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FEATURE ARTICLE

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Widespread occurrence of the anammox reaction in estuarine sediments

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ABSTRACT: We assayed sediment for the anammox reaction at 40 sites from 9 estuaries during the summer of 2004. The anammox reaction was detected at all sites with its potential contribution to the production of N_2 (*ra*, %) ranging from <1 to 11%. Overall, a higher contribution from anammox was positively correlated with both the concentration of NO_3^- in the overlying water and the organic carbon content of the sediment. Whilst the organic carbon content of the sediment decreased towards the coast, its reactivity remained essentially constant (k = 0.6 y^{-1}), which suggested that the amount of organic carbon rather than its reactivity or quality was more important. In addition, our large dataset enabled us to critically assess the 2 assays for measuring anammox ($^{15}NH_4^+$ or $^{15}NO_3^-$). While the 2 assays gave good agreement at *ra* > 3% (>2 nmol N_2 ml^{-1} wet sediment for anammox after 24 h), below this, though still detectable, anammox was underestimated with $^{15}NH_4^+$. The decrease in the estimate for anammox with $^{15}NH_4^+$ relative to $^{15}NO_3^-$ was partly explained by a decrease in the recovery of N_2 gas, probably as a result of significant dissimilatory nitrate reduction to ammonium (DNRA) in some of the slurries. The potential interference from DNRA in the anammox assay, however, would be low, with the probability of anamox making $^{30}N_2$ ($A^{30}N_2$) being about 1.4%. Our findings provide firm evidence that the anammox reaction is widespread in estuarine sediments.

KEY WORDS: Anammox · Estuary · Sediment · Organic carbon · Nitrate

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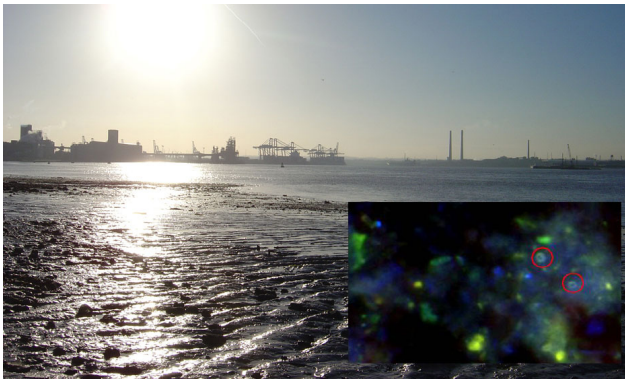

An organically enriched estuarine mudflat, combined with plentiful nitrate, provides an ideal environment for anammox bacteria. *Photos: JC Nicholls, W Mehsana*

INTRODUCTION

The discovery of anaerobic ammonium oxidation (anammox) in the laboratory and the environment has lead to a redefining of the classic nitrogen cycle (Mulder et al. 1995, Thamdrup & Dalsgaard 2002). Anammox has been shown to be widespread in coastal marine sediments (Rysgaard et al. 2004, Dalsgaard et al. 2005, Engström et al. 2005, Schmid et al. 2007), yet knowledge of its distribution and regulation in estuarine systems is comparatively scarce, with data reported from just 4 estuaries around the world so far (Trimmer et al. 2003, Risgaard-Petersen et al. 2004, Meyer et al. 2005, Rich et al. 2008). Two factors which regulate anammox are temperature for marine sediments, with greater anammox activity at lower temperatures relative to denitrification (Dalsgaard & Thamdrup 2002, Rysgaard et al. 2004), and an *in situ* supply of NO_2^- modulated by organic carbon and/or the concentration of NO_3^- in the overlying water column of estuarine (Trimmer et al. 2003, 2005, Meyer et al. 2005, Risgaard-Petersen et al. 2005) and arctic marine sediments (Rysgaard et al. 2004).

A general increase in the significance of anammox to N_2 production with increasing water depth has been shown for marine sediments (Thamdrup & Dalsgaard 2002, Engström et al. 2005), which was hypothesized to be due to limitation of denitrification by reactive organic matter in deeper waters. In our first study of

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anammox in an estuarine environment (Thames estuary, UK; Trimmer et al. 2003), we showed a clear trend of increasing contribution of anammox to N_2 (*ra*, %) with increasing organic carbon and NO_3^- further upstream. This pattern was in stark contrast with that reported for the coastal sediments, but since then has also been reported for the subtropical Logan-Albert estuary system in Australia (Meyer et al. 2005) and for parts of Chesapeake Bay (Rich et al. 2008). Meyer et al. (2005) were able to correlate the greater contribution of anammox to the production of N_2 with a greater availability of NO_2^- in the pore water. Together this corroborated a link between organic carbon and a supply of NO_2^- via NO_3^- reduction (Trimmer et al. 2003, Meyer et al. 2005). The importance of NO_3^- was confirmed by Risgaard-Petersen et al. (2004), who found no significant anammox activity in Norsminde Fjord but an *ra* of up to 22% in Randers Fjord; in Rander Fjord, NO_3^- was abundant in the water column and penetrated into the sediment, but was absent in Norsminde Fjord. Given the ubiquity of NO_3^- towards the landward end of UK estuaries, such limitation is unlikely (Nedwell et al. 2002).

The aim of the present study was to explore the distribution of anammox in the estuaries of southeast England and thus extend the knowledge of the biogeography of the anammox process in relation to the distribution of both organic carbon and water column NO_3^-.

MATERIALS AND METHODS

Sample sites and screening for anammox. Samples of sediment (oxic and suboxic layers 0 to 2 cm) and estuary water (filtered 0.2 µm Minisart Plus™, Sartorius) were collected from intertidal flats at low tide from 40 sites over 9 estuaries in southeast England in summer 2004 (Fig. 1). Sediment samples were stored in plastic bags and returned to the laboratory as quickly

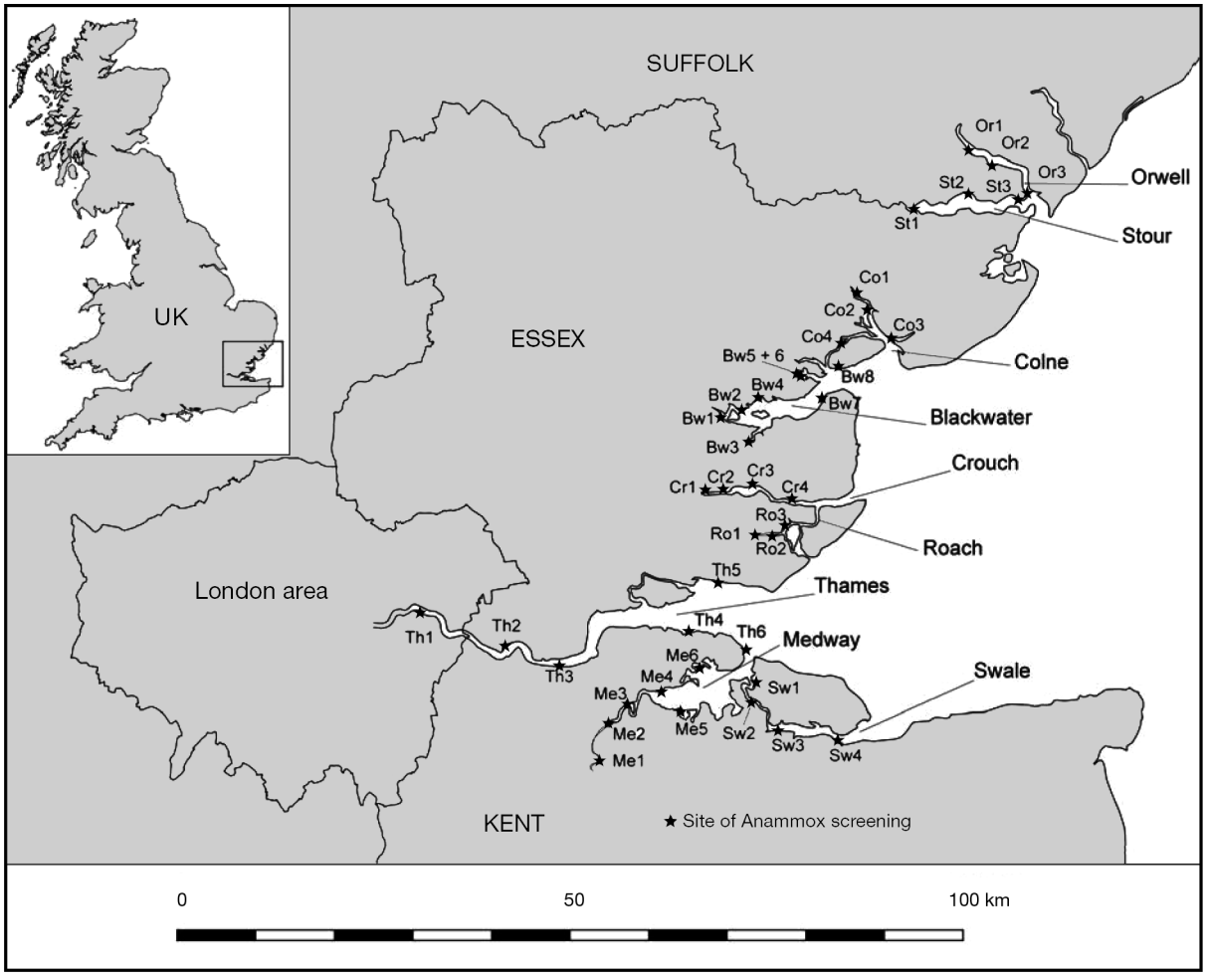

Fig. 1. Southeast England (inset on UK map), with sampling sites (★) and codes. The key to the site codes is given in Table 1

as possible. Upon return to the laboratory, water samples were measured for salinity with a hand-held refractometer, then frozen at −20°C until later nutrient analysis for NO_3^-, NO_2^- and NH_4^+ (see section 'Analyses and calculations' below). A standardised experiment was used to assay the sediment for the anammox reaction using anaerobic sediment slurries as described previously (Trimmer et al. 2003). For each site, sediment slurries (20 ml homogenised sediment, plus 14 ml of degassed low nutrient sea water [LNS] adjusted to *in situ* salinity with distilled water) were prepared and sealed in 37 ml serum bottles (Alltech Associates) inside an anaerobic glove box (Belle Technologies). Slurries were preincubated on rollers in the dark in a constant temperature room at 15°C overnight to ensure that all traces of potential oxidants (e.g. NO_3^-, NO_2^- and O_2) were removed prior to the start of any experiments.

Subsequently, the slurries were enriched by injection through the septa using microlitre syringes (Hamilton) with degassed concentrated stocks of either $^{15}NH_4Cl$ (120 mM $^{15}NH_4Cl$ [99.3 ^{15}N atom %], Sigma-Aldrich) plus $Na^{14}NO_3$ (93 mM, VWR International) or $^{14}NH_4Cl$ (120 mM $^{14}NH_4Cl$, VWR International) plus $Na^{15}NO_3$ (93 mM [99.2 ^{15}N atom %], Sigma-Aldrich) to give a final concentration of 500 µM NH_4^+ (in the case of $^{15}NH_4^+$ this represented an ~60% labelling of the NH_4^+ pool on average) and 100 µM NO_3^- or 0 in the case of controls. All of the slurries were then incubated overnight as above, after which samples of the headspace gas were collected (1 ml) using a gas-tight syringe (SGE, gastight Luer-lock syringe, Alltech Associates) and stored in water-filled gas-tight vials (12 ml Exetainer, Labco). The ^{15}N-labelling of the NH_4^+ pool was estimated by the difference in pore water concentration of NH_4^+ before (non-enriched reference) and after enrichment with ^{15}N-NH_4^+. Appropriate slurries were centrifuged, the supernatant filtered (0.2 µm, Minisart Plus™, Sartorius) and then analysed for NH_4^+ (see section ' Analyses and calculations' below).

A relative measure of sediment reactivity. We have already shown that both anammox activity and its contribution to the production of N_2 are correlated with the organic carbon content of the sediment (Trimmer et al. 2003). In the present study we wanted to test whether organic content was actually representative of reactivity across a broad range of sediment types. In a subsequent trip to the original anammox survey sites, a further 1 ml of sediment was collected from the top 1 cm of sediment (using a truncated hypodermic syringe) at 5 positions (by proportion of exposed intertidal) on the intertidal flats at 10 of the original sites (4 Medway, 3 Stour and 3 Thames, i.e. where we had measured most of the highest *ra* values) and transferred to a gas-tight vial (as above). The 50 vials were then incubated at 22°C and the headspace of each was repeatedly sampled every ~90 min for 83 h and analysed for CO_2 by gas chromatography-flame ionization detection (GC-FID) after catalytic reduction (hot nickel NiCat, Agilent Technologies) to CH_4, as described in Sanders et al. (2007). Rates of CO_2 production were calculated using linear regression as the change in amount of CO_2 as a function of time. Subsequently, each vial was opened and the sediment dried to a constant weight and treated for analysis of organic carbon content as described in the section 'Analyses and calculations' below. A relative measure of sediment or organic carbon reactivity (k) in terms of carbon turnover per unit time was calculated according to (Middelburg et al. 1996, Moodley et al. 2005):

$$k = \frac{R}{C} \quad (1)$$

where R is the measured rate of CO_2 produced per gram of dry sediment per day and C the organic carbon content of the sediment (µmol C g^{-1} dry sediment, which is not strictly a concentration). Hence the turnover of the carbon pool per day was represented by:

$$k\ d^{-1} = \frac{\mu molCO_{2prod}\ g\ dry\ sed^{-1}\ d^{-1}}{\mu molC_{Org}\ g\ dry\ sed^{-1}} \quad (2)$$

In addition, we checked whether our measurement of the accumulation of CO_2 in the headspace was representative of the majority of CO_2 produced in the sediment, i.e. what fraction, if any, accumulated as HCO_3^- and CO_3^-. To do this we repeated the assay at the seaward and landward ends of the Medway estuary and compared the change in each respective pool over 76 h. Twenty replicate 1 ml samples of sediment were placed in gas-tight vials (12 ml, as above) and the concentration of CO_2 in the headspace was measured in half of them by GC-FID; the remainder were incubated for 76 h. After measuring the initial CO_2, the vials were centrifuged at ca. 1500 × g for 3 min, and a 100 µl sample of pore water was collected and transferred to a smaller gas-tight vial (3 ml); its pH was measured using a narrow tip probe (pHC4000-8, Radiometer Analytical). All the 3 ml vials were then sealed and the headspaces sampled for CO_2 as before. Subsequently, the 100 µl of pore water was acidified (pH < 0.8) by injecting HCl (100 µl of 1 M) through each septa and the vials were then shaken vigorously for 2 min and allowed to equilibrate before again measuring the CO_2 in the headspace. This procedure was repeated on the other half of the samples after 76 h. At each point, the equilibration for CO_2 between the headspace and the pore water in the sediment and that extracted (100 µl) was calculated according to Weiss (1974).

Analyses and calculations. All nutrient analyses (NO_3^-, NO_2^- and NH_4^+) were carried out using a continuous flow autoanalyser (Skalar, SAN^{++}, De-Breda) and standard colorimetric techniques. Water content, bulk density and porosity were determined from the dry and wet weights of known volumes of sediment. Samples of this sediment were subsequently treated according to the methods of Hedges & Stern (1984) before combustion in an elemental analyser, and analysis of organic carbon via a continuous flow-isotope ratio mass spectrometer (CF-IRMS) was standardised against known quantities of urea (Delta Matt Plus, Thermo-Finnigan). Samples of the headspace (25 µl) from the gas-tight vials were injected using an autosampler into the elemental analyser interfaced with the CF-IRMS, and the mass to charge ratios for m/z 28, 29 and 30 nitrogen ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) were measured (Delta Matt Plus, Thermo-Finnigan). The total production of N_2 due to either anammox (with $^{15}NH_4^+$ and $^{15}NO_3^-$) or denitrification ($^{15}NO_3^-$) was calculated as described previously (Thamdrup & Dalsgaard 2002, Trimmer et al. 2003). Note that here we did not mea-

Table 1. Estuaries where sediment was assayed for the anammox reaction, water column (salinity, NO_3^-) and sediment (porosity, organic carbon) characteristics and the potential contribution of anammox to the production of N_2 gas (*ra*, %). Code: refers to the sites in Fig. 1; blank cells: samples inadvertently lost before analysis

Estuary	Site	Code	Salinity	NO_3^- (µM)	Porosity	Organic C (% dry wt)	Anammox (*ra*, %)
Thames	Grays	Th2	19	317	0.82	2.38	7.44
	Grays	Th2	17	314	0.82	1.99	5.01
	Gravesend	Th3	21	261		1.82	7.06
	Southend	Th5	33	23	0.62	0.77	1.45
	Southend	Th5	34	37	0.59	0.41	1.76
Medway	Wouldham	Me1	5	340	0.83	2.39	7.48
	Strood	Me3	5	216	0.73	2.01	10.93
	Hoo	Me4	29	16	0.68	1.92	4.49
	Horrid Hill	Me5	34	31	0.70	1.03	3.09
	Stoke	Me6	25	7	0.88	2.57	5.39
Swale	Queenborough	Sw1	24	65	0.80	1.30	2.41
	Kingsferry	Sw2	20	66	0.77	1.04	1.57
	Saxon Way	Sw3	30	40	0.79	1.31	2.31
	Harty Ferry (S)	Sw4	32	87	0.77	1.89	1.74
Roach	Broomhills	Ro1	5	183	0.77		1.90
	Bolts Farm	Ro2	21.5	54	0.70		0.57
	Paglesham	Ro3	23	36	0.65		1.13
Crouch	Clementsgreen	Cr1	23	36	0.83	1.77	2.92
	Fambridge	Cr2	31	44	0.79	1.09	1.37
	Althorne	Cr3	33	55	0.79	0.96	1.64
	Burnham	Cr4	35	20	0.76	0.93	2.48
Blackwater	Maldon	Bw1	31	109	0.82	2.06	7.32
	Decoy Point	Bw2	34.5	106	0.73	2.05	1.97
	Maylandsea	Bw3	34.5	13	0.66	1.26	1.86
	Goldhanger	Bw4	34.5	9	0.72	1.15	0.69
	Tollesbury	Bw5	34.5	3	0.83	1.21	0.45
	Tollesbury	Bw6	34.5	6	0.78	3.25	0.76
	Bradwell	Bw7	34.5	29	0.74	1.64	1.72
	West Mersea	Bw8	34.5	29	0.77	1.50	1.72
Colne	The Hythe	Co1	1	719	0.80	1.78	7.85
	Wivenhoe	Co2	28	212	0.81	1.51	7.56
	The Ford	Co3	26	211	0.74	1.41	5.30
	The Strood	Co4	32	62	0.80	1.65	1.07
	Brightlingsea	Co5	34.5	67	0.58	0.59	0.70
Stour	Sluice	St1	1	790	0.75	2.32	8.03
	Holbrook	St2	3	612	0.78	2.09	1.55
	Shotley Gate	St3	34.5	99	0.75	1.06	2.46
Orwell	Orwell Bridge	Or1	31	44	0.78	2.18	3.06
	Butt & Oyster	Or2	32	167	0.82	1.17	3.57
	Shotley Gate	Or3	34.5	78	0.57	0.41	0.89

sure actual rates or the specific activity of N_2 production by either anammox or denitrification but merely their respective yields of N_2 from the total reduction of either ~116 nmol of ^{14}N- or ^{15}N-NO_3^- ml^{-1} of wet sediment after incubating to completion (Risgaard-Petersen et al. 2004). The relative yield of N_2 due to anammox was then expressed as percentage of the total yield (*ra*). All statistical analyses were performed using SPSS version 15, and any data which were not normally distributed were first log_{10}-transformed before any relationships between potential controlling variables (e.g. NO_3^- and organic carbon) and anammox or reactivity were explored, using Pearson product-moment and partial correlation or linear regression where appropriate.

Table 2. Pearson and partial correlation analyses on log-transformed data between anammox (*ra*, %) and water column NO_3^-, salinity or sedimentary organic carbon. cv: controlling variable

	NO_3^- (µM)	Salinity	Organic C (% dry wt)
Pearson correlation coefficient (r)	0.621	−0.499	0.479
p	0.000	0.003	0.002
Partial correlation coefficient (r)	0.505	cv	cv
p	0.002		
Partial correlation coefficient (r)	cv	cv	0.440
p			0.008

RESULTS

Anammox potential

The anammox reaction was found at all 40 sites assayed across the 9 estuaries, with the potential contribution of anammox to N_2 production ranging from <1% (at only 5 sites) to 11% (Table 1). There were strong axial gradients of NO_3^- and organic carbon along the estuaries, with both typically negatively correlated with salinity (r = −0.673 and −0.373, p < 0.001, 0.023 and n = 40 and 37, respectively; data not shown). The anammox potential was positively correlated with both the organic carbon content of the sediment and the concentration of NO_3^- in the overlying water across all of the estuaries (Fig. 2A,B). Further exploration of the data, using partial correlation to control for organic carbon, nitrate and salinity, in turn suggested that the relationships between anammox, organic carbon and nitrate were both significant, though the relationship with NO_3^- was stronger than that for organic carbon (Table 2).

We used both the $^{15}NH_4^+$ and $^{15}NO_3^-$ assays to screen sediment for the anammox reaction, with the latter used to directly estimate the respective contributions from either anammox or denitrification to the production of N_2 (*ra*). It is important to bear in mind that our data represent a relative yield of N_2 after complete reduction of a known amount of NO_3^- (~116 nmol ml^{-1} wet sediment) and not a rate. Above 2 nmol N_2 ml^{-1} wet sediment (i.e. *ra* > 3.4%; [2/(116/2)] × 100) there

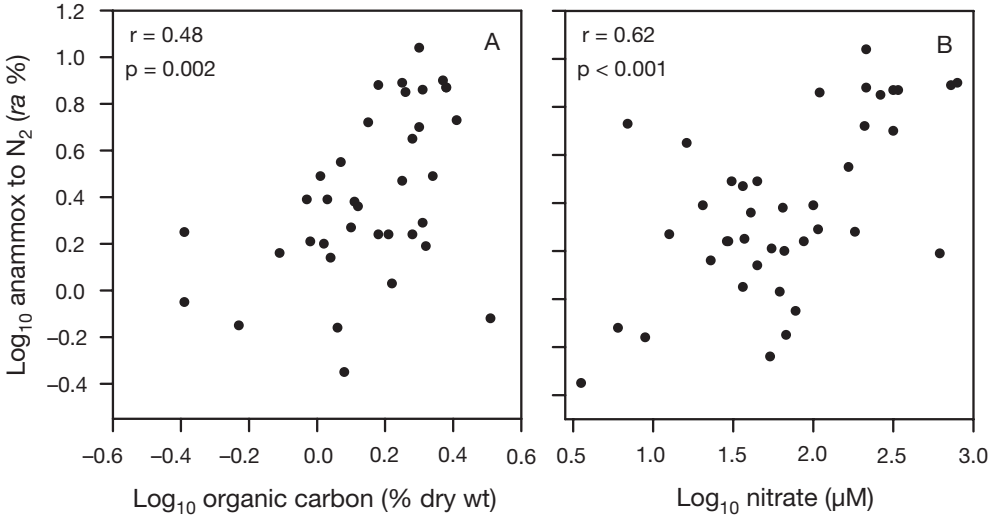

Fig. 2. Potential contribution of anammox to N_2 production (*ra*, %) scattered against (A) the organic carbon content of the sediment and (B) the concentration of nitrate in the overlying water, all as log_{10}-transformed data. The correlation coefficient r and its significance p are also shown. Partial correlations are given in Table 2

was good agreement between the 2 assays, but it was clear that $^{15}NO_3^-$ was more sensitive than $^{15}NH_4^+$ at the lower yields for anammox (Fig. 3A). Although it was still possible to detect anammox with $^{15}NH_4^+$ below a yield of 2 nmol N_2, the slope was much shallower and would underestimate the potential for anammox by ~8-fold relative to $^{15}NO_3^-$. It is worth noting that not all of the ^{15}N from $^{15}NO_3^-$ was recovered as ^{15}N gas (range = 11 to 68 %, mean = 34 ± 2, n = 40) and this loss could be indicative of considerable dissimilatory reduction of NO_3^- to ammonium (DNRA) in the slurries. Further, this loss of gas (be it $^{15}N_2$ or its analogue, $^{14}N_2$) could explain some of the change in the ratio of anammox measured with $^{15}NH_4^+$ relative to that measured with $^{15}NO_3^-$ (Fig. 3B).

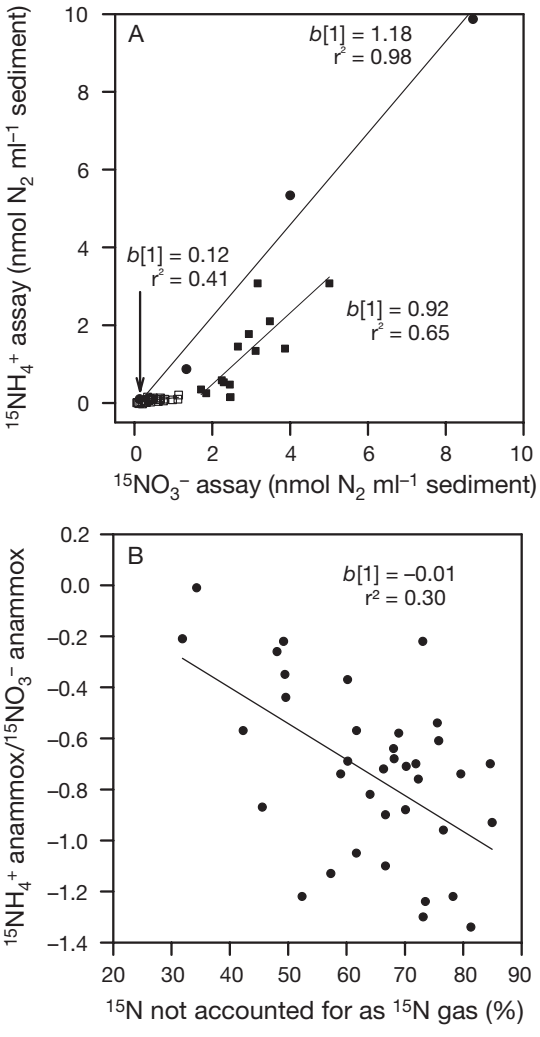

Fig. 3. Comparison between the 2 assays for measuring anammox. (A) Estimate of total anammox yield (nmol N_2 ml^{-1} wet sediment) measured with the $^{15}NH_4^+$ assay as a function of the yield measured with $^{15}NO_3^-$. (●): data from our original survey of the Thames estuary (Trimmer et al. 2003); (□, ■): data from the present study split between yields of approximately ≤2 and ≥2 nmol N_2 ml^{-1} wet sediment, respectively. (B) Ratio of anammox yield with $^{15}NH_4^+$ to $^{15}NO_3^-$ as a function of the amount of ^{15}N not accounted for as ^{15}N gas (%). The regression coefficient (r^2) and slope (b[1]) of each line is indicated; $p < 0.05$ in each case

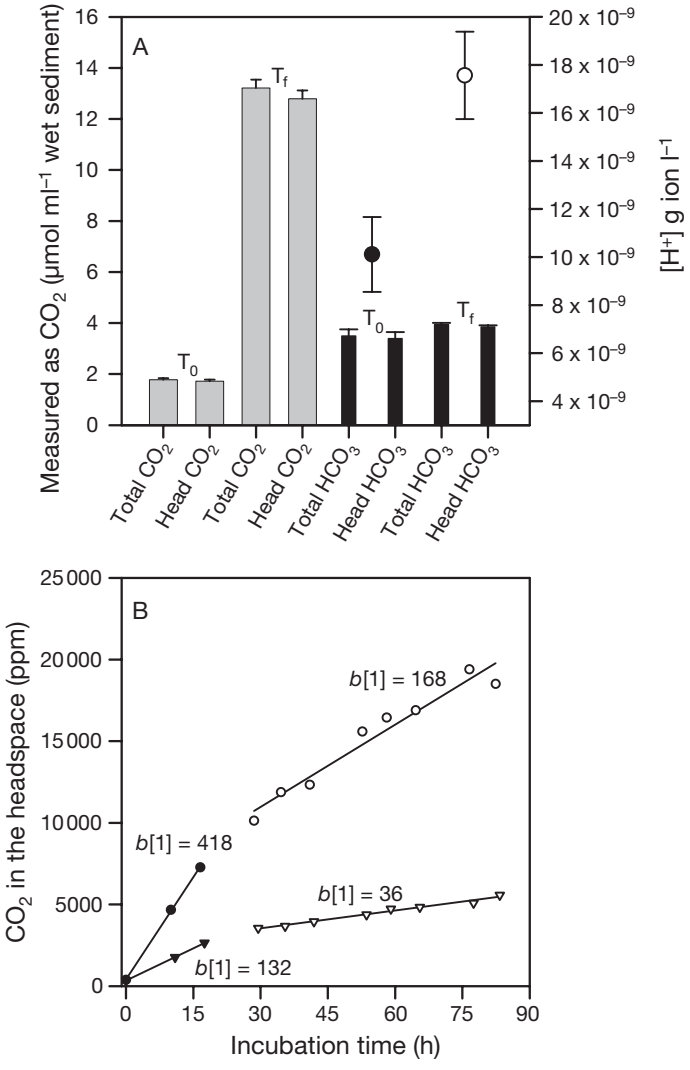

Fig. 4. Testing the simple assay for sediment reactivity. (A) Inventory of CO_2 as that measured directly as CO_2 (grey bars) in the head space (head CO_2) plus that dissolved (as CO_2) in the pore water (total CO_2) and that in 100 µl of pore water after acidification (black bars), again as CO_2 in the headspace (head HCO_3) plus that dissolved in the 100 µl of pore water (total HCO_3). The initial (T_0) and final (T_f) amounts of CO_2 after 76 h are indicated, as is the increase in H^+ ions in the pore water (decrease in pH) over the same period (●, ○). Data are from the seaward site of the Medway estuary (n = 10, ±SE). (B) Evolution of CO_2 from the sediment into the headspace of the gas-tight vials for the most (●, ○) and least (▼, ▽) active sediment, with closed and open symbols representing data during and after the first 16 h of incubation, respectively. Note the marked change in rate (slope, b[1]) after 16 h; the steady evolution over the subsequent 2 d was used to estimate rates of CO_2 production

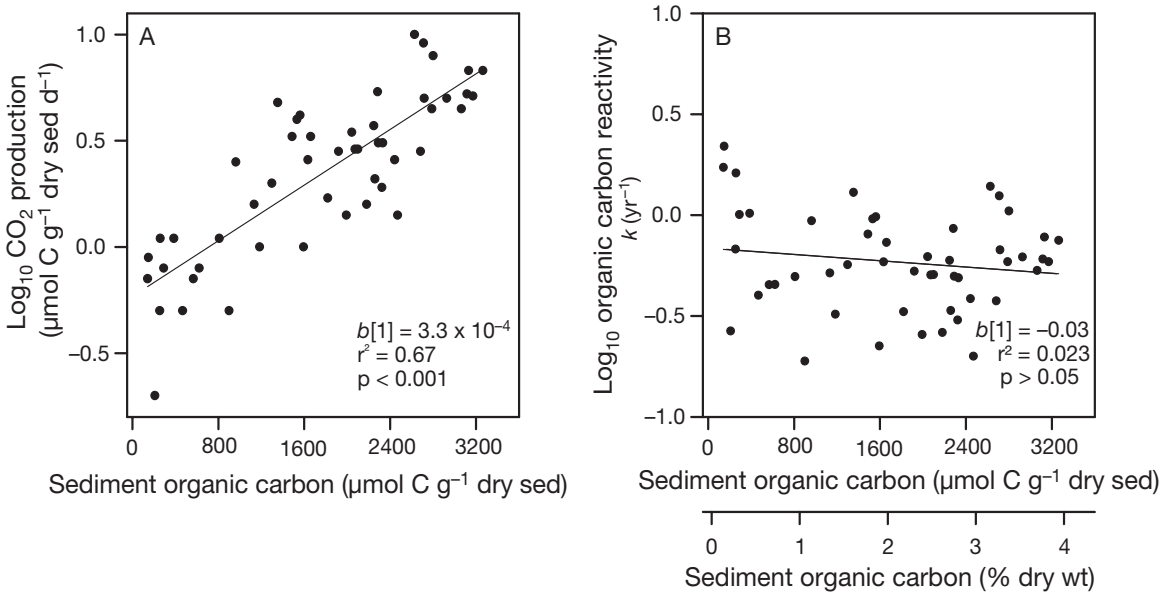

Fig. 5. Relative reactivity of organic carbon at a subsample of 10 of the original survey sites. (A) Production of CO_2 as a function of the organic carbon content of the sediment and (B) the product of these as an estimate of reactivity, *k* (here scaled to turnovers yr^{-1} to make it more readily comparable to other datasets). The *x*-axis is given in terms of molar amounts and percent for comparison. The regression coefficient (r^2), slope (*b*[1]) and p-value is indicated in each case

Organic reactivity

At each stage of the reactivity assay the vast majority (≥96%) of the CO_2 was recovered directly in the headspace (Fig. 4A, head versus total). In addition, at both the landward and seaward end of the Medway estuary, the vast majority of the measured flux (≥96%) of total dissolved inorganic carbon (CO_2, HCO_3^- and CO_3^-) was also measured directly as an accumulation of CO_2 gas in the headspace (Fig. 4A, seaward site only), with the small difference in the dissociated pool between T_0 (initial time) and T_f (final time; 76 h) not significant ($p > 0.05$). There was also a marked decrease in the pH of the pore water over the 76 h, depicted here as a significant increase in the average H^+ concentration. The release of CO_2 from the sediment was almost twice as great in the first 16 h of incubation compared to that in the remaining 55 h (Fig. 4B), and we used the second half of the time series to estimate the rates of CO_2 production.

Production of CO_2 by the sediment (at a subsample of 10 of the original sites) ranged from 0.2 to 10 µmol CO_2 g^{-1} dry sediment d^{-1} ($\log_{10}CO_{2prod} = -0.7$ to 1; Fig. 5A), with the rate increasing as a function of the organic carbon content of the sediment. As a consequence of this approximately linear relationship, the reactivity (or rate of turnover of organic carbon) was found to be approximately constant across all of the sites at 0.6 yr^{-1} (±95% CI = 0.5 to 0.7 yr^{-1}, $\log_{10}k = -0.23$; Fig. 5B).

DISCUSSION

Here we have demonstrated quite clearly that the potential for the anammox reaction is widespread in the estuaries of southeast England, but it is important to appreciate that the significance of anammox to the production of N_2 is likely to be both seasonal and potentially even greater in intact sediment cores (Trimmer et al. 2005, 2006). The simple trend in decreasing anammox contribution to N_2 production from the land to the sea found in the Thames (UK) and the Logan-Albert (Australia) estuarine systems was not observed within all the estuaries studied here, and this probably reflects differences in the morphologies of the estuaries sampled (Trimmer et al. 2003, Meyer et al. 2005, Rich et al. 2008). For example, the Thames and Logan-Albert estuaries are classic coastal plain estuaries, with clear axial gradients in NO_3^- and probably also organic carbon in the sediment, whereas the Swale and Blackwater estuaries are more complex (Fig. 1, Table 1).

Despite these differences in estuarine morphology, taken across all 9 of the estuaries, the data suggest that the anammox potential was positively related to both NO_3^- in the overlying water and the organic carbon content of the sediment (Table 2). Although these are only observational data, the maintenance of an anammox potential by both NO_3^- and organic carbon is both logical and consistent with previous data for estuarine sediments (Trimmer et al. 2003, Risgaard-Petersen et al. 2004, Meyer et al. 2005, Rich et al. 2008). For exam-

ple, it is well established that anammox is reliant on a supply of NO_2^- to fuel the oxidation of NH_4^+ and that this NO_2^- is produced within the suboxic sediment by the reduction of NO_3^- (Meyer et al. 2005). Further, Risgaard-Petersen et al. (2005) demonstrated that decreasing the concentration of NO_3^- in the overlying water from 600 µM to 5–10 µM reduced the sediment's capacity for anammox by 85%. In turn, the heterotrophic reduction of NO_3^- to NO_2^- cannot proceed without a source of electron donors. Hence, in the present study, the greatest potential for anammox was predominantly measured in the upper reaches of the estuaries, where the organic carbon content of the sediment and concentration of NO_3^- in the overlying water were both greatest, and, together, could maintain the critical supply of NO_2^-. Although others have shown the contribution of anammox to N_2 production to be independent of the concentration of NO_2^- (Thamdrup & Dalsgaard 2002, Dalsgaard & Thamdrup 2002), the overall supply of NO_2^- regulates the overall amount of anammox (Meyer et al. 2005, Trimmer et al. 2005).

Interestingly, our simple assay for comparing the reactivity of organic carbon along the estuarine gradient suggested that reactivity was approximately constant (~0.6 yr^{-1}). Such high and uniform reactivity across 3 of our estuarine systems suggested that the organic carbon was only a couple of months old (Middelburg 1989). Middelburg et al. (1996) reported an exponential drop in reactivity between salinities of 1 and 10 in the Westerscheldt estuary, but *k* was approximately uniform seaward of these sites. At their upper sites, however, the total flux of C gas (CO_2 + CH_4) was dominated by methanogenesis, which was not the case at any of our sites. In the estuaries sampled in the present study, if the reactivity of the organic carbon was constant, then the increase in CO_2 production, as a function of organic carbon content, could be linked to a greater abundance of bacteria supported by a greater availability of organic carbon. This supports the idea that it is the total availability of organic carbon, rather than its quality, coupled to a supply of NO_3^-, which maintains the potential of a sediment to support the anammox reaction in these estuaries.

We used both $^{15}NH_4^+$ and $^{15}NO_3^-$ to assay for the anammox reaction. Whereas only $^{15}NO_3^-$ can be used to directly calculate *ra*, both assays should give similar estimates for total anammox (Thamdrup and Dalsgaard 2002, Trimmer et al. 2003), although others have not always found this to be the case (Rich et al. 2008). We found good agreement between the 2 assays (in line with our initial findings; Trimmer et al. 2003) for *ra* > 3%, but a marked decrease in anammox measured with $^{15}NH_4^+$ relative to that for $^{15}NO_3^-$ at *ra* < 3% (2 nmol N_2 $m;^{-1}$ wet sediment, Fig. 3A,B). Some of this difference can be explained by simple probability. The chance of anammox making $^{29}N_2$ in the presence of $^{15}NO_3^-$ and $^{14}NH_4^+$ is 100%, i.e. all of the NH_4^+ in the sediment pool can combine with $^{15}NO_3^-$ to make $^{29}N_2$. In contrast, with $^{15}NH_4^+$, only 30 to 80% of the NH_4^+ can combine with $^{14}NO_3^-$ to produce $^{29}N_2$, and this ^{15}N-labelling of the NH_4^+ pool will be further diluted by ammonification during the incubation. In addition, the ratio of $^{15}NH_4^+$ anammox to $^{15}NO_3^-$ anammox decreased with the decrease in the recovery of ^{15}N gas (Fig. 4B), which would have exacerbated the decreased sensitivity of the $^{15}NH_4^+$ assay at the lower yields for anammox. If this loss of ^{15}N added as $^{15}NO_3^-$ was indicative of DNRA (though it may be an artefact of sediment slurries; Revsbech et al. 2006), then this could potentially complicate the measurement of anammox with $^{15}NO_3^-$. The problem arises from the potential production of $^{15}NH_4^+$ from $^{15}NO_3^-$ via DNRA which could then, in the presence of anammox, combine with $^{15}NO_2^-$ to form $A^{30}N_2$. The central tenet for the calculation of anammox is that denitrification is the only source of $^{30}N_2$ and that $P^{30}N_2 = D^{30}N_2$ (Thamdrup & Dalsgaard 2002). It should be clear that spurious production of $A^{30}N_2$ would undermine this, though the chances of this appear slight. The probability of $A^{30}N_2$ in 100 units of N_2 is given by $A^{30}N_2 = 100 \times ra \times q \times s$, where *q* is the proportion of ^{15}N in the NO_3^- pool (in our case 0.992) and *s* the ^{15}N in the NH_4^+ pool. Our average amount of missing $^{15}NO_3^-$ was 62 µM and our starting pool of $^{14}NH_4^+$ was 364 µM. If we assume a maximum *ra* in slurries of 10% (checked with $^{15}NH_4^+$ which is not interfered with) then $A^{30}N_2 = 100 \times 0.1 \times 0.992 \times 0.145$, or 1.4%, which is not likely to cause any serious interference with the assumption that $P^{30}N_2 = D^{30}N_2$.

CONCLUSIONS

The present study provides firm evidence that the anammox metabolism is widespread in estuarine sediments and that its significance is associated with both high NO_3^- in the overlying water and a greater availability of organic carbon, which, in turn, could regulate the supply of NO_2^- via the reduction of NO_3^-.

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