N₂ production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica

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In oxygen-depleted zones of the open ocean, and in anoxic basins and fjords, denitrification (the bacterial reduction of nitrate to give N₂) is recognized as the only significant process converting fixed nitrogen to gaseous N₂. Primary production in the oceans is often limited by the availability of fixed nitrogen such as ammonium or nitrate, and nitrogen-removal processes consequently affect both ecosystem function and global biogeochemical cycles. It was recently discovered that the anaerobic oxidation of ammonium with nitrite—the ‘anammox’ reaction, performed by bacteria—was responsible for a significant fraction of N₂ production in some marine sediments. Here we show that this reaction is also important in the anoxic waters of Golfo Dulce, a 200-m-deep coastal bay in Costa Rica, where it accounts for 19–35% of the total N₂ formation in the water column. The water-column chemistry in Golfo Dulce is very similar to that in oxygen-depleted zones of the oceans—in which one-half to one-third of the global nitrogen removal is believed to occur—and we therefore expect the anammox reaction to be a globally significant sink for oceanic nitrogen.

Golfo Dulce is connected to the equatorial tropical North Pacific Ocean through a 14-km-wide opening with a sill at 60 m depth. The bay is about 50 km long, with anoxic, nitrate-rich water in the bottom depths of the basin. During the sampling reported here, the pycnocline was located at 40–55 m depth, and the water column was anoxic at depths greater than about 100 m, although sulphide did not accumulate until the bottom 10–20 m (Fig. 1a). Nitrate reduction, and not sulphate reduction, dominated water-column processes through most of the anoxic zone, similar to well-developed oxygen-minimum zones in the eastern Pacific and Indian oceans. Curiously, ammonium (a normal product of anoxic organic matter mineralization) did not accumulate in the anoxic waters until nitrate was depleted and H₂S appeared (Fig. 1a). This observation alone indicates a significant role for anaerobic ammonium oxidation, where the ammonium liberated in the water column during denitrification is further oxidized with nitrate (through nitrite) as shown in equation (1) (refs 10, 11):

\[
\text{(CH}_2\text{O)}_{10n}(\text{NH}_3)_{16}\text{H}_3\text{PO}_4 + 94.4\text{HNO}_3 \rightarrow 106\text{CO}_2 + 55.2\text{N}_2 + 177.2\text{H}_2\text{O} + \text{H}_3\text{PO}_4
\]

(1)

This stoichiometry can be explained by the simultaneous operation of two known bacterial processes, denitrification (equation (2)), and the anammox reaction (equation (3) (refs 2, 12, 13)).

\[
\text{(CH}_2\text{O)}_{10n}(\text{NH}_3)_{16}\text{H}_3\text{PO}_4 + 94.4\text{NO}_3^- + 94.4\text{H}^+ \rightarrow 16\text{NH}_4^+ + 16\text{NO}_2^- + 39.2\text{N}_2 + 145.2\text{H}_2\text{O} + \text{H}_3\text{PO}_4
\]

(2)

\[
16\text{NH}_4^+ + 16\text{NO}_2^- \rightarrow 2\text{N}_2 + 32\text{H}_2\text{O}
\]

(3)

The stoichiometric expression for denitrification (equation (2)) is somewhat modified from the ‘standard’ formulation, showing the production of nitrite, a free intermediate of denitrification, in equal proportions to ammonium liberation. This is because anaerobic bacteria utilize nitrite and ammonium in a 1:1 ratio to form N₂ gas (equation (3)). It follows from equations (2) and (3) that the anammox reaction is responsible for 29% of the total N₂ production if all of the ammonium liberated during denitrification is subsequently oxidized by anammox. This possibility was recognized many years ago, although it was not then known whether organisms in nature could in fact conduct the anammox process.

We used ¹⁵N-labelling techniques to explore for anammox in the water column of Golfo Dulce. Samples were retrieved from four depths in the anoxic water column at two sites (stations A and B) in the 200-m-deep central part of the basin, and amended with different combinations of ¹⁵N- and ¹⁴N-labelled nitrate and ammonium. At both sites and all depths, we observed anaerobic oxidation of NH₄⁺ to N₂, as illustrated by the formation of ¹⁴N¹⁵N from added ¹⁵NH₄⁺ (Fig. 2a). The formation of ¹⁵N²N started immediately after addition of ¹⁵NH₄⁺, and the rate was constant over time. The formation of ¹⁵N²N was at the limit of detection. Therefore, the added ¹⁵NH₄⁺ was combined with nitrogen from the natural ¹⁴NO₃⁻ or ¹⁴NO₂⁻ pools to form N₂, which is characteristic of the anammox reaction. It has been suggested that anaerobic...
ammonium oxidation could occur with manganese oxide as oxidant producing either N₂ or nitrate\(^{16-17}\). Under denitrifying conditions the nitrate would subsequently be reduced to N₂. However, those mechanisms cannot explain the oxidation of ammonium to N₂ in this study, as both mechanisms would lead to the formation of \(^{15}N\)N—which was not observed. Furthermore, if ammonium was oxidized to nitrate with manganese oxide, this would lead to a gradual increase in the \(^{15}N\) abundance in the nitrate pool and the rate of production of \(^{14}N\)N would thus increase over time. The linearity of the \(^{15}N\)N production from \(^{14}N\)H\(^+_2\) is incompatible with that mechanism (Fig. 2a).

With the addition of \(^{15}NO_3^-\), both \(^{14}N\)\(^{15}N\) and \(^{15}N\)\(^{15}N\) were produced with rates increasing during the incubations (Fig. 2b, c). This time dependence is explained by the gradual build-up of \(^{15}N\) in the nitrite pool. Initial concentrations of unlabelled nitrite were 0.5–1.3 \(\mu M\) in all incubations, except for Station B at 160 m where it was 2.4 \(\mu M\). Nitrite is the electron acceptor for anammox, and a free intermediate in N₂ formation by denitrification, and added \(^{15}NO_3^-\) must be transferred into the nitrite pool before \(^{15}N\)-labelled N₂ can be produced.

In incubations with \(^{15}NO_3^-\) and unlabelled, native H\(^+_2\) and NO\(_2^- + \)NO\(_3^-\), anammox will produce \(^{14}N\)\(^{14}N\) and \(^{14}N\)\(^{15}N\), whereas denitrification produces \(^{14}N\)\(^{14}N\), \(^{14}N\)\(^{15}N\) and \(^{15}N\)\(^{15}N\) through random isotope pairing\(^{12,13,18}\). In our experiments, the production rate of \(^{14}N\)\(^{15}N\) was considerably higher than expected for denitrification alone, indicating another source for \(^{15}N\), consistent with anammox activity. For the experiment depicted in Fig. 2b, a contribution of anammox to total N₂ production of 28% was determined from the specific labelling of \(^{15}N\) in the nitrate pool, and the production rates of \(^{14}N\)\(^{14}N\) and \(^{15}N\)\(^{15}N\) (ref. 2). As the propagation of the \(^{15}N\) label from nitrate to nitrite was greatest at the end of the experiment, the production rates of \(^{15}N\)\(^{15}N\) and \(^{15}N\)\(^{15}N\) were calculated as the slope of the concentration curves through the data points from the last two samplings. Rates of both anammox and denitrification may be slightly overestimated owing to the elevated nitrate concentrations in the \(^{15}NO_3^-\) amendment experiments. However, owing to the tight coupling of the two processes, and the NH\(_2\)\(^+_2\) limitation of the anammox process, the relative importance of the two processes should not be affected.

At the three upper sampling depths of both stations, the ammonium concentration was around the detection limit (0–0.3 \(\mu M\), Fig. 1a), and the low ammonium concentration apparently limited the rates of anammox. The limitation of ammonium on rates of anammox was substantiated by a stimulation of anammox when unlabelled ammonium was added together with the \(^{15}NO_3^-\) (Fig. 2c). At the deepest sampling depth, where ammonium concentrations were higher, ammonium limitation was less pronounced, as indicated by either no stimulation of ammonium oxidation (Station B) or a much lower stimulation than in the three upper depths (Station A). Integrated through the whole anoxic anoxic water column, the ammonium addition stimulated anammox by a factor of 4 at Station A and by a factor of 2 at Station B. As anammox is ammonium limited in Golfo Dulce, there is the capacity for anammox organisms to oxidize all the ammonium produced by carbon mineralization in the anoxic water column. Therefore, the ammonium deficiency in the anoxic water column can be explained by the coupling between denitrification and anammox as expressed in equations (2) and (3).

Rates of anammox and denitrification were calculated from the \(^{15}NO_3^-\) labelling experiments (Fig. 1b, c), as anammox in the \(^{15}NH_4^+\) experiments was stimulated by the added ammonium (see above). The relative importance of anammox was greatest in the upper three sampling depths, where it accounted for on average 58% and 32% of the total N₂ production on stations A and B, respectively. The significance of anammox thus matched, or even exceeded, what is predicted by the equations above. Higher than expected proportions of anammox may be explained by the preferential decomposition of nitrogen-rich organic matter\(^{19}\) through denitrification, releasing more ammonia than predicted by standard Redfield stoichiometry (equation (2)). At the deepest sampling depth, anammox accounted for only 28% and 13% of total N₂ production at stations A and B, respectively. This relatively low contribution of anammox, particularly at Station B, could be a result of significant denitrification coupled to sulphide oxidation (Fig. 1a). Alternatively sulphide could inhibit the anammox process, but this has not been tested. Integrating the rates for both anammox and denitrification for the whole anoxic water column, we find that anammox contributed between 19\% (Station B) and 35\% (Station A) of the N₂ production (Table 1).

Our experiments demonstrate a tight coupling between the ammonium liberated during denitrification, and further transformation of this ammonium by the anammox process. Such a coupling has been considered\(^8\), but has not previously been documented experimentally in a marine water column. The water chemistry of Golfo Dulce has many similarities to oxygen-depleted oxygen-minimum zones, which also display extensive nitrate reduction, but without the build-up of ammonium\(^{14,17,20-22}\). We therefore suspect that anammox is coupled to denitrification, and is responsible for ammonium oxidation in those environments as well. By current estimates, between about one-third and one-half of the total denitrification in the oceans occurs in oxygen-minimum zones\(^3,4\).

![Figure 2](image_url) Isotopic enrichment experiments. a-c, Concentrations of \(^{15}NH_4^+\) (open circles) and \(^{15}N\)\(^{15}N\) (squares) in experiments with addition of \(^{15}NH_4^+\) a, \(^{15}NO_3^-\) b and \(^{15}NO_3^- + \)NH\(_2\)\(^+_2\) c. The concentrations of NO\(_2^-\) and NH\(_2\)\(^+_2\) were 7.2 \(\mu M\) and 0.2 \(\mu M\), respectively, before amendments. Data from Station B at 120 m depth. Each data point represents the average of three incubations.

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<th>Table 1: Integrated N₂ production rates</th>
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Rates were calculated from experiments with \(^{15}NO_3^-\) addition and integrated for the whole anoxic water column. Relative anammox is calculated as anammox/(anammox + denitrification). Denitrification rates were calculated as: \(R_D = \frac{1}{1-R_{NH_2}} R_{NO_3^-}\), where \(R_D\) is the integrated rate, \(R_{NH_2}\) is the measured rate for depth interval \(n\), \(h_n\) is the height of the water column for interval \(n\). The boundaries for each interval was set as the midpoint between two consecutive measurements, the upper boundary was set at 100 m (Station A) or 90 m (Station B), and the lower boundary was set at 250 m.
Therefore, ammonia in these waters could account for 10–15% of the marine $N_2$ production. The anammox process may be more important than this, with particularly favourable conditions occurring in upwelling areas where anoxic nitrate-rich water reaches the sediment. A good example would be upwelling areas off the Peruvian and Chilean coasts, where sedimentary sulphide is oxidised by sulphide-oxidizing bacteria with nitrate, producing ammonium. This ammonium is released to the water column, probably enhancing the significance of anammox, and $N_2$ production, in these areas. Thus, the anammox process, with its distinct regulatory characteristics, should be included in studies of nitrogen cycling in the marine environment.

A

Methods

Sampling was performed at Station A (8° 34.15'N, 83° 14.69'W) and Station B (8° 32.99'N, 20° 62.82'W) of Golfo Dulce in November 2001. Profiles of salinity, temperature and oxygen were measured at 10-m intervals with a DataSonde 4 (Hydrotel). All water samples were retrieved with a 5-l Niskin bottle (KC Denmark). For nutrient profiles, water was sampled from the Niskin bottle with a plastic syringe and filtered through a cellulose acetate filter (pore size 0.22 μm) into polypropylene vials that were stored on ice until return to the laboratory where they were frozen for later analysis. For the 15N-experiment, water samples was collected with the Niskin bottle via Tygon tubing into the bottom of a 250-ml glass bottle and allowed to overflow for half a volume change. The bottle was closed with a Viton stopper taking care to exclude bubbles, and stored in ice until return to the laboratory. Experiments were started no later than 6 h after sampling.

For each of the four depths from each station experiments were started by the addition of 15N labelled and unlabelled nitrate and ammonium to the 250-ml bottles to the following final concentrations from concentrated stock solutions: 10 μM $^{15}$NO3-, 10 μM $^{15}$NO2- + 10 μM $^{15}$NH4+, 10 μM $^{15}$NO3- + 10 μM $^{15}$NH4+ and 10 μM $^{15}$NH4+. After addition the amended water was bubbled with helium for 15 min to facilitate the detection of $N_2$ production, and then transferred to 12-ml glass vials with a 5-mm butyl rubber septum (Exetainers, Labco) through a glass tube leading from the bottle into the bottom of the Exetainer and allowed to overflow. The Exetainers were incubated at site temperature in a water bath kept anoxic with an alkaline sodium ascorbate solution. The oxygen concentration in the Exetainers was measured with a microelectrode designed for measuring low oxygen concentrations (Unisense) and was below the detection limit of 0.2 μM throughout the experiment. At each time point Exetainers were sampled in triplicate by removing 5 ml of water before replacing it with helium and adding 0.1 ml of 50% (w/v) ZnCl2 to stop biological activity in the Exetainer. The removed water was filtered for nutrient analysis and stored as described above.

The concentration of $N_2O + NO_2$ was determined using the vanadium chloride reduction method35 (NOx analyser model 42c, Thermo Environmental Instruments Inc.). Nitrite was analysed spectrophotometrically36 and $N_2$H4 was determined using the flow injection method with conductivity detection37. The isotopic composition of the nitrate and ammonium pool was estimated from their concentrations before and after amendment. Concentrations of 15N-N and 15N-N$^2$ were determined by isotope ratio mass spectrometry and calculated as excess above their natural abundance.

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Letters to Nature

Anaerobic ammonium oxidation by anammox bacteria in the Black Sea

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The availability of fixed inorganic nitrogen (nitrate, nitrite and ammonium) limits primary productivity in many oceanic regions. The conversion of nitrate to $N_2$ by heterotrophic bacteria (denitrification) is believed to be the only important sink for fixed inorganic nitrogen in the ocean. Here we provide evidence for bacteria that anaerobically oxidize ammonium with nitrite to $N_2$ in the world's largest anoxic basin, the Black Sea. Phylogenetic analysis of 16S ribosomal RNA gene sequences shows that these bacteria are related to members of the order Planctomycetales performing the anammox (anaerobic ammonium oxidation) process in ammonium-removing bioreactors. Nutrient profiles, fluorescently labelled RNA probes, $^{15}$N tracer experiments and the distribution of specific 'ladderane' membrane lipids indicate that ammonium diffusing...