Anaerobic Ammonium Oxidation Measured in Sediments along the Thames Estuary, United Kingdom

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Until recently, denitrification was thought to be the only significant pathway for N_2 formation and, in turn, the removal of nitrogen in aquatic sediments. The discovery of anaerobic ammonium oxidation in the laboratory suggested that alternative metabolisms might be present in the environment. By using a combination of ^{15}N -labeled NH_4^+ , NO_3^- , and NO_2^- (and ^{14}N analogues), production of $^{29}N_2$ and $^{30}N_2$ was measured in anaerobic sediment slurries from six sites along the Thames estuary. The production of $^{29}N_2$ in the presence of $^{15}NH_4^+$ and either $^{14}NO_3^-$ or $^{14}NO_2^-$ confirmed the presence of anaerobic ammonium oxidation, with the stoichiometry of the reaction indicating that the oxidation was coupled to the reduction of NO_2^- . Anaerobic ammonium oxidation proceeded at equal rates via either the direct reduction of NO_2^- or indirect reduction, following the initial reduction of NO_3^- . Whether NO_2^- was directly present at 800 μM or it accumulated at 3 to 20 μM (from the reduction of NO_3^-), the rate of $^{29}N_2$ formation was not affected, which suggested that anaerobic ammonium oxidation was saturated at low concentrations of NO_2^- . We observed a shift in the significance of anaerobic ammonium oxidation to N_2 formation relative to denitrification, from 8% near the head of the estuary to less than 1% at the coast. The relative importance of anaerobic ammonium oxidation was positively correlated ($P < 0.05$) with sediment organic content. This report of anaerobic ammonium oxidation in organically enriched estuarine sediments, though in contrast to a recent report on continental shelf sediments, confirms the presence of this novel metabolism in another aquatic sediment system.

Since the 1970s, substantial research has focused on the ability of estuarine sediments to attenuate riverine nitrogen (N) loads before they affect coastal seas (4, 18, 19, 31, 33). Estuarine sediments are essentially anaerobic below a few surface millimeters, and the mineralization of organic matter proceeds via alternate electron acceptors such as NO_3^- and SO_4^{2-} (20, 27). In turn, the reduction of NO_3^- removes NO_3^- from the overlying waters. Until recently, it was largely thought that NO_3^- could be either reduced to N_2 gas via denitrification (a facultative metabolism mediated by a variety of bacteria) and lost from the system or reduced to ammonium (NH_4^+) by fermentative metabolisms and hence conserved within the sediments (dissimilatory nitrate reduction to ammonium [DNRA]) (8, 23). It had been demonstrated that changes in sediment organic loadings and estuarine NO_3^- concentrations may affect the partitioning between these two end products of NO_3^- reduction (11, 12). The discovery within the laboratory (17) of anaerobic ammonium oxidation revealed a novel metabolism that could short circuit the N cycle, bypassing what was previously thought to be a critical aerobic nitrification phase and potentially providing an alternative pathway for N_2 gas formation in the environment (Fig. 1).

Originally, it was thought (17) that anaerobic ammonium oxidation coupled the oxidation of NH_4^+ to the reduction of NO_3^- :

Further work, however, showed that the oxidation of ammonium was actually coupled to the reduction of nitrite rather than nitrate (34, 35):

The application of this process to the treatment of nitrogenous waste has received a great deal of attention (9, 10), and more recently, the organism responsible has been classified as a new autotrophic planctomycete (28).

Anaerobic ammonium oxidation was recently reported to account for as much as 24 and 60% of N_2 formation in continental shelf sediments in relatively deep water (380 and 695 m, respectively) but less than 2% of N_2 formation in eutrophic shallow coastal bay sediments (30). The drop in the significance of anaerobic ammonium oxidation for N_2 formation relative to denitrification was attributed to changes in organic matter availability and hence sediment reactivity. If this trend continues inshore, where organic content and reactivity further increase, anaerobic ammonium oxidation might be assumed to be insignificant in organically enriched estuarine sediments (33).

The purpose of this research was to look for direct evidence of environmental anaerobic ammonium oxidation in estuarine sediments. Initial trials were designed to simply assay sediment at one site in the Thames estuary. Having established that anaerobic ammonium oxidation was detectable in estuarine sediments, the trials were extended downstream to reflect the changing sediment characteristics of both organic content and reactivity.

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FIG. 1. Simplified N cycle showing how anaerobic ammonium oxidation (Anammox) (broken arrows) bypasses the classic coupling of aerobic nitrification to denitrification, with the oxidation of NH_4^+ and N_2 formation being driven by the reduction of NO_2^- . Note the process of DNRA and that NO_2^- is an intermediate of both DNRA and nitrification.

MATERIALS AND METHODS

Sampling sites. The Thames estuary is a major macrotidal estuary on the southeastern coast of England which flows into the North Sea at Southend-on-Sea (Fig. 2). Oxygen saturation in the Thames estuary generally drops to 20% in the summer at site 1 and does not return to full saturation until some 50 km seaward at Southend-on-Sea (33). In contrast, the waters are highly enriched with NO_3^- and NO_2^- , and their concentrations are on average for the year ~550 and 9 μM , respectively, at 0 salinity. At the outset of this study, no other studies of anaerobic ammonium oxidation in the environment had been carried out, and until techniques were proven to be reliable, experiments were restricted to the most- NO_3^- -enriched and -oxygen-depleted sediments near the major sewage discharge point at Crossness (site 1). Sediments from along the estuary were collected from sites close to those described in detail earlier (33). At sites 1 and 2, the sediments have a high organic and silt-clay content, both of which then decrease seaward along the estuary from sites 3 to 6 (Table 1 and see below).

Sediment preparation. Sediment samples (oxic and suboxic layers, 0 to 2 cm) were collected from intertidal flats along the Thames estuary at low tide (Fig. 2), stored in plastic bags, and returned to the laboratory within 1 h. Sediment slurries (30 ml; 50%, vol/vol) were prepared in serum bottles (37 ml) with low-nutrient seawater, adjusted to in situ salinity with distilled water, sealed, degassed (oxygen-free nitrogen, 20 min), and preincubated on rollers in the dark at 15°C for 6 h in a constant-temperature room. All subsequent experiments were carried out in a constant-temperature room at 15°C. Anoxia was checked by sampling the headspace in each serum bottle and measuring the O_2 with a gas chromatograph fitted with an electron capture detector. Preliminary trials showed that ambient NO_3^- and NO_2^- were reduced to less than 1 μM during the preincubations.

FIG. 2. Map of the Thames estuary on the southeastern coast of England showing locations of the six sample sites (filled circles) and the two major STWs (open circles).

TABLE 1. Sediment characteristics and representative salinity of the overlying water at the six sites in the Thames estuary

Site	Particle size ^a (0–5 cm [$\geq 50\%$, wt/wt])	Organic carbon (% of dry wt)	Porosity	Specific gravity (g cm^{-3})	Salinity (%)
1 Crossness	Silts and clays	2.91	0.76	1.21	2
2 Grays	Silts and clays	3.59	0.76	1.24	10
3 Gravesend	Very fine sands	2.70	0.67	1.45	10
4 Allhallows	Very fine sands	1.18	0.65	1.46	26
5 Southend	Fine sands	0.45	0.50	1.68	30
6 Grain	Fine sands	0.26	0.47	1.74	30

^a For detailed descriptions, see reference 33.

Measuring anaerobic ammonium oxidation. Slurries from site 1 were enriched to approximately 10% above the ambient level with concentrated stocks of labeled $^{15}\text{NH}_4^+$ (18 mM $^{15}\text{NH}_4\text{Cl}$ [99.3 ^{15}N atom%]; Sigma-Aldrich, Poole, United Kingdom) and either $^{14}\text{NO}_2^-$ or $^{14}\text{NO}_3^-$ (or both) at either 800 μM or a range up to 3,200 μM and incubated as described above. Some additional trials were carried out with autoclaved sediment in serum bottles to confirm that the N transformations were biologically mediated. Initial trials at 200 μM NO_2^- or NO_3^- had demonstrated a very rapid turnover (1 to 2 h) in these sediments, and concentrations were increased to enable production of $^{29}\text{N}_2$ to be measured with time. Under anaerobic conditions and the above-described isotopic labeling, any anaerobic ammonium oxidation coupled to the reduction of NO_2^- or NO_3^- would yield ^{15}N -labeled gas according to the following formulas:

The sole production of $^{29}\text{N}_2$ would confirm anaerobic ammonium oxidation coupled to the reduction of NO_2^- rather than NO_3^- in these sediments. Assuming that the pool of $^{14}\text{NH}_4^+$ turned over at the same rate as $^{15}\text{NH}_4^+$, the total rate of anaerobic ammonium oxidation could then be calculated with the equation (29)

$$\text{dN}_2/\text{dt} = \text{d}^{29}\text{N}_2/\text{dt} \times [\text{NH}_4^+_{\text{total}}]/[^{15}\text{NH}_4^+] \quad (5)$$

(where dN_2 is total N_2 production by anaerobic ammonium oxidation per unit of time [dt], d^{29}N_2 is the measurement of $^{29}\text{N}_2$ per unit of time, $\text{NH}_4^+_{\text{total}}$ is the total soluble NH_4^+ pool, and $[^{15}\text{NH}_4^+]$ is the concentration of $^{15}\text{NH}_4^+$ determined by difference from the nonenriched reference samples) and expressed as a proportion of the total NO_2^- or NO_3^- reduction in the slurries. Separate slurries were enriched with their respective analogues, e.g., $^{14}\text{NH}_4^+$, $^{15}\text{NO}_2^-$, and $^{15}\text{NO}_3^-$ (>99% of the ambient level, 93 mM stock $\text{Na}^{15}\text{NO}_3^-/\text{NO}_2^-$ [99.2 ^{15}N atom%]; Sigma-Aldrich), which gives a measure of total ^{15}N gas production ($^{29}\text{N}_2$ and $^{30}\text{N}_2$) from either $^{15}\text{NO}_2^-$ or $^{15}\text{NO}_3^-$. With $^{15}\text{NO}_2^-$ or $^{15}\text{NO}_3^-$, the production of $^{29}\text{N}_2$ can be due to both anaerobic ammonium oxidation (A_{29}) and denitrification (D_{29}) (30). Essentially, the proportion due to each respective process can be calculated from the equation $A_{29} = P_{29} - D_{29}$, where A_{29} equals the production of $^{29}\text{N}_2$ gas due to anaerobic ammonium oxidation, D_{29} equals the production of $^{29}\text{N}_2$ gas due to denitrification, and P_{29} equals the total production of $^{29}\text{N}_2$ measured by isotope ratio mass spectrometry (see below). In contrast, the production of $^{30}\text{N}_2$ (P_{30}) is assumed to be due solely to denitrification of $^{15}\text{NO}_2^-/\text{NO}_3^-$; hence, P_{30} equals D_{30} and, from this, D_{29} can be calculated, assuming random pairing of ^{14}N and ^{15}N from the labeled NO_2^- or NO_3^- pools, which, with only trace amounts of $^{14}\text{NO}_2^-/\text{NO}_3^-$ and enrichment with 99.2% atom $^{15}\text{NO}_2^-/\text{NO}_3^-$, would usually be less than 1.5% (6). Overall, the calculations (30) give an estimate of total anaerobic ammonium oxidation and denitrification in the sediment slurries. These estimates can then be compared to $^{29}\text{N}_2$ production in the presence of $^{15}\text{NH}_4^+$ after taking into account the proportionate labeling of the ^{14}N - and ^{15}N - NH_4^+ pools. Trials were extended along the estuary by using labeling with $^{15}\text{NO}_3^-$, as this gives a measure of total denitrification and anaerobic ammonium oxidation in the same serum bottle.

During the incubations, independent slurries were sacrificed with time, and a gas sample (4 ml) was collected from the slightly pressurized headspace with a gastight syringe (SGE gastight Luer lock syringe; Alltech Associates Ltd., Carnforth, Lancashire, United Kingdom), with allowance for equilibration to atmospheric pressure, and transferred through a septum to an inverted water-filled vial (12-ml Exetainer vial; Labco Ltd., High Wycombe, United Kingdom), with

venting of water to atmosphere through a small-bore needle. Microbial activity was then inhibited by injecting ZnCl_2 (250 μl ; 50%, wt/vol) through the septum. Pore waters were recovered by centrifugation of the slurry, filtered (0.2- μm -pore-size Minisart Plus filter; Sartorius UK Ltd.), and frozen (-20°C) until analysis. Exchangeable ammonium was recovered by using a KCl extraction technique, which recovered >95% of adsorbed NH_4^+ by a double extraction with 2 M KCl (16).

Analytical procedures. All nutrient analyses (NO_3^- , NO_2^- , and NH_4^+) were carried out with a continuous-flow autoanalyzer (SAN++; Skalar, De-Breda, The Netherlands) and standard colorimetric techniques (13). Salinity was measured with a handheld refractometer. Water content, specific gravity, and porosity were determined from the dry weights and wet weights of known volumes of sediment. Acidified dried sediment samples (with allowance for hygroscopic adsorption [see reference 7]), were analyzed for organic carbon with an elemental analyzer coupled to a continuous-flow isotope ratio mass spectrometer calibrated with known quantities of urea (Delta Matt Plus; Thermo Finnigan, Bremen, Germany).

Mass/charge ratios for m/z 28, m/z 29, and m/z 30 nitrogen ($^{28}\text{N}_2$, $^{29}\text{N}_2$, and $^{30}\text{N}_2$) were measured by the Natural Environment Research Council (NERC) ^{15}N stable isotope facility (Centre for Ecology and Hydrology-Merlewood, Cumbria, United Kingdom). The headspace of each respective vial was sampled (20 μl) with a gastight precision syringe and directly injected into a N_2 preparative interface coupled to an Isoprime isotope ratio mass spectrometer (Micromass U.K., Wythenshawe, United Kingdom) via an open split. Instrument stability checks were performed prior to each analysis by running a series of 10 reference pulses of N_2 until a standard deviation of better than 10^{-6} was achieved.

RESULTS

Anaerobic ammonium oxidation at site 1. Production of $^{29}\text{N}_2$ from the oxidation of $^{15}\text{NH}_4^+$ occurred at equal rates in the presence of either $^{14}\text{NO}_3^-$, $^{14}\text{NO}_2^-$, or both (Fig. 3) but could not be measured in control slurries enriched with only $^{15}\text{NH}_4^+$ (data not shown). Production of $^{29}\text{N}_2$ was linear with time until the pools of either NO_2^- or NO_3^- were depleted and, hence, was independent of the decreasing concentration of either acceptor. Measurable production of $^{29}\text{N}_2$ ceased after 8 h, when either electron acceptor had been depleted, reconfirming the production measured in the controls. When the slurries were enriched with NO_3^- , little, if any, transient accumulation of NO_2^- could be detected as the NO_3^- diminished. When spiked together with $^{14}\text{NO}_2^-$ and $^{14}\text{NO}_3^-$, production of $^{29}\text{N}_2$ occurred at the same rate as in the independent trials, but NO_3^- was consumed preferentially to NO_2^- by the NO_3^- -reducing community (Fig. 3c). In addition, the production of $^{29}\text{N}_2$ continued to the end of the trial (12 h), being sustained by the NO_2^- pool, which remained largely untouched until after 8 h, when the concentration of NO_3^- had been reduced to approximately 200 μM . The rates of turnover were consistently equal for NO_3^- and NO_2^- , being equivalent to 0.11 and 0.12 $\mu\text{mol ml of wet sediment}^{-1} \text{ h}^{-1}$, respectively, at a starting concentration of 800 μM . Anaerobic ammonium oxidation produced 0.16 and 0.15 nmol of $^{29}\text{N}_2 \text{ ml of wet sediment}^{-1} \text{ h}^{-1}$ for NO_3^- and NO_2^- , respectively. With 10.3% labeling of the NH_4^+ pool, this gives an average total anaerobic ammonium oxidation for both acceptors of 1.5 nmol of $\text{N}_2 \text{ ml of wet sediment}^{-1} \text{ h}^{-1}$, equivalent to 1.3% of the total NO_3^- or NO_2^- reduction.

The sediments at site 1 reached an average maximum NO_3^- or NO_2^- reduction rate of 383 $\mu\text{M h}^{-1}$ at 3,200 μM (Fig. 4a). Because the pool of either substrate from 200 to 800 μM was completely turned over during the incubation, the data presented in Fig. 4 are for the whole 4-h incubation period, and rates are estimated from data points above 800 μM . The yield of $^{29}\text{N}_2$ from anaerobic ammonium oxidation increased with

FIG. 3. Production of $^{29}\text{N}_2$ from labeled $^{15}\text{NH}_4^+$ oxidation in the direct presence of $^{14}\text{NO}_3^-$ (a), $^{14}\text{NO}_2^-$ (b), or both (c). Values are means $\pm$ 1 standard error of the mean (SEM) ($n = 4$). The same symbols are used for each panel. Data were collected July 2002.

the turnover of substrate, up to a maximum of 0.8 or 1 nmol of $^{29}\text{N}_2 \text{ ml of wet sediment}^{-1}$ for approximately 1.5 $\mu\text{mol of NO}_3^-$ or $\text{NO}_2^- \text{ ml of wet sediment}^{-1}$ (Fig. 4b). Though NO_2^- accumulated in the NO_3^- -enriched slurries at a constant 20

FIG. 4. (a) Total rate of turnover for either NO_3^- or NO_2^- as a function of the initial enrichment concentration; (b) production of $^{29}\text{N}_2$ from labeled $^{15}\text{NH}_4^+$ oxidation; (c) ratio of $^{29}\text{N}_2$ per mole of NO_3^- or NO_2^- reduced as a function of total NO_3^- or NO_2^- reduction. Values are means $\pm$ 1 SEM (four replicates were performed). The same symbols were used for each panel. Data were collected August 2002.

μM between 800 and 3,200 μM NO_3^- , this had no effect on the yield of $^{29}\text{N}_2$. Although total NO_3^- or NO_2^- reduction increased above 1.5 $\mu\text{mol ml}^{-1}$ of wet sediment⁻¹, production of $^{29}\text{N}_2$ did not change significantly, and in turn, the yield of $^{29}\text{N}_2$ as a proportion of total NO_3^- or NO_2^- reduction decreased (Fig. 4b and c).

The addition of $\sim 9 \mu\text{mol}$ of $^{15}\text{NH}_4^+$ to the slurries increased the soluble NH_4^+ concentration at site 1 by 150 μM , i.e., 10% (or half that predicted), which indicated that half of the label was exchanged and bound to the sediments. Throughout all the slurry experiments, the total NH_4^+ pool remained essentially constant, suggesting that NH_4^+ oxidation (and/or assimilation) was balanced by ammonification (data not shown). The $^{15}\text{NH}_4^+$ pool was diluted during these incubations, but as $^{29}\text{N}_2$ production remained linear, this dilution was not significant and did not affect our measure of anaerobic ammonium oxidation. There was no significant production of $^{30}\text{N}_2$ in the $^{15}\text{NH}_4^+$ incubations and, in addition, no ^{15}N gases were produced in slurries that had previously been autoclaved, confirming the theory that anaerobic ammonium oxidation is a biological process.

Anaerobic ammonium oxidation along the estuary. Slurries spiked with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ produced $>90\%$ $^{30}\text{N}_2$ (Table 2). With >98 atom% ^{15}N labeling of the NO_3^- pool, the predicted proportion of $^{29}\text{N}_2$ was $<2\%$; but under these conditions, $^{29}\text{N}_2$ contributed up to 9.42% of the total ^{15}N gas production at site 2, and anaerobic ammonium oxidation could account for 7.82% of total ^{15}N gas formation. Overall, there was good agreement between the rates of anaerobic ammonium oxidation measured with ^{15}N labeling of either the NH_4^+ or the NO_3^- pool (Table 2) ($P = 0.497$).

Maximum rates of anaerobic ammonium oxidation were measured at site 2 in October and November 2002; the rates then decreased significantly between sites (by analysis of variance, P was <0.05) to a minimum at site 6 at the mouth of the estuary (Fig. 5a). Denitrification rates followed a similar pattern, though the peak was found further downstream at site 3, and were significantly positively correlated with rates of anaerobic ammonium oxidation ($P < 0.05$). In a similar pattern, maximum N_2 formation via anaerobic ammonium oxidation was measured at site 2 (8%); the proportion then decreased steadily to $<1\%$ at site 6 at the mouth of the estuary (Fig. 5b). Overall, there was good positive correlation ($P < 0.05$) between sediment organic carbon content and the significance of anaerobic ammonium oxidation to N_2 formation along the estuary, although site 1 was a distinct outlier in this relationship (Fig. 5c; Table 1). In November 2002, when all six sites were visited, there was a distinct decrease in the rate of NO_3^- reduction along the estuarine gradient and a distinct shift in the fate of reduced NO_3^- (Fig. 5d). At sites 1 and 2, 60 to 70% recovery of reduced NO_3^- as ^{15}N gas (as in the initial trials at site 1 [data not shown]) suggested significant DNRA, while 100% recovery at sites 3 to 6 suggested the dominance of denitrification. Nitrite accumulated in the sediments to 3 μM during the incubations with $^{15}\text{NO}_3^-$ (800 μM) at all sites (except site 1, as in the initial trials). A large accumulation of NO_2^- (150 μM) at site 5 explains the difference between NO_3^- reduction and ^{15}N gas formation but does not necessarily suggest significant DNRA.

TABLE 2. Anaerobic ammonium oxidation measured by production of $^{29}\text{N}_2$ with ^{15}N labelling of either the NO_3^- or NH_4^+ pools

Site	Actual $^{30}\text{N}_2$ (% ^{15}N gas)	Predicted $^{29}\text{N}_2$ (% ^{15}N gas) ^a	Actual $^{29}\text{N}_2$ (% ^{15}N gas) ^b	A_{29} (% ^{15}N gas) ^c	Probability of ^{15}N from $^{15}\text{NH}_4^{+d}$	AAO ^e from:	
						$^{15}\text{NO}_3^-$ (μmol)	$^{15}\text{NH}_4^+$ (μmol)
2 Grays	90.58	1.86	9.42	7.82	0.49	0.261 (0.019)	0.296 (0.0289)
3 Gravesend	93.77	1.99	6.23	4.37	0.56	0.120 (0.0228)	0.160 (0.0164)
4 Allhallows	95.44	1.93	4.56	2.71	0.27	0.040 (0.0045)	0.026 (0.0033)
6 Grain	97.60	1.85	2.40	0.57	0.54	0.004 (0.0006)	0.003 (0.0005)

^a From >98 atom % ^{15}N - NO_3^- and random pairing of ^{14}N and ^{15}N .

^b As a proportion of total measured ^{15}N gases.

^c $A_{29} = P_{29} - D_{29}$ (see the text and reference 30).

^d $[^{15}\text{NH}_4^+]/[\text{total } \text{NH}_4^+]$.

^e Anaerobic ammonium oxidation (AAO) with ^{15}N -labeled NO_3^- ($n = 4$) or NH_4^+ ($n = 3$). Figures in parentheses are SEM.

DISCUSSION

Biogeography of anaerobic ammonium oxidation. The results presented here confirm the presence of anaerobic ammonium oxidation in another aquatic system and extend the findings recently reported for marine sediments and anoxic marine basins (2, 15, 30). However, the pattern reported here is in stark contrast to that reported for marine sediments. For example, the significance of anaerobic ammonium oxidation to N_2 production decreased along the estuarine gradient with decreasing organic content to <1% at the most seaward site (Fig. 5c; Table 1). Though a similar low proportion of anaerobic ammonium oxidation was reported at an inshore coastal site (30), an increase in the significance of anaerobic ammonium oxidation was measured with decreasing organic content and decreasing sediment reactivity in an offshore direction, i.e., in the opposite direction to that of our estuarine gradient. In addition, the maximum proportion of anaerobic ammonium oxidation to N_2 production in the Thames sediments was 8%; therefore, denitrification still dominated N_2 production overall, while up to 60% of N_2 production via anaerobic ammonium oxidation was reported to occur in offshore marine sediments (30).

Anaerobic ammonium oxidation via nitrite reduction. The sole production of $^{29}\text{N}_2$ from sediments incubated with $^{15}\text{NH}_4^+$ and either $^{14}\text{NO}_3^-$ or $^{14}\text{NO}_2^-$ agreed with the 1:1 stoichiometry of equation 3. With the stoichiometry of equation 4, if any oxidation of ammonium was directly coupled to the reduction of NO_3^- , then at least 25% of the ^{15}N -labeled gases should have been $^{30}\text{N}_2$; as this was not the case, it was concluded that the anaerobic ammonium oxidation in these estuarine sediments was coupled to the reduction of NO_2^- , which agrees with the original hypotheses and that recently reported for marine sediments (3, 30, 34).

In the presence of both electron acceptors, the concentration of NO_2^- remained essentially the same (Fig. 3c), which can be explained only if the rate of NO_2^- reduction was balanced by the supply of NO_2^- from NO_3^- reduction. Given that anaerobic ammonium oxidation could account for only 1% of NO_2^- reduction at site 1, this change remained unnoticeable. Even if obligate NO_2^- respirers, as well as anaerobic ammonium oxidizers, were present (5), their impact on the NO_2^- pool while in the presence of NO_3^- was minimal. After 8 h, NO_3^- became limiting, and the community that reduced NO_3^- changed to a community that directly reduced NO_2^- . Despite the increase in the rate of turnover, and therefore

competition, for NO_2^- , the rate of anaerobic ammonium oxidation remained unaffected. Saturation of anaerobic ammonium oxidation at low concentrations of NO_2^- would explain why there was no measurable difference between the rates measured with either trace or significant ($\sim 20 \mu\text{M}$) accumulation of NO_2^- from NO_3^- (or in the direct presence of NO_2^-) but also suggests that the availability of NO_2^- would seldom be limiting for anaerobic ammonium oxidation in estuarine sediments. Anaerobic ammonium-oxidizing bacteria are reliant on the in situ formation of NO_2^- via NO_3^- reduction, and as concentrations of NO_2^- are usually less than $5 \mu\text{M}$ in upper sediment layers (M. Trimmer, unpublished results), such an affinity is suited to scavenging NO_2^- at low concentrations. Whereas the NO_3^- - and NO_2^- -reducing community has a high capacity to reduce NO_2^- (up to $383 \mu\text{M h}^{-1}$), anaerobic ammonium oxidation appears to be saturated at a comparatively low NO_2^- concentration, which agrees with K_m estimates ($3 \mu\text{M}$) for NO_2^- consumption via anaerobic ammonium oxidation in marine sediments (3).

In the only other published environmental study of anaerobic ammonium oxidation in aquatic sediments, the vast majority (>91%) of NO_3^- or NO_2^- reduced was recovered as N_2 gas (30). This finding suggests that DNRA was insignificant and that anaerobic ammonium oxidation was reliant on NO_2^- formed intracellularly by the denitrifying community "leaking" into the sediment. In contrast, both the denitrification and DNRA pathways may support anaerobic ammonium oxidation at sites 1 and 2. In previous studies on the end products of NO_3^- reduction in organically enriched sediments, it was speculated that NO_2^- formed by DNRA may later be respired by denitrifying bacteria (11). Yet this may be an additional pathway for NO_2^- formation and, in turn, a pathway for NO_2^- coupling to anaerobic ammonium oxidation in estuarine sediments. Hence, N transformations in estuarine sediments may actually be more complex than originally thought, with potential interplay between DNRA, denitrification, and anaerobic ammonium oxidation. At the remaining sites, however, anaerobic ammonium oxidation must have been coupled to the in situ formation of NO_2^- via the denitrifying community.

The quantification of anaerobic ammonium oxidation using $^{15}\text{NO}_3^-$ is based on the assumption that the process produces only $^{28}\text{N}_2$ and $^{29}\text{N}_2$ (30). This assumption may be violated in the presence of DNRA, which, via the reduction of $^{15}\text{NO}_3^-$ to $^{15}\text{NH}_4^+$, may in turn generate $^{30}\text{N}_2$ by anaerobic ammonium oxidation. At site 2, where the contribution of anaerobic am-

FIG. 5. (a) Anaerobic ammonium oxidation (AAO) and denitrification (Den) at six sites along the Thames estuary in November 2002; (b and c) anaerobic ammonium oxidation as a proportion of total ¹⁵N gas formation in October and November 2002 (b) and as a scatter against sediment organic carbon content (c); (d) total NO_3^- reduction and ¹⁵N gas formation in November 2002. Values are means $\pm$ 1 SEM ($n = 4$). sed, sediment. Note that a large accumulation of NO_2^- at site 5 explains differences between reduction and gas formation (see the text).

monium oxidation to N_2 formation was greatest, DNRA may account for approximately 30% of the reduced $^{15}\text{NO}_3^-$. The starting concentration for $^{15}\text{NO}_3^-$ was 800 μM , and with a 50% turnover and 30% DNRA, these conditions would produce 120 μM $^{15}\text{NH}_4^+$. As only approximately 50% of $^{15}\text{NH}_4^+$ remains in the soluble pool, 60 μM $^{15}\text{NH}_4^+$ was left, which represents about 5% of the total soluble NH_4^+ pool of $\sim 1,200$ μM . At the start, the probability of $^{29}\text{N}_2$ production with $^{15}\text{NO}_3^-$ and $^{14}\text{NH}_4^+$ was 100%, but with a 50% turnover, this estimate dropped to 95%, and in turn, our estimate of anaerobic ammonium oxidation from our measured production of $^{29}\text{N}_2$ is underestimated by $\sim 5\%$. The magnitude of this viola-

tion via DNRA depends upon its contribution to NO_3^- reduction and the relative size of the nonlabeled NH_4^+ pool.

Anaerobic ammonium oxidation along the estuary. The distribution of anaerobic ammonium oxidation along the Thames estuary may simply reflect changes in the abundance of bacteria per unit of wet sediment. At site 1, despite having the highest concentrations of NO_2^- in the water column throughout the estuary, the sediments are highly reduced (visible observations) and concentrations of NO_2^- are permanently low (< 1 μM), which may in part be due to denitrification, substantial DNRA, and the reoxidation of sulfides. Such a low NO_2^- supply may be capable of sustaining only a relatively small

population of anaerobic ammonium-oxidizing bacteria; hence, anaerobic ammonium oxidation accounted for only ~1% of N_2 formation. Though anaerobic ammonium oxidation was saturated at low concentrations of NO_2^- , it accumulated at all sites except for site 1, which was a clear outlier in the overall relationship between organic content and anaerobic ammonium oxidation (Fig. 5c). At site 2, conditions must have been favorable for both anaerobic ammonium-oxidizing bacteria and denitrifying bacteria, e.g., sustained though low supplies of NO_2^- and organic substrates and NO_3^- , respectively. Despite the saturation of anaerobic ammonium oxidation at a low NO_2^- concentration, a sustained population would still be reliant on an in situ supply of NO_2^- , hence the correlation between sediment organic C and the significance of anaerobic ammonium oxidation. Higher organic loadings, i.e., the loadings at site 2, stimulated NO_3^- reduction (26) and, in turn, the in situ availability of NO_2^- , which sustains a greater population of anaerobic ammonium-oxidizing bacteria. The overall positive correlation between rates of denitrification and anaerobic ammonium oxidation supports this hypothesis, though DNRA may help support the maximum rates of anaerobic ammonium oxidation at site 2. The marked decrease in anaerobic ammonium oxidation and denitrification seaward of site 2 suggested a decline in both populations as organic C, NO_3^- , and NO_2^- decreased along the estuary.

The organism responsible for anaerobic ammonium oxidation in the original bioreactor studies has been identified as a novel planctomycete (17, 28), but the organism(s) that is responsible for anaerobic ammonium oxidation in the Thames estuary, as well as what conditions regulate its significance relative to denitrification, remains unknown. Given that the anaerobic ammonium oxidation reaction was first discovered in a sewage bioreactor (17) and that two very large sewage treatment works (STWs) (among many others) discharge into the Thames at site 1 (combined output, $1.3 \times 10^6 \text{ m}^3 \text{ day}^{-1}$), the sediments along the estuary may have been seeded by these STWs, yet the activity was most significant some 13 km downstream from the main discharge area. In addition, the well-studied classic nitrifier *Nitrosomonas eutropha* can perform anaerobic ammonium oxidation under anaerobic or O_2 -limiting conditions and, as such, is distinct from the anaerobic ammonium-oxidizing bacteria (1). Indeed, anaerobic ammonium oxidation may in fact be due to a combination of both of these metabolisms (24).

Although it is possible to measure the proportion of N_2 production from either anaerobic ammonium oxidation or denitrification in anaerobic slurries, regulation of either process maybe more complex under in situ conditions, and such measurements should be taken as estimates of potential rates. For example, anaerobic ammonium oxidation is an obligate anaerobic process, while denitrification is facultative, and because of this, denitrification is likely to occur along the gradient of decreasing oxygen concentrations and below the oxic-anoxic interface within sediments (14). Oxygen penetration (typically 0.2 to 2 mm) into intact estuarine sediment cores will directly affect the demand for NO_3^- from the overlying water and, in turn, the in situ rates of NO_2^- formation via nitrification and reduction (21, 22, 23). Oxygen penetration is likely to be more significant where the dominant source of NO_2^- and NO_3^- within sediments is that of nitrification, as in unpolluted estu-

aries or coastal seas (25, 32). While total anaerobic ammonium oxidation is unlikely to be limited by the availability of NO_2^- , oxygen penetration will affect the source of NO_2^- and overall rates of denitrification.

The organically enriched sediments of the Thames estuary, although representing only 18% of the total sediment area, are responsible for 34% of the total mineralization of organic material (33). While the maximum contribution from anaerobic ammonium oxidation to N removal was only 8%, this maximum activity occurred within these organically enriched sediments, which again highlights the significance of the depositional zone (i.e., around the turbidity maxima) to N processing—including novel pathways—in estuarine sediments.

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