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Direct measurement of anaerobic ammonium oxidation (anammox) and denitrification in intact sediment cores

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ABSTRACT: Anammox, i.e. the anaerobic oxidation of NH_4^+ with NO_2^- to N_2, has redefined our understanding of nitrogen cycling in aquatic ecosystems. The isotope pairing technique (IPT) is the dominant tool for quantifying denitrification in intact sediments, but it cannot distinguish anammox from denitrification as sources of N_2 and may, where anammox is significant, lead to large errors in the estimate of true N_2 production. In a previous study, the IPT was revised in theory and a solution was proposed whereby the parameter r_{14}, i.e. the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ in the NO_3^- reduction zone is used to correct the IPT in the presence of anammox. We begin by exploring the limitations of the 2 indirect techniques previously proposed for estimating r_{14}. The first, based on the contribution of anammox to N_2 production (*ra*) in sediment slurry incubations, underestimates anammox and cannot fully correct the IPT. The second, derived from the production of ^{15}N-N_2 gas as a function of $^{15}NO_3^-$ concentration, although valid in sieved sediment, was ineffective in natural intact sediments. In contrast, a newly developed direct technique based on the ^{15}N-labelling of N_2O, corrects the IPT in the presence of significant anammox (48% of N_2 formation) in natural sediment and can even distinguish between anammox and denitrification in estuarine sediment with a lower anammox contribution (21%). We contrast these findings with sediments where anammox is minimal (<1%). The ^{15}N-N_2O technique allows denitrification and anammox to be quantified in sediment cores using techniques similar to those already established with the original IPT and, importantly, shows that the contribution of anammox to N_2 production is greater than previously measured using slurries.

KEY WORDS: Anammox · Denitrification · Sediments · Isotope pairing · ^{15}N-N_2O technique

INTRODUCTION

The discovery of anammox, i.e. the anaerobic oxidation of NH_4^+ with NO_2^- to N_2, has redefined our understanding of N cycling in aquatic ecosystems and it has been established that this novel process can contribute significantly to benthic N_2 production in marine and estuarine systems (Dalsgaard et al. 2005).

Most sediment anammox rates presented in the literature (Dalsgaard & Thamdrup 2002, Thamdrup & Dalsgaard 2002, Trimmer et al. 2003, 2005, Risgaard-Petersen et al. 2004a, Rysgaard et al. 2004) are, however, potential rates measured with slurry incubations. Although the use of anaerobic slurries was essential in discovering anammox in the environment and the data obtained so far has increased our knowledge about both the biogeography and regulation of the anammox process, their use disrupts the natural gradients of substrates and redox in sediments and thereby destroys the chemical microenvironment of the bacteria to be studied. There is, therefore, a need for techniques that can directly quantify both anammox and denitrification in intact sediments. Only measurement of anammox and denitrification in intact sediments will enable us to fully elucidate and understand the cycling of N in aquatic systems.

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Since its introduction by Nielsen in 1992, the ^{15}N isotope pairing technique (IPT) has become one of the most widely used techniques for measuring true N_2 production (i.e. p_{14} or ^{14}N production as it would occur without the addition of ^{15}N) in intact aquatic sediments (Steingruber et al. 2001). Furthermore, the IPT calculations, in conjunction with the relative ^{15}N-labelling of the NO_3^- in the overlying water, can be used to determine the contribution of coupled nitrification/denitrification (D_n) and denitrification of water-column derived NO_3^- (D_w) (Nielsen 1992). The IPT cannot, however, distinguish between anammox and denitrification as sources of N_2 production and is, therefore, not applicable for direct quantification of anammox in intact sediments. More seriously though, the presence of anammox violates the central assumptions on which the IPT is built and, as a consequence, the IPT will overestimate true N_2 production (Risgaard-Petersen et al. 2003). The magnitude of this error is positively correlated with the contribution of anammox to N_2 production (*ra*) and negatively correlated with the ratio between $^{14}NO_3^-$ and $^{15}NO_3^-$ undergoing reduction (r_{14}). Hence, the error imposed by the IPT in estimating true N_2 production is more likely to be significant in a coastal environment where *ra* is high and ambient $^{14}NO_3^-$ is low, and to increase with increasing concentrations of $^{15}NO_3^-$ (Dalsgaard et al. 2005). This may constitute a serious problem for the continued use of the IPT and there is, therefore, a need for a revision of this technique.

A recently proposed revision (Risgaard-Petersen et al. 2003, 2004b) to the classical IPT should, in theory, enable more accurate quantification of true N_2 production where anammox and denitrification coexist. Moreover, this revision can distinguish between anammox and denitrification as sources of N_2. The revised IPT (r-IPT) assay is the same as the classical IPT using additions of $^{15}NO_3^-$ to overlying water of intact sediment cores, although a more complex calculation procedure is applied. Input variables in these revised equations are rates of $^{29}N_2$ and $^{30}N_2$ production plus estimates of the ratio between $^{14}NO_3^-$ and $^{15}NO_3^-$ undergoing reduction (r_{14}).

In their original revision to the IPT, Risgaard-Petersen et al. (2003) proposed that while $^{29}N_2$ and $^{30}N_2$ production could be measured directly, the term r_{14} could only be estimated indirectly and they, in turn, suggested 2 procedures to do this. One procedure is based on the measurement of ^{15}N-N_2 production in $^{15}NO_3^-$ amended sediment cores, combined with a measurement of the contribution of anammox to total N_2 production (*ra*) estimated from slurry incubations. The other is based on measurement of ^{15}N-N_2 production as a function of increasing $^{15}NO_3^-$ concentration.

The aim of the present study was to explore the limits of this methodology and to devise necessary improvements. The study was performed in 2 steps. First, we applied the r-IPT in natural bioturbated sediments and in sediments where the fauna had been removed in order to reduce heterogeneity. Although both of the above procedures for indirectly estimating r_{14} could potentially quantify true p_{14} in these sediments, they encountered problems in the natural bioturbated sediments. We then devised a technique for directly measuring r_{14} and thereby more accurately quantifying true N_2 production from both anammox and denitrification in heterogeneous and more homogenous intact sediments. This technique is based on the assumption that denitrification, but not anammox, will produce ^{15}N-N_2O in a $^{15}NO_3^-$ sediment core experiment and that the isotopic composition of the ^{15}N-N_2O ($p^{44}N_2O$, $p^{45}N_2O$, $p^{46}N_2O$) will be binomially distributed which, as a consequence, directly reflects the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ undergoing dissimilatory reduction, i.e. r_{14}.

MATERIALS AND METHODS

Study sites and sampling. This study was conducted using 3 sediment types with a known range of potential anammox activity. Alsbäck, in the deepest part of Gullmarsfjorden (116 m), Sweden (where anammox contributes 30 to 60% of N_2 production) and 2 sites, Gravesend and Southend, in the Thames Estuary, England (where anammox contributes about 5 and <1% of N_2 production, respectively) (Trimmer et al. 2003, Engström et al. 2005). At Alsbäck, the bottom water has a salinity of ca. 34 and a temperature of 6°C. The sediment is muddy with a porosity of 0.86 (v/v). Sediment was collected from Alsbäck using an Olausen box-corer followed by direct sampling of subcores in 300 mm long Plexiglas tubes (inner diameter 55 mm). The cores were placed in an insulated box until their return to the laboratory. Another batch of surface sediment (top 2 cm) from the box cores was collected, sieved (1 mm) to remove macrofauna, and transferred to a plastic container (~500 l, 12 cm sediment depth). This reconstituted sediment was then preincubated for 3 wk in a dark room at 8°C under a laminar flow of seawater to re-establish redox and substrate gradients. Prior to the ^{15}N incubations, intact sediment subcores were sampled in Plexiglas tubes as mentioned above.

Intact sediment cores were collected at low tide from the intertidal flats at Gravesend and Southend using Plexiglas tubes and bungs and returned to the laboratory within 2 h as described by Trimmer et al. (2000). At Gravesend the sediments are very fine sands with relatively high organic matter (2.7% dry weight) and

a porosity of 0.67 (Trimmer et al. 2003). At Southend the sediments are fine sands with low organic matter (<0.5% dry wt) and porosity of 0.5 (Trimmer et al. 2003). Seawater (salinity 30) with 80 to 100 µM NO_3^- was collected from Southend at high tide for topping-up and storing the cores. For the sediment cores collected from Gravesend (salinity 17, ambient NO_3^- 200 µM), the Southend seawater was diluted with distilled water and enriched with $^{14}NO_3^-$. All cores were stored in an aerated barrel of respective water overnight at a constant 16°C.

Measuring anammox and denitrification potentials in sediment slurries. A standard procedure was used at all sites to measure anammox and denitrification potentials as described in Engström et al. (2005) and Trimmer et al. (2003). Briefly, this consists of screening preincubated anaerobic sediment for anammox activity using $^{15}NH_4^+$ and $^{14}NO_3^-$; the production of $^{29}N_2$, measured by mass spectrometry, is taken as positive proof of anammox activity. The potential contribution to N_2 formation of either anammox or denitrification is calculated from the measured production of $^{29}N_2$ and $^{30}N_2$ following enrichment and incubation with 50 to 100 µM $^{15}NO_3^-$ (Thamdrup & Dalsgaard 2002).

Measuring anammox and denitrification in intact sediment cores. In Alsbäck, the natural and sieved sediment cores were placed in groups of 8 into 4 aerated aquaria of site water (Risgaard-Petersen & Rysgaard 1995). The 4 aquaria were then amended with $^{15}NO_3^-$ (113 mM $Na^{15}NO_3$ [99.3 ^{15}N at. %] Sigma-Aldrich) to approximately 50, 100, 150 and 200 µM. The water column in each core was stirred by small magnets driven by a large external rotating magnet, and the cores were preincubated for 24 h. Water samples (10 ml) were collected before and after the addition of $^{15}NO_3^-$ and frozen for later determination of the ^{15}N-labelling of the NO_3^- pool (Dalsgaard et al. 2000).

We then sacrificed 3 of the cores (time zero, t_0) from each aquarium and slurrified these by gently mixing the sediment and overlying water with a Plexiglas rod. A slurry sample (20 ml) was carefully drawn off, allowed to overflow into gas-tight vials (Exetainer, 12 ml), fixed using formaldehyde solution (100 µl, 38% w/v), and sealed for later ^{15}N-N_2 analysis. For N_2O analysis, a larger sample of slurry (60 ml) was taken using a syringe and gently transferred to a serum bottle (125 ml), which was then sealed and crimped. The serum bottle was shaken vigorously by hand for 2 min and then connected via butyl tubing with a needle on either end to an inverted smaller serum bottle (55 ml) filled with water. The headspace in the larger bottle was then transferred to the smaller bottle by displacing the gas in the large bottle with water introduced by a needle and syringe, while the water in the smaller bottle was displaced by the gas and vented through a needle. The remaining 5 cores (time final, t_f) in each aquarium were then capped, incubated for a further 24 h and then treated as t_0.

$^{15}NO_3^-$ time series experiments with estuarine sediments. The sediment from the Thames is about 10 times more reactive than that at Alsbäck, i.e. a slurry prepared with sediment from the Thames will consume NO_3^- at a rate of 100 nmol ml^{-1} h^{-1}, compared to <10 nmol ml^{-1} h^{-1} at Alsbäck (Trimmer et al. 2003, Engström et al. 2005). It was, therefore, unnecessary to have a 24 h preincubation period or to collect such a large gas sample for N_2O analysis (see later subsection). Oxygen penetrates to 2–4 mm into the sediment at Southend (Trimmer unpubl. data) and, with a porosity of 0.5 and at 16ºC it should, in theory, take a spike of $^{15}NO_3^-$ between 0.9 and 3.4 h to diffuse to the NO_3^- reduction zone. To check that our 50 min preincubation was sufficient for $^{15}NO_3^-$ to fully mix with $^{14}NO_3^-$ in the reduction zone, and that ^{15}N-labelling remained constant with time, a single time series experiment was performed at Southend. After overnight storage the sediment cores were transferred to a single holding tank and just the overlying water in 32 cores was enriched to about 30% ^{15}N, i.e. 50 µM $^{15}NO_3^-$ (50 µl of 113 mM $Na^{15}NO_3$ [99.3 ^{15}N at. %] Sigma-Aldrich); 4 cores were left unamended as references. The overlying water was then gently mixed and the cores preincubated for 50 min. After this preincubation period a water sample (5 ml) was collected from each core, filtered (0.2 µm Minisart Plus™, Sartorius) and frozen at –20°C until later analysis. We immediately sacrificed 3 cores (t_0) and treated them as those at Alsbäck, but 2 equal-volume slurry samples (20 ml) were carefully drawn off into gas-tight vials (as in preceding subsection) for later ^{15}N-N_2 and N_2O gas analyses. The remainder of the $^{15}NO_3^-$ enriched cores were then sealed and incubated in the dark at 16°C with gentle stirring (~60 rpm) of the water column, and sacrificed in batches of 3 every hour for 6 h. The 4 reference cores were processed in the same way as the $^{15}NO_3^-$ enriched cores.

$^{15}NO_3^-$ concentration series experiment with estuarine sediments. Concentrations of ambient $^{14}NO_3^-$ are much higher in the water of the Thames estuary (100 to 600 µM) than Alsbäck (<10 µM). Hence, r_{14} is relatively high and, in the former, with the lower contribution of anammox to N_2 production in the Thames estuary (*ra* slurries <10%), any potential dependency between p_{14} and $^{15}NO_3^-$ as a result of anammox is harder to detect. Therefore, to measure any effect of anammox on the estimate of p_{14} with the original IPT in the Thames, the design of the $^{15}NO_3^-$ concentration series experiment was changed to increase the sensitivity. Cores were collected (as in the preceding paragraph for time) and the overlying water in 32 sediment cores from each site was enriched incrementally with $^{15}NO_3^-$ (7 to 224 µM

for Southend, 15 to 448 µM for Gravesend), i.e. n = 32; df = 30 for linear regression. The cores were then pre-incubated for 50 min, capped, run for 3 to 4 h, and then treated as described above, with 4 unamended cores as references.

^{15}N-N_2 and N_2O gas analyses. For N_2 analysis a headspace (2 ml analytical grade He) was introduced into the gas-tight vials using a 2-way valve and a syringe. The vials were then shaken vigorously, inverted, and stored upright at 22°C to allow N_2 to equilibrate between the water phase and headspace. Samples of the headspace (50 µl) were then injected using an auto-sampler into an elemental analyser but bypassing the reduction/oxidation columns, so that ^{15}N-labelled N_2O would not be reduced to ^{15}N-N_2. Gases were separated on the elemental analyser's GC column prior to passing to the continuous-flow isotope ratio mass spectrometer. Calibration was performed with N_2 in helium over air-equilibrated water at 22°C and the specific mass to charge ratios for m/z 28, 29 and 30 nitrogen ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) measured (Delta Matt Plus, Thermo-Finnigan). For N_2O isotope ratio analysis the gas from the 55 ml serum bottles from Alsbäck was swept, using a 2-way needle and analytical grade He, to a trace gas pre-concentrator (Cryo-Focusing, PreCon, Thermo-Finnigan). For the Thames N_2O analysis, a headspace (3 ml analytical grade He) was introduced into the gas-tight vials as above. Samples of the headspace (0.5 to 1 ml) were subsampled using a gas-tight syringe (SGE Gastight Luer Lock syringe) into an empty gas-tight vial for subsequent sweeping via the 2-way needle. The gases were then dried and scrubbed free of most of the CO_2 before being cryo-focused twice in liquid N_2 and final separation of N_2O from CO_2 on a PoraPLOT Q capillary column. The sample was then passed to the continuous-flow isotope ratio mass spectrometer (Finnigan MAT Delta^{Plus}) via an interface (ConFlo III Interface, Thermo-Finnigan) and the specific mass to charge ratios for m/z 44, 45 and 46 ($^{44}N_2O$, $^{45}N_2O$ and $^{46}N_2O$) were measured. Calibration was performed with known amounts of N_2O (98 ppm; Scientific and Technical Gases) over the range 0.41 to 13.25 nmol N_2O ($\sum$ $^{44}N_2O$, $^{45}N_2O$ and $^{46}N_2O$) and was linear between 0.8 and 99 pmol for $^{45}N_2O$ and $^{46}N_2O$. Here, for clarity, we refer to ^{15}N-labelled N_2O as $^{45}N_2O$ and $^{46}N_2O$, and not as $^{29}N_2{}^{16}O$ and $^{30}N_2{}^{16}O$.

Calculations. We used 2 different procedures to estimate true N_2 production (p_{14}): the IPT (Nielsen 1992) and the revised IPT proposed by (Risgaard-Petersen et al. 2003). The IPT estimates N_2 production as:

$$p_{14}IPT = \frac{p^{29}N_2}{2 \cdot p^{30}N_2} \cdot (2 \cdot p^{30}N_2 + p^{29}N_2) \quad (1)$$

where $p^{29}N_2$ and $p^{30}N_2$ are production rates of $^{29}N_2$ and $^{30}N_2$, respectively. The revised IPT estimates total N_2 production (N_2 from both anammox and denitrification) as:

$$\text{r-IPT}\, p_{14} = 2r_{14} \cdot (p^{29}N_2 + p^{30}N_2 \cdot (1 - r_{14})) \quad (2)$$

and anammox (p_{14} AAO) as

$$p_{14}\, AAO = 2r_{14} \cdot (p^{29}N_2 - 2 \cdot r_{14} \cdot p^{30}N_2) \quad (3)$$

Denitrification is then estimated as the difference between Eqs. (2) and (3). As above, $p^{29}N_2$ and $p^{30}N_2$ are the production of $^{29}N_2$ and $^{30}N_2$ after $^{15}NO_3^-$ amendment and r_{14} is the ratio between $^{14}NO_3^-$ and $^{15}NO_3^-$ in the NO_3^- reduction zone. In Eqs. (1), (2) and (3), $p^{29}N_2$ and $p^{30}N_2$ are directly quantifiable using mass spectrometry; the difference being that Eqs. (2) and (3) hinge on the parameter r_{14}. Here, 3 different methods were used to estimate r_{14}: firstly 2 indirect methods proposed by (Risgaard-Petersen et al. 2003), and then a third, more direct, method.

Method 1: Here r_{14} is calculated from the contribution of anammox to total N_2 production (*ra*) measured in anaerobic slurries and $p^{29}N_2$ and $p^{30}N_2$ according to:

$$r_{14} = \frac{(1 - ra) \cdot (\frac{p^{29}N_2}{p^{30}N_2}) - ra}{(2 - ra)} \quad (4)$$

Method 2: Here r_{14} is calculated using $p^{29}N_2$ and $p^{30}N_2$ measured in sediment cores amended with a range of $^{15}NO_3^-$ concentrations according to:

$$r_{14}{}^{(1)} = \frac{p^{29}N_2{}^{(1)} - V \cdot p^{29}N_2{}^{(2)}}{2 \cdot (p^{30}N_2{}^{(1)} - V^2 \cdot p^{30}N_2{}^{(2)})} \quad (5)$$

where $p^{29}N_2{}^{(1)}$ and $p^{30}N_2{}^{(1)}$ are the production of $^{29}N_2$ and $^{30}N_2$ in Incubation 1 and $p^{29}N_2{}^{(2)}$ and $p^{30}N_2{}^{(2)}$ are the production of $^{29}N_2$ and $^{30}N_2$ in Incubation 2 — a parallel incubation with different $^{15}NO_3^-$ concentrations in the overlying water column. V is the ratio between the $^{15}NO_3^-$ concentration in the water column in Incubations 1 and 2, which can be estimated from the production of ^{15}N gas in the 2 incubations:

$$V = \frac{p^{29}N_2{}^{(1)} + 2 \cdot p^{30}N_2{}^{(1)}}{p^{29}N_2{}^{(2)} + 2 \cdot p^{30}N_2{}^{(2)}} \quad (6)$$

Note that in the paper of Risgaard-Petersen et al. (2003), Eq. (5) is not correct. The correct equation is given in Risgaard-Petersen et al. (2004b).

Method 3: Here r_{14} is estimated from the ^{15}N-labelling of the N_2O produced during the incubation, i.e. $^{14}N^{15}N_2O$ ($p^{45}N_2O$) and $^{15}N^{15}N_2O$ ($p^{46}N_2O$). For the purposes of this technique, denitrification is assumed to be the only quantitative significant source of ^{15}N-N_2O in a $^{15}NO_3^-$ labelling experiment (see 'Results and Discussion' for full rationale). If the ratio between $^{15}NO_3^-$ and $^{14}NO_3^-$ is constant in the NO_3^- reduction zone, the produced isotopic N_2O species will be bi-

nomially distributed (equivalent to the fundamental assumption of the IPT regarding the distribution of ^{15}N-N_2 isotopic species) and the ratio between $p^{45}N_2O$ and $p^{46}N_2O$ can be expressed as:

$$\frac{p^{45}N_2O}{p^{46}N_2O} = \frac{2 \cdot x \cdot y}{y^2} = 2 \cdot r_{14} \quad (7)$$

where x and y are the mole fractions of $^{14}NO_3^-$ and $^{15}NO_3^-$, respectively in the NO_3^- reduction zone (e.g. $x = {}^{14}NO_3^- / [{}^{14}NO_3^- + {}^{15}NO_3^-]$), and r_{14} is the ratio between $^{14}NO_3^-$ and $^{15}NO_3^-$ in the NO_3^- reduction zone. Rearranging Eq. (7), r_{14} can be expressed as

$$r_{14} = \frac{p^{45}N_2O}{2 \cdot p^{46}N_2O} \quad (8)$$

The term r_{14} can be converted into a more familiar parameter which is useful when interpreting these calculations, i.e. q. The term q is the proportion of ^{15}N in the total N gas pool and is directly related to r_{14}:

$$q = \frac{1}{r_{14} + 1} \quad (9)$$

Using q enables better statistical analysis of the data, since q is constrained between 0 and 1, where r_{14} is not and, therefore, the data are better suited to regression analysis. The term q is calculated for both ^{15}N gas species, i.e. q N_2 vs. q N_2O, and subjected to regression analysis. If the slope deviates significantly from 1, then the change in the predicted distribution of ^{15}N is taken as being due to anammox. To use the q N_2 vs. q N_2O slope effectively, it is essential to have a good spread of data, too few replicates or too few $^{15}NO_3^-$ concentrations, and/or incubations with very low ambient $^{14}NO_3^-$ concentrations are likely to give an inaccurate slope. The slope of q N_2 vs. q N_2O is directly related to *ra*, i.e. the contribution of anammox to N_2 production according to:

$$ra = \frac{2 - 2 \cdot \text{slope}}{2 - \text{slope}} \quad (10)$$

and can be used to illustrate the effects of anammox on the distribution of ^{15}N in both the N_2 and N_2O pools.

RESULTS AND DISCUSSION

Evaluation of Methods 1 and 2: indirect quantification of r_{14} from *ra* and *V* in a $^{15}NO_3^-$ concentration series

According to the data from the slurry incubations in Alsbäck, anammox accounted for 36 and 40% of total N_2 production in the natural and the sieved sediment, respectively, which agreed well with previous measurements for this site (Engström 2004). True N_2 production (p_{14}) estimated with the original IPT was dependent on the $^{15}NO_3^-$ concentration in the water column for both the sieved (Fig. 1A) and the natural (Fig. 1B) sediment. The effect of bioturbation on the accuracy of each estimate of p_{14} was clear from the difference in the errors bars for the respective graphs (Fig. 1). Such a dependency between p_{14} and $^{15}NO_3^-$ is to be expected, since the IPT includes $^{29}N_2$ production from anammox in the calculation of ^{14}N-N_2 production (p_{14}) and this production represents an additional oxidation of $^{14}NH_4^+$, which would not have taken place in the absence of $^{15}NO_3^-$ addition (Risgaard-Petersen et al. 2003). Although using method 1 (*ra*) in the r-IPT reduced the estimate of p_{14} compared to that estimated with the original IPT, there was still dependency be-

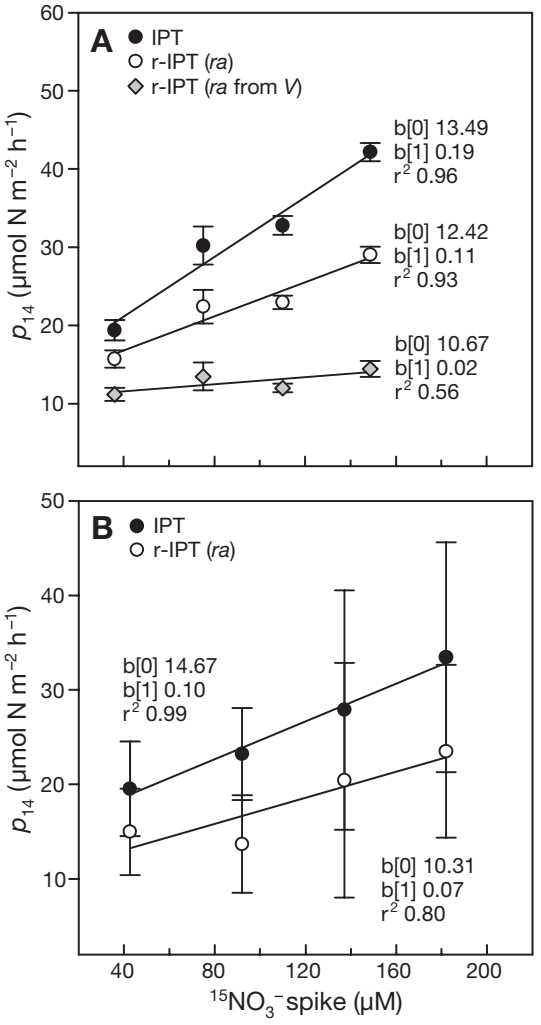

Fig. 1. Estimated (means ± 1 SEM; n = 5) true production of N_2 gas (p_{14}) in (A) sieved sediment using original isotope pairing technique (IPT) and r-IPT corrected using Method 1 (*ra* from slurries) and combination of Methods 1 and 2 (*ra* from *V*), and (B) natural sediment from Alsbäck, using original IPT and r-IPT corrected using Method 1 (*ra* from slurries) only. Regression constants: *b*[0] = intercept, *b*[1] = slope. See 'Materials and methods' for parameter details

tween p_{14} and $^{15}NO_3^-$ (Fig. 1). Theoretically, however, such a correlation should not be seen, because in Eq. (2) the 'false' $^{29}N_2$ production from anammox should have been taken into account.

Calculating the r-IPT with Method 2 (*V*) produced negative values for p_{14} in the natural sediment (data not shown), probably because of excessive sediment heterogeneity and, whereas this method assumes equal *ra* for each core, it became clear that *ra* varied across the cores. In light of this, it was only possible to apply the r-IPT with Method 2 to the sieved sediment from Alsbäck and, accordingly, p_{14} ranged from 5 to 22 µmol N m^{-2} h^{-1} for all combinations of the different $^{15}NO_3^-$ concentrations and was, on average, 12.8 ± 2.2 (SEM) µmol N m^{-2} h^{-1} (data not shown). Anammox ranged from 3.8 to 11 µmol N m^{-2} h^{-1}, with an average of 7.6 ± 0.95 (SEM) µmol N m^{-2} h^{-1}, which suggests that anammox accounted for approximately 59% of N_2 production, i.e. somewhat more than inferred from the slurry incubations (see next subsection). Using the *ra* value of 0.59 derived with Method 2 (*V*) as input to Eq. (4) (i.e. Method 1) for estimation of r_{14} for the individual cores, we then recalculated p_{14} for the sieved sediment using the r-IPT (Eq. 2). This resulted in no significant dependency (p = 0.13) between the estimated value of p_{14} and the concentration of $^{15}NO_3^-$ (Fig. 1A) and is indicative of a valid result (Risgaard-Petersen et al. 2003). This suggests that the slurry method underestimated the actual contribution of anammox to N_2 production (*ra*) and thereby did not fully take into account the 'false' $^{29}N_2$ production from anammox.

Thus the results from the first half of the study support the use of a $^{15}NO_3^-$ concentration series experiment to estimate p_{14} from anammox and denitrification in sediments where the processes coexist. This method, however, requires minimal sediment heterogeneity and this criteria was not met in the natural sediment from Alsbäck. To measure true N_2 production from both anammox and denitrification in this sediment, and probably in most natural bioturbated sediment, an alternative procedure is required.

Evaluation of Method 3: quantification of r_{14} from the ^{15}N isotopic signature of N_2O

If there was no anammox and denitrification was solely responsible for the production of ^{15}N-N_2 and ^{15}N-N_2O, then it is valid to calculate r_{14} from ^{15}N-N_2, that is:

$$r_{14} = \frac{p^{29}N_2}{2 \cdot p^{30}N_2} \quad (11)$$

(Risgaard-Petersen et al. 2003) and, consequently, r_{14} would be the same regardless of which ^{15}N-N gas species it was calculated from. In the presence of anammox, however, r_{14} estimated from $^{29}N_2$ and $^{30}N_2$ would be numerically higher than that estimated from ^{15}N-N_2O, because the $^{29}N_2$ from anammox represents induced oxidation of $^{14}NH_4^+$ from the addition of $^{15}NO_3^-$, which does not occur in denitrification.

The natural sediment from Alsbäck produced ^{15}N-N_2O in the presence of $^{15}NO_3^-$ (Fig. 2A), which could be used to independently calculate r_{14}. On average, r_{14} calculated from ^{15}N-N_2O was 0.47 ± 0.06 (SEM) and was significantly (paired *t*-test: $p < 0.001$) lower than r_{14} calculated from ^{15}N-N_2 (1.21 ± 0.22). As the site has significant anammox activity (Engström 2004, Engström et al. 2005), this finding agrees well with the expectations outlined above. The correction term r_{14}

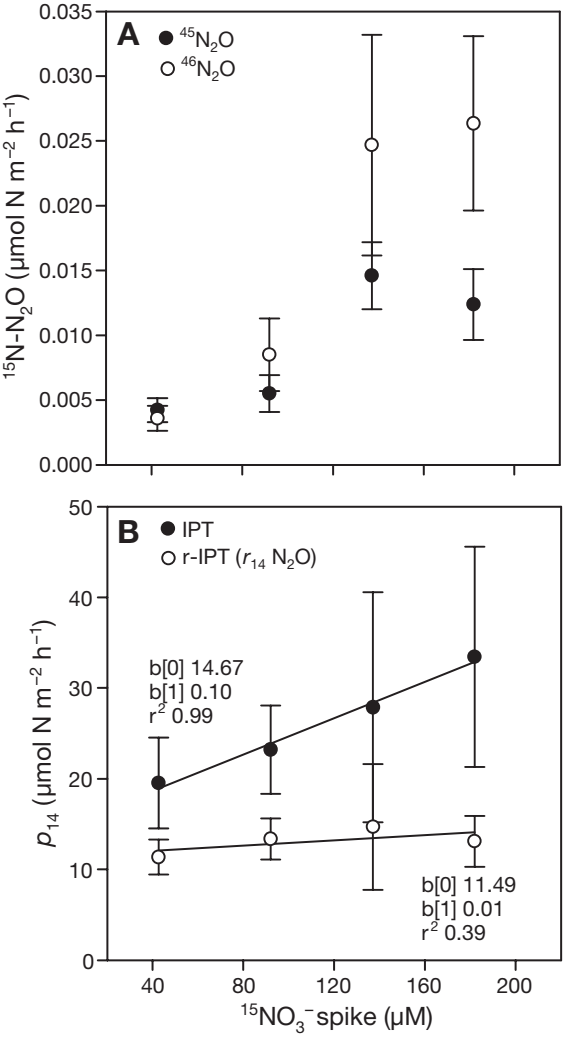

Fig. 2. Use of ^{15}N-N_2O to derive direct measure of r_{14} for use in Method 3 in natural sediment from Alsbäck, Sweden. (A) Production of ^{15}N-N_2O as a function of $^{15}NO_3^-$ addition; (B) estimates of true production of N_2 gas (p_{14}) as a function of $^{15}NO_3^-$ addition using original IPT and r-IPT corrected using Method 3. Values are means ± 1 SEM (n = 5); regression constants: *b*[0] = intercept, *b*[1] = slope

from ^{15}N-N_2O was calculated using Eq. (8) for each individual core and used as input to Eq. (2). This produced near constant values for p_{14} that were virtually independent from the $^{15}NO_3^-$ added (Fig. 2B). Furthermore the estimate of p_{14} (12.72 ± 1.47 (SEM) µmol N m^{-2} h^{-1}) was 50% lower ($p < 0.001$) than the estimate obtained with the classical IPT (26.01 ± 4.50 (SEM) µmol N m^{-2} h^{-1}) (Table 1). Importantly, Method 3 corrects the IPT even in the natural and heavily bioturbated sediments of Alsbäck and reduces the variation around the estimate of p_{14}. This is primarily due to the fact that each measure of r_{14} is derived directly from ^{15}N gas production in each core, whereas the indirect estimate (Method 1) is based on the assumption that *ra* is constant for all cores, irrespective of clear heterogeneity (*ra* estimated in individual cores using the N_2O method ranged from 30 to 76%). Of the average p_{14} of 12.72 µmol N m^{-2} h^{-1}, anammox and denitrification accounted for 6.64 and 6.08 µmol N m^{-2} h^{-1}, respectively, although average *ra* from all cores was 48% (Table 1).

Anammox apparently contributed more to N_2 production in intact cores relative to the slurry experiments. With the combination of Methods 1 and 2 in the first Alsbäck experiment, with sieved sediment, anammox accounted for 59% of N_2 production in intact sediment compared to 42% in slurries, and, with Method 3, 48% for intact natural sediment compared to 36% in slurries. This is an important finding and highlights the need for a technique to measure anammox and denitrification in intact sediment cores. Recently published estimates of r-IPT p_{14} in intact cores relied upon Method 1 and the generation of *ra* from slurries to correct for anammox (Risgaard-Petersen et al. 2004a, Rysgaard et al. 2004). In most cases these estimates of p_{14} showed that there was no significant difference between the original IPT p_{14} and r-IPT p_{14} using *ra*; however, it is likely that these were underestimates of *ra* and therefore p_{14} was not sufficiently corrected. The slurry-based method for measuring *ra* integrates the activity of denitrifiers and anammox bacteria in a volume of sediment and the facultative denitrifiers are, therefore, likely to be overrepresented in anaerobic slurries prepared from mixed aerobic and anaerobic strata. In contrast, this distribution will be more accurately captured by whole-core incubations. In addition, in slurries enriched with 100 µM NO_3^-, transient concentrations of NO_2^- can be generated, which may exceed the optimal NO_2^- concentration for anammox (Trimmer et al. 2005).

Time series experiment in the Thames estuary

The production of ^{15}N-labelled N_2 and N_2O was linear throughout the 6 h incubation at Southend (Fig. 3A, B) and, importantly, the parameter r_{14} (derived from the ^{15}N distribution in the labelled N_2O) was constant with time in these sediments (Fig. 3C: $r^2 = 0.08$, $p = 0.214$). p_{14} was calculated using both the original IPT expression (Eq. 1) and r-IPT (Eq. 2) and there was very good agreement between these 2 estimates for p_{14} (Fig. 3D), with no significant difference between either the intercepts ($b[0]$: $p = 0.706$) or slopes ($b[1]$: $p = 0.983$) (ANCOVA). Under these conditions the application of Nielsen's classic IPT to estimate p_{14} is still perfectly valid and, as there is only a negligible contribution to N_2 production from anammox at this site, this is what we expected. In addition, this experiment confirms that denitrification is the only source of ^{15}N-N_2O production in these estuarine sediments because, if this were not the case, the r-IPT-estimate would deviate from that of the classic IPT, which was clearly not the case (Fig. 3D).

Table 1. Estimates (means ± 1 SEM) of true N_2 production p_{14}; (µmol N m^{-2} h^{-1}) from $^{15}NO_3^-$ concentration series experiments in intact sediment cores, using original isotope pairing technique (IPT_{orig}) calculation and revised IPT (*r*-IPT) with Method 3 to determine r_{14} (ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ in NO_3^- reduction zone). *ra*: contribution of anammox to N_2 production; na: not applicable; Note: mean p_{14} from Alsbäck has been calculated across all concentrations, despite the dependency of p_{14} on $^{15}NO_3^-$, as this represents what would be taken as the mean were the IPT to be applied in its original form

Site	p_{14}		n	*t*-statistic	p	p_{14} *r*-IPT		*ra*
	IPT_{orig}	*r*-IPT total				anammox	denitrification	%
Alsbäck	26.01 ± 4.50	12.72 ± 1.47	18	4.11	<0.001	6.64 ± 1.17	6.08 ± 0.62	48 ± 4
Southend	73.63 ± 2.85	na	29	na	na	na	na	0
Gravesend	290.76 ± 17.68	241.84 ± 18.20	30	9.65	<0.001	48.94 ± 4.40	192.90 ± 16.03	21 ± 2

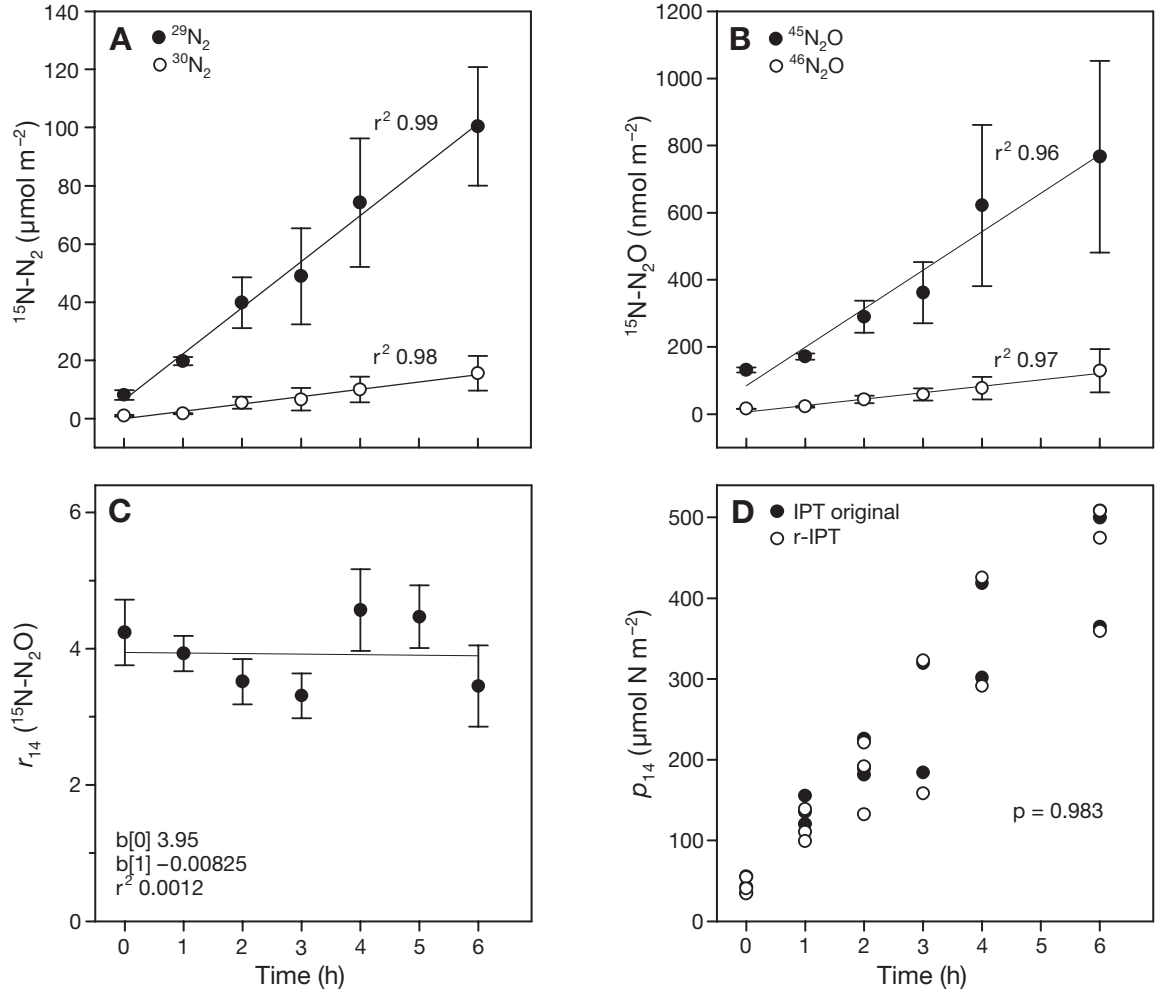

Fig. 3. Time series incubations for sediment with minimal anammox activity from Southend in the Thames estuary. (A, B) Production of $^{29}N_2$ and $^{30}N_2$ and $^{45}N_2O$ and $^{46}N_2O$ as a function of time, respectively; (C) r_{14} derived from ^{15}N labelling of N_2O as a function of time; (D) estimates of true production of N_2 gas (p_{14}) as a function of time using original IPT and the r-IPT corrected using Method 3. Values are means ± 1 SEM (n = 4); regression constants: *b*[0] = intercept, *b*[1] = slope

Concentration series experiments in the Thames estuary

The results for Alsbäck showed that a direct measure of r_{14} from ^{15}N-N_2O using the r-IPT could remove the dependency between p_{14} and $^{15}NO_3^-$, and we wanted to show that this held where anammox was less significant. Although there was a significant correlation between the production of ^{15}N-N_2 gas at both Southend and Gravesend with $^{15}NO_3^-$ concentration (r^2 = 0.58 $p < 0.001$, $r^2 = 0.50$ $p < 0.001$, respectively), as would be expected, there was no such relationship with ^{15}N-N_2O production. Given that N_2O is an intermediate in the denitrification pathway and that it is, in turn, metabolised to N_2, such a correlation between ^{15}N-N_2O production and $^{15}NO_3^-$ addition need not be expected in the more reactive sediments of the Thames estuary. The power of this approach (Method 3) lies in the ability to discern a difference between the distribution of ^{15}N in both the N_2 and N_2O pools, and the actual pattern of production is irrelevant. Using the parameter *q* and by rearrangement of Eq.(10) it is possible to predict the expected deviation from 1 in the slope *q* N_2 vs. *q* N_2O at any *ra*. The expected slopes for a range of *ra* (*ra* % = 0, 25, 50, 75) are shown in Fig. 4A. Previous estimates of the contribution of anammox from slurry incubations at Southend and Gravesend were < 1 and 5%, respectively (Trimmer et al. 2003). The *q* N_2 vs. *q* N_2O plots of the data from Southend and Gravesend, together with the expected *q* N_2 vs. *q* N_2O plots from the slurry estimate of *ra* are shown in Fig. 4B and C, respectively. At Southend, the slope is not significantly different from 1 (*b*[1] = 1.05 ± 0.10 (95% CI), df = 28, $t = 1.81$, $p > 0.05$), there is no difference in the distribution of ^{15}N in the two ^{15}N gas species, and p_{14} using the

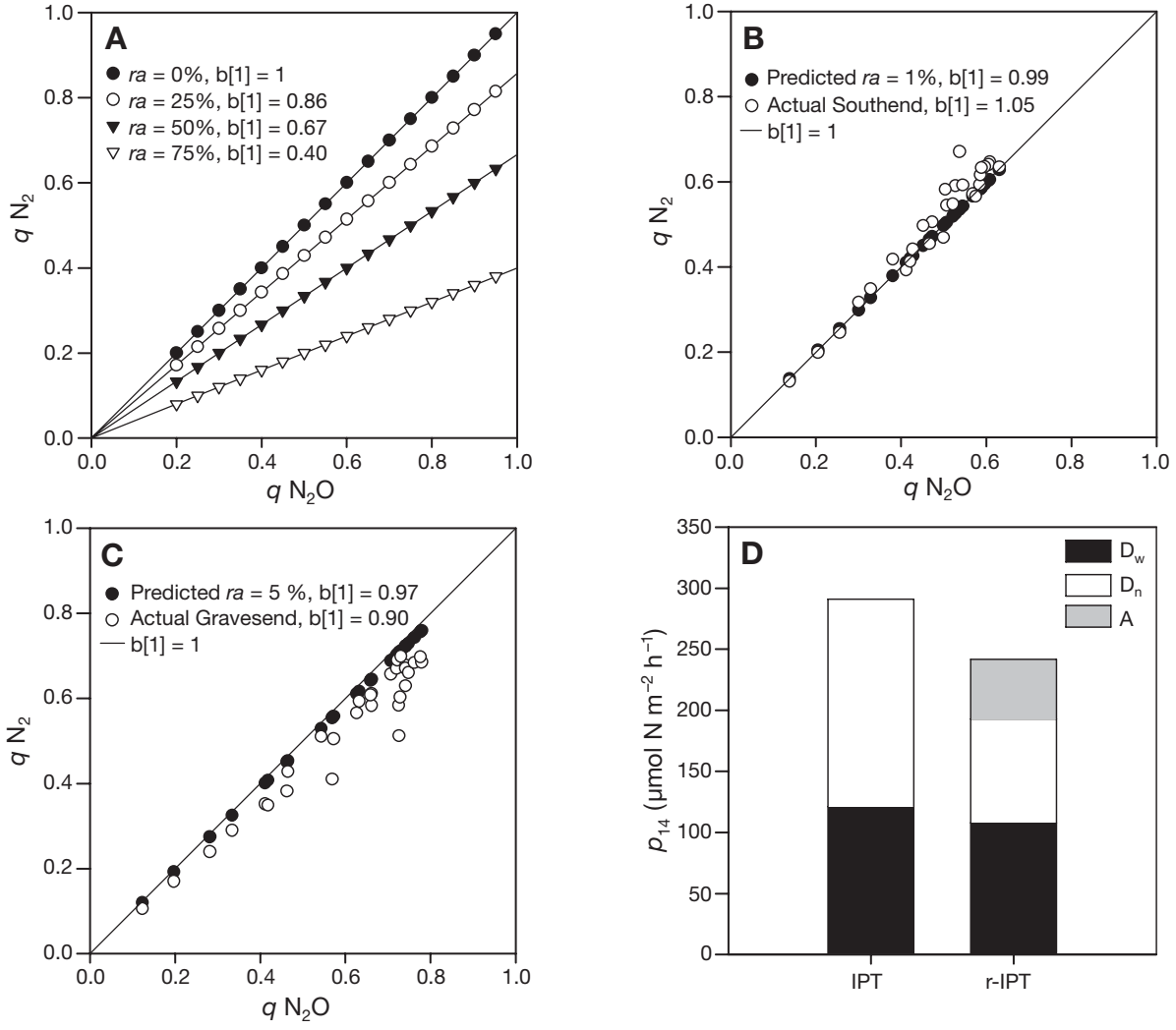

Fig. 4. Concentration series incubations for sediment with minimal and low anammox activity from the Thames estuary. (A) Theoretical deviations in $q\,N_2$ vs. $q\,N_2O$ slope (b[1]) with changing ra; (B) scatter of $q\,N_2$ vs. $q\,N_2O$ at Southend as predicted with ra from slurries, actual data and b[1] = 1 for comparison; (C) scatter of $q\,N_2$ vs. $q\,N_2O$ at Gravesend as predicted with ra from slurries, actual data and b[1] = 1 for comparison; (D) estimates of true production of N_2 gas (p_{14}) at Gravesend and subsequent distribution into D_w, D_n and anammox, using original IPT and r-IPT corrected with Method 3

original IPT was, on average, 73.63 ± 2.85 (SEM) µmol N m^{-2} h^{-1} (Table 1).

In contrast, at Gravesend, where anammox has been shown to be significant, the slope of the 2 derivations of q was significantly different from 1 (b[1] = 0.90 ± 0.09 (95% CI), df = 29, t = 2.43, $p < 0.05$) and, more importantly, significantly different from the data at Southend ($p < 0.001$, ANCOVA). Unlike Alsbäck (Fig. 1), however, there was no significant dependency between p_{14} and $^{15}NO_3^-$ (b[1]: $p > 0.05$). As previously discussed, in an estuarine environment, where r_{14} is high and ra is low, this deviation is unlikely to be visibly apparent (Risgaard-Petersen et al. 2003). However, using Method 3, the estimate for p_{14} with the r-IPT of 241.84 ± 18.20 (SEM) is significantly lower than the original IPT p_{14} estimate of 290.76 ± 17.68 (SEM) (paired t test, $p < 0.001$) (Table 1) and, hence, the production of false $^{29}N_2$ from anammox is enough to cause the original IPT to overestimate p_{14}. The percentage contribution of anammox to N_2 production in intact sediment was calculated to be 21.36 ± 1.65 (SEM), which is greater than the previous estimate from slurries of 5%.

In the presence of anammox, an additional problem with the original IPT, apart from overestimating p_{14} and not accounting for anammox, is that of overestimation of coupled nitrification–denitrification (D_n), since part of the 'false' $^{29}N_2$ could be interpreted as D_n rather than anammox. As stated above, however, this excess $^{29}N_2$ represents neither true anammox nor denitrification. The distributions of p_{14} as D_n, D_w and anammox

from Gravesend, calculated using the equations of Nielsen (1992) with and without the IPT revision, are shown in Fig. 4D. On average, the original IPT calculation overestimated D_n by 100%, clearly illustrating the significance of the problem.

Clearly the robustness of our r_{14} ^{15}N-N_2O technique (Method 3) hinges on denitrification being the only significant source of ^{15}N-N_2O from $^{15}NO_3^-$, and we have argued that the good agreement between the estimates for p_{14} with either the r-IPT or the original IPT at Southend supports this (Fig. 3D). In principle, however, isotopic discrimination during denitrification could violate our central assumption. Middelburg et al. (1996) dealt with the potential impact of ^{15}N discrimination during the application of the IPT technique and showed that it would affect the results at most by <0.5%, with a discrimination factor of 40‰. Our situation is slightly different from this, since we are using differences in the distribution of ^{15}N in the ^{15}N-N_2 pool and ^{15}N-N_2O pool to discern an effect of anammox on this distribution and, in theory, this difference could be affected by isotopic fractionation. On average, the fractionation between NO_3^- and N_2 is 24‰ and therefore, between N_2O and N_2, it is approximately 12 to 13‰ (Barford et al. 1999). A simple comparison between this magnitude of fractionation (12‰, i.e. 0.012) and the confidence intervals (90‰, i.e. 0.090) for q N_2 vs. q N_2O for the data from Southend and Gravesend shows that any effect from fractionation is unlikely to be discernible in our estimates of r_{14}. It could also be argued that N_2O production during nitrification could affect our estimate of r_{14}. Shaw et al. (2006) showed that *Nitrosospira* spp. produced N_2O under aerobic conditions and that 50% of the resultant ^{15}N-N_2O must have come from a coupling between $^{15}NO_2^-$ and $^{14}NH_4^+$, although it represented ~0.2% of the net NO_2^- production. If this were happening to any appreciable degree in our sediments, then nitrification-based ^{15}N-N_2O production would increase $r_{14}(N_2O)$ and $r_{14}N_2O$ would exceed r_{14} 'true', i.e. our estimate of the ratio between $^{14}NO_3^-$ to $^{15}NO_3^-$ in the NO_3^- reduction zone would be wrong. We know that nitrification is significant at Southend, with 50% of denitrification being fuelled by nitrification, i.e. D_n (Trimmer et al. 2000), and it is implicit in Fig. 3C (i.e. r_{14} [N_2 or N_2O] equals 3.95 with only 30% ^{15}N in the overlying water). Even with 50% of the $^{29}N_2$ production (Fig. 3A) coming from D_n, and 0.2% of this yielding $^{45}N_2O$, this could only explain about 14% of the measured amount of $^{45}N_2O$ (Fig. 3B). Quite simply, however, the data in Fig. 3D show that any effect from nitrification is minimal; otherwise the 2 estimates of p_{14} would deviate from each other, which they do not. In addition, any overestimate of r_{14}, i.e. $r_{14}N_2O > r_{14}$ true, would underestimate anammox (*ra*) and anaerobic slurry incubations would give higher estimates for *ra* than those in intact cores; yet we see the opposite trend, despite the fact that nitrification is relatively high at both Gravesend (Fig. 4D) and Alsbäck (where pore water $NO_3^- \geq$ water column NO_3^-). Hence, aerobic production of $^{15}N_2O$ from $^{15}NO_2^-$ can be ignored in practice.

The production of N_2O during NO_3^- or NO_2^- reduction to ammonium (DNRA) has also been reported in pure culture (Smith & Zimmerman 1981, Smith 1982). However, N_2O production dropped by 90% when NO_2^- changed from 15 000 to 150 µM in these pure culture experiments, and the highest NO_2^- concentration we have ever measured in sediments is 16 µM. We suggest, therefore, that this process is negligible. Further, ^{15}N gas can account for 100% of the $^{15}NO_3^-$ reduced in our slurry incubations (Trimmer et al. 2003), showing that DNRA is insignificant in the sediment studied here. Obviously, N_2O production via anammox would weaken our case and it would underestimate anammox with Method 3, although (according to present knowledge) anammox does not produce N_2O or, if it does, only in trace amounts (i.e. <0.01% of the anammox rate) that may be due to NO_2^- toxicity at high concentrations (van de Graaf et al. 1997, Strous et al. 1998).

CONCLUSION

In the present study we have explored the limits of a methodology for measuring anammox and denitrification in intact cores proposed in a previous study (Risgaard-Petersen et al. 2003), and our findings with data for sediments with contrasting anammox activity fully support the theoretical proposals for correcting the IPT. However, the methodology originally proposed by Risgaard-Petersen et al. (2003) for quantifying the central input parameter r_{14} based on Method 2 (V), although effective, has obvious limitations. The derivation of the parameter *ra* and, in turn, r_{14} (Method 1) from sediment slurry incubations has been shown not to be applicable to intact sediment cores, since it significantly underestimates the contribution from anammox to N_2 production and, therefore, cannot correct the IPT. Previous estimates of p_{14} using *ra* from slurries (Risgaard-Petersen et al. 2004a, Rysgaard et al. 2004) are likely to have been overestimates, and therefore, this study represents the first true measurements of p_{14} from intact sediment cores where anammox and denitrification coexist. The ^{15}N-N_2O-based methodology presented here represents a much more rigorous approach, as it enables direct measurements of r_{14} in individual cores. This technique, in combination with the general revised procedure for calculation of true N_2 production (p_{14}: Eq. 2), allows denitrification (and sub-

sequently D_n and D_w) and anammox to be quantified in intact sediments using similar techniques to those already established with the original IPT.

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