visited the oxygen-minimum zones in the central Arabian Sea to measure nitrous oxide

(Law and Owens, 1990; Nicholls *et al.*, 2007) and collected water samples from 125 to 295 m at two sites, targeting the characteristic peaks and troughs in nitrite concentration (Codispoti and Christensen, 1985; Morrison *et al.*, 1999; Nicholls *et al.*, 2007). We carried out a suite of ^{15}N incubations with and without the addition of allylthiourea (ATU), an organic-N compound known to inhibit nitrification (Figure 1a and Supplementary Material). Although the role of nitrification in the production of nitrous oxide was minor (Nicholls *et al.*, 2007), the inhibitor measurements themselves provided us with a fortuitous and potentially important finding.

The production of N_2 via anammox has a unique 1:1 stoichiometry, which provides a very sensitive ^{15}N 'tool' to detect anammox in ecosystems, with the stoichiometry proceeding according to (van de Graaf *et al.*, 1995):

$$^{14 \text{ or } 15}NH_4^+ + {}^{15 \text{ or } 14}NO_2^- \rightarrow {}^{29}N_2 + 2H_2O \text{ (reaction 1)}$$
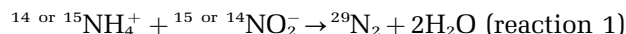
The presence of anammox was confirmed at our sites by the production of $^{29}N_2$ in our control incubations with either inorganic $^{15}NH_4^+ + {}^{14}NO_2^-$ or its analogue $^{15}NO_2^- + {}^{14}NH_4^+$ (reaction 1). Although the production of $^{29}N_2$ was low, at less than 70 and 20 nmol $^{29}N_2$ per litre with either $^{15}NO_2^-$ or $^{15}NH_4^+$, respectively, after 96 h (Figures 1b and c), it was roughly equivalent to that previously reported for the Arabian Sea (e.g., ~ 18 versus ~ 10 nmol l^{-1} per day; Ward *et al.*, 2009). In contrast, when we added organic ATU along with $^{15}NO_2^-$ and $^{14}NH_4^+$, there was a much greater production of $^{29}N_2$ at all depths at both sites, peaking at 1.42 µmol $^{29}N_2$ per litre over 96 h, on average

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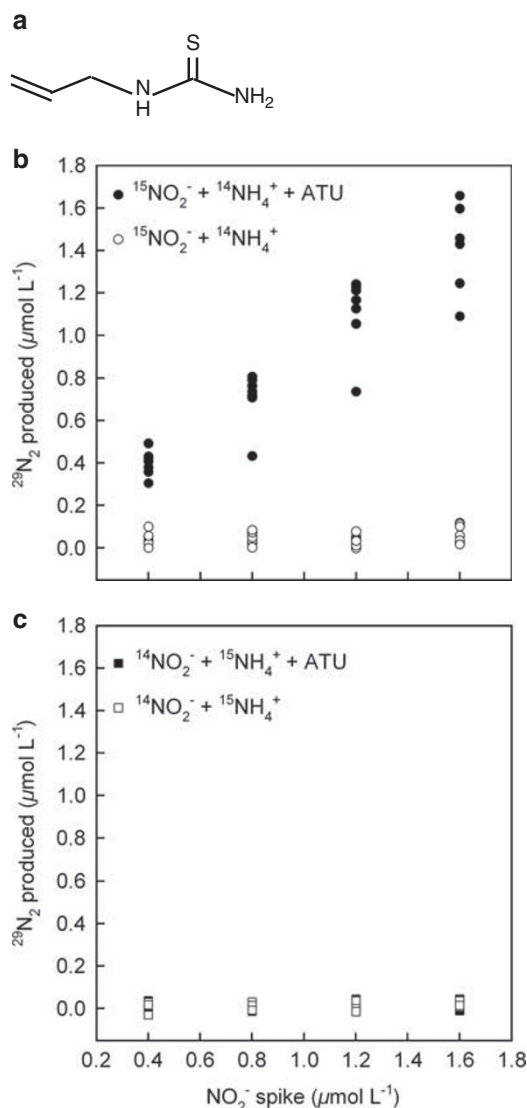E-mail: m.trimmer@qmul.ac.uk

Figure 1 Organic-N and production of $^{29}N_2$. (**a**) Molecular formula of ATU showing the primary and secondary amine groups. (**b**) Production of $^{29}N_2$ in the presence of $^{15}NO_2^-$ plus $^{14}NH_4^+$ with and without ATU (filled and open circles, respectively), and (**c**) the same as (**b**), but in this case the ^{15}N-label is on the NH_4^+ molecule instead of the NO_2^- (filled and open squares, respectively). We present all of the data from each depth at both sites to show the reproducibility of the response.

(Figure 1b). Indeed, with $^{15}NO_2^-$ the turnover of ^{15}N was almost complete and, across all of the incubations, 92% ($\pm$3, s.e.m., $n = 28$) of the ^{15}N-label was recovered as $^{29}N_2$. This production of $^{29}N_2$ indicated a 1:1 pairing of $^{15}NO_2^-$ and ^{14}N, as in anammox (reaction 1). However, here the formation of $^{29}N_2$ could not be due to the oxidation of NH_4^+ present in the water, as the addition of ATU, along with labelled $^{15}NH_4^+$ (and $^{14}NO_2^-$), produced $^{29}N_2$ only in small amounts equivalent to that in the controls (Figure 1c). It could be argued that the measured production of $^{29}N_2$ was due to a simple organic stimulation of denitrification, for example:
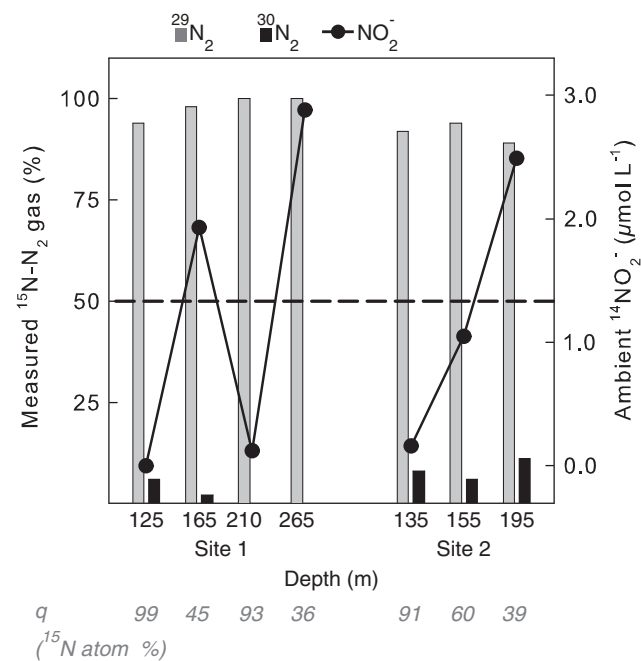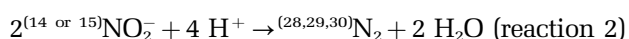
$$2^{(14 \text{ or } 15)}NO_2^- + 4\,H^+ \rightarrow {}^{(28,29,30)}N_2 + 2\,H_2O \quad \text{(reaction 2)}$$

Figure 2 Predicted and measured $^{29}N_2$ and $^{30}N_2$ production. Measured proportions of $^{29}N_2$ and $^{30}N_2$ (grey and black bars, respectively) at the maximum enrichment for $^{15}NO_2^-$ ($\sim$1.6 μmol l^{-1}) with allylthiourea. The 'q' term for each site is the ^{15}N atom (%) of the NO_2^- pool (below the x axis) at each depth, which gives the 'chances' of denitrification producing $^{30}N_2$ and reflects the mix of ambient $^{14}NO_2^-$ (filled circles) and $^{15}NO_2^-$ spike. Dashed line indicates the predicted maximum 50% $^{29}N_2$ contribution from denitrification.

The relative amounts of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ in denitrification are governed by a binomial distribution reflecting the frequencies of ^{15}N and ^{14}N in the NO_2^- pool being reduced, according to:

$$[p + q]^2 = p^2 + 2pq + q^2 \quad (1)$$

where p and q are the proportions of ^{14}N and ^{15}N, respectively, in the NO_2^- pool ($p + q$ is equal to unity) and p^2, $2pq$, and q^2 are the predicted proportions of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ produced through denitrification. Accordingly, not more than 50% of the ^{15}N added can ever be recovered as $^{29}N_2$, the remainder being $^{30}N_2$. Quite clearly that was not the case as, at all depths at both sites, $^{29}N_2$ dominated in the ATU-amended samples, with $^{30}N_2$ contributing far less than that predicted for denitrification (Figure 2).

The excess of $^{29}N_2$ in the ATU-amended samples was not due to denitrification or anammox. Our observations were fortuitous and we did not fully test the nature of the reaction in the field. We have, however, since tested whether or not ATU 'spontaneously' breaks down in the presence of the oxidising agents nitrite and $ZnCl_2$ in both oxic and anoxic filter-sterilised seawater in the laboratory. After 2 weeks the ATU remained unchanged ($\sim$79 μM, Supplementary Table S1) and there was no significant production of $^{29}N_2$ from $^{15}NO_2^-$ either

with or without ATU (Supplementary Table S2). Thus, ATU appears to be stable in sterile seawater. One reasonable explanation for our observations is, therefore, that the ATU was partially metabolised and one of its amine groups provided the ^{14}N for the production of $^{29}N_2$ in the incubations with $^{15}NO_2^-$, where the ATU acted as an analogue of urea. Urea is a main component of the complex pool of dissolved organic-N cycling in the ocean (Painter et al., 2008). Some may argue that definitive proof could only be provided by the production of $^{29}N_2$ after the addition of ^{15}N-labelled dissolved organic nitrogen (DON). However, as $^{29}N_2$ can only be produced through the one-to-one pairing of ^{14}N and ^{15}N, the production of $^{29}N_2$ from ^{14}N-DON and $^{15}NO_2^-$ is just as equally valid.

Recently, denitrification was reported to dominate the production of N_2 gas in the central Arabian Sea, whereas we had previously argued for 'multiple pathways of N_2 production' that could not be ascribed categorically to either anammox or denitrification (Nicholls et al., 2007; Ward et al., 2009). Here we present evidence for an uncharacterised metabolism, potentially capable of oxidising DON (e.g. NH_2 groups) directly to N_2 gas with NO_2^-.

Acknowledgements

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)