

Application of the isotope pairing technique in sediments where anammox and denitrification coexist

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Abstract

The isotope pairing technique (IPT) is a well-established ¹⁵N method for estimation of denitrification. Presence of anammox, the anaerobic oxidation of NH₄⁺ to N₂ with NO₂⁻ results in violation of central assumptions on which the IPT is built. It is shown that anammox activity causes overestimation of the N₂ production calculated by the IPT. However, experiments with different additions of ¹⁵NO₃⁻ will reveal the problems posed by anammox. Two alternative calculation procedures are presented, which enable a more accurate quantification of anammox and denitrification activity in sediments where the processes coexist. One procedure is based on measurements of ¹⁵N-N₂ production in ¹⁵NO_x⁻-amended intact sediment cores and data addressing the contribution of anammox to total N₂ production estimated from slurry incubations. The other procedure is based on measurements of ¹⁵N₂ production in at least two parallel series of sediment cores incubated with different ¹⁵NO_x⁻ additions. The calculation procedure presented is used on field data from four studies where the IPT was used and the potential anammox rate measured. The IPT overestimated total ¹⁴N-N₂ production rates by 0%, 2.5%, 31%, and 82% relative to the revised estimates from the 4 different sites, where anammox accounted for 0%, 6%, 18%, and 69.8%, respectively, of N₂ production. The overestimation of true denitrification was, however, up to several hundred percent. Our analysis suggests however that the IPT does not seriously overestimate N₂ production in estuarine sediments because anammox accounts for <6% of N₂ production in such sediments, according to present knowledge.

The isotope pairing technique (IPT) (Nielsen 1992) is a well-established ¹⁵N technique for estimating denitrification of bottom-water NO₃⁻ + NO₂⁻ (NO_x⁻) and of NO_x⁻ produced via sedimentary nitrification. During the past decade, this technique has been used in numerous studies, including temperate (Cabrita and Brotas 2000; Dong et al. 2000; Ogilvie et al. 1997; Ottosen et al. 2001; Trimmer et al. 2000a), tropical (Kristensen et al. 1998), and arctic marine sediments (Glud et al. 1998; Glud et al. 2000; Rysgaard et al. 1998), lake sediments (Mengis et al. 1997; Risgaard-Petersen et al. 1999; Svensson et al. 2001), rivers (Pind et al. 1997;

Trimmer et al. 2000b), and wetlands (Davidsson et al. 1997; Hoffmann et al. 2000; Stepanauskas et al. 1996). A review of the method and its applications is given by Steingruber et al. (2001).

The IPT aims to quantify the genuine production rate of N₂ gas, i.e., ¹⁴N-N₂ production as it would occur without the addition of ¹⁵NO_x⁻. According to the technique, this corresponds to the production rate of ²⁸N₂ (¹⁴N¹⁴N) times 2 plus the production rate of ²⁹N₂ (¹⁴N¹⁵N) after addition of ¹⁵NO_x⁻ to the system. This production (*p*₁₄) is estimated from the production rates of ²⁹N₂ (*p*²⁹N₂) and ³⁰N₂ (*p*³⁰N₂) using the following expression of Nielsen (1992):

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

The supposition that the genuine production rate of N₂ equals 2 · ²⁸N₂ + ²⁹N₂ production and that this production can be calculated from Eq. 1 is based on a number of assumptions. First, it is assumed that addition of ¹⁵NO₃⁻ does not affect the production of ¹⁴N-N₂. This assumption is valid if denitrification is

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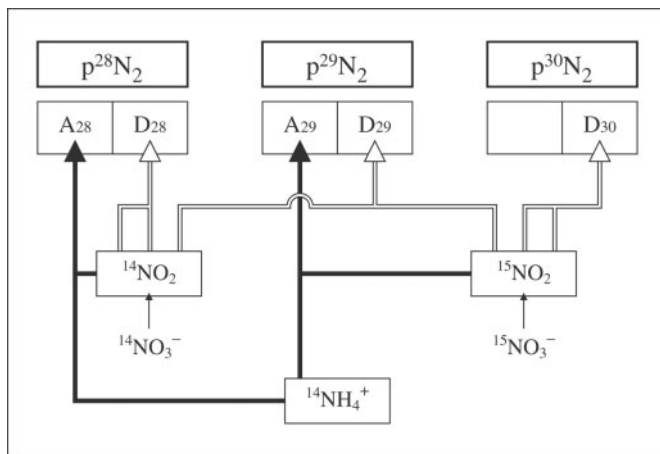


Fig. 1. Distribution of N_2 isotopes in ^{15}N labeling experiments where both anammox and denitrification occurs. A_{28} and A_{29} are the produced pools of $^{28}N_2$ and $^{29}N_2$ originating from anammox, whereas D_{28} , D_{29} , and D_{30} denote the pools of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ produced via denitrification. $p^{28}N_2$, $p^{29}N_2$, and $p^{30}N_2$ are the integrated pools of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$, respectively. Dark arrows represent the anammox process and light arrows denitrification. Thin arrows represent NO_3^- reduction.

the only N_2 -producing process and if the process is NO_x^- limited. Under these conditions, $^{15}NO_x^-$ addition will not affect the rate of $^{14}NO_x^-$ reduction but only the manner in which reduced $^{14}NO_x^-$ is distributed between $^{28}N_2$ and $^{29}N_2$. Second, it is assumed that the three isotopic N_2 species produced, $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$, are binomially distributed (Nielsen 1992). This requirement is met in denitrifying environments if the ratio between $^{15}NO_x^-$ and $^{14}NO_x^-$ is constant throughout the NO_x^- reduction zone, which is the third assumption of the IPT.

Several tests including time and $^{15}NO_3^-$ concentration series are available as documentation for these critical assumptions. If the assumptions hold, the results of the IPT should demonstrate the following: There should be a linear relationship between the concentration of added $^{15}NO_x^-$ and the rate of $^{15}N-N_2$ production. The production of $^{14}N-N_2$ as estimated from Eq. 1 should, however, be independent of the amount of $^{15}NO_x^-$ added (see Nielsen and Glud 1996; Rysgaard et al. 1995; Dong et al. 2000). Comparison of the rates measured in identical experimental systems, where denitrification was presumed to be the only N_2 -producing process, has shown good agreement between estimates obtained by the IPT, the N_2 flux method (Risgaard-Petersen et al. 1998a), and the N_2/Ar method (Eyre et al. 2002), respectively. This is a strong indication of the validity of the technique.

Anammox represents an alternative N_2 -producing process, where NH_4^+ is oxidized to N_2 with NO_2^- under strictly anoxic conditions (Strous et al. 1997). The process was first discovered in a wastewater treatment plant by Mulder et al. (1995), and recent studies have shown that anammox may be a significant process in marine sediments too, where it can account

for more than 60% of anaerobic N_2 production (Thamdrup and Dalsgaard 2002).

Presence of anammox is a challenge to the isotope pairing technique, because it leads to violation of both the assumption of independence between added $^{15}NO_3^-$ and $^{14}N-N_2$ production and the assumption of binomial distribution of produced $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$.

Bacteria with anammox capacity will only produce two of three possible isotopic N_2 species, $^{28}N_2$ and $^{29}N_2$, following addition of $^{15}NO_x^-$ (Fig. 1). In systems where denitrification and anammox coexist, the resultant pool of N_2 produced after $^{15}NO_x^-$ addition will therefore be a mixture of pools with different isotopic compositions (Fig. 1) and binomial distribution of produced $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ can no longer be assumed. This implies that the genuine production rate of N_2 estimated as $2 \cdot ^{28}N_2 + ^{29}N_2$ production cannot be calculated from Eq. 1.

Violation of the assumption of independence between the amount of added $^{15}NO_3^-$ and $^{14}N-N_2$ production can be illustrated with the following example: Consider a sediment without nitrification and without NO_x^- in the overlying water. In such a system, no N_2 production would take place. If there is a capacity for denitrification alone, addition of $^{15}NO_x^-$ will result in production of only $^{30}N_2$. In this case, the IPT will correctly estimate the $^{14}N-N_2$ production rate as zero from Eq. 1. However, if the capacity for both anammox and denitrification exists, $^{15}NO_x^-$ addition will result in production of both $^{29}N_2$ and $^{30}N_2$ (Fig. 1), and the IPT would erroneously estimate a $^{14}N-N_2$ production according to Eq. 1. The problem is more general. In systems exhibiting anammox where anaerobic N_2 production is limited by the availability of NO_x^- , which is a prerequisite for extrapolation from ^{15}N tracer results to the unamended state, $^{15}NO_x^-$ addition will increase the rate of $^{14}NH_4^+$ oxidation via the reaction $^{14}NH_4^+ + ^{15}NO_2^- \rightarrow ^{29}N_2$ (Fig. 1). The alternative isotopic N_2 species, $^{28}N_2$, is produced by the reaction $^{14}NH_4^+ + ^{14}NO_2^- \rightarrow ^{28}N_2$. In such a system, the two reactions are independent of one another, and the rate of $^{28}N_2$ production therefore remains constant whereas the production of $^{29}N_2$ increases as more $^{15}NO_x^-$ is added. This implies that $^{14}N-N_2$ production estimated as $2 \cdot ^{28}N_2 + ^{29}N_2$ is correlated with the amount of $^{15}NO_x^-$ added and therefore does not reflect genuine N_2 production of the nonmanipulated sediment.

In the present study, we focus on solutions to the problems associated with the application of the IPT in sediments where anammox and denitrification coexist. We develop mathematical expressions that enable evaluation of results obtained with the IPT, and we furthermore develop alternative calculation procedures that enable quantification of $^{14}N-N_2$ production from data obtained through isotope pairing experiments. It should be stressed that these equations are based on a set of assumptions, and we cannot guarantee that these are valid in all systems because of the limited present knowledge of the distribution and regulation of anammox. However, the equations facilitate test and quality assurance procedures that can be applied to accept or reject results.

Table 1. List of variables used in the equation system

Parameter	Designation
p_{14}	Genuine N_2 production (units N units time ⁻¹)
p_{14IPT}	Genuine N_2 production (units N units time ⁻¹) estimated with the classical IPT
$p^{29}N_2$	Total production of $^{29}N_2$
$p^{30}N_2$	Total production of $^{30}N_2$
D_{28}	Production of $^{28}N_2$ via denitrification
D_{29}	Production of $^{29}N_2$ via denitrification
D_{30}	Production of $^{30}N_2$ via denitrification
A_{28}	Production of $^{28}N_2$ via anammox
A_{29}	Production of $^{29}N_2$ via anammox
r_{14}	Ratio between $^{14}NO_x^-$ and $^{15}NO_x^-$ in the NO_x^- reduction zone
r_{14W}	Ratio between $^{14}NO_x^-$ and $^{15}NO_x^-$ in the water column
ra	Contribution of anammox to N_2 production
V	Ratio between $^{15}NO_x^-$ concentration in the water for two different incubations with different $^{15}NO_x^-$ amendments
R_{29}	Ratio between $p^{29}N_2$ and $p^{30}N_2$

Within the concepts developed, we evaluate some of the very limited data comprising information on both anammox and N_2 production measured by isotope pairing. These data originate from studies in the Skagerrak (Risgaard et al. 2001; Thamdrup and Dalsgaard 2002); two shallow-water Danish estuaries: Norsminde Fjord and Randers Fjord (Risgaard-Petersen et al. pers. comm. unref.); and sediments from the high-arctic fjord Young Sound, Greenland (Risgaard et al. pers. comm. unref.).

Materials and procedures

Definitions and assumptions—In the following discussion, we will use a series of terms that are listed in Table 1. A central variable in our discussion is p_{14} . This variable represents genuine N_2 production expressed in units of produced N atoms per unit time, i.e., the ^{14}N - N_2 production which, in the IPT, is presumed to be unaffected by addition of $^{15}NO_3^-$.

Our discussion is based on the following assumptions:

1. The ratio between ^{15}N -labeled and unlabeled NO_x^- species in the NO_x^- reduction zone is constant after $^{15}NO_x^-$ amendment. As mentioned above, this assumption is also central to the IPT and according to results from $^{15}NO_3^-$ concentration series experiments (Nielsen and Glud 1996; Risgaard et al. 1995; Dong et al. 2000; Eyre et al. 2002), it appears to be valid for a variety of sediments. The assumption implies that any isotopic N_2 species produced via denitrification or anammox can be estimated from the mole fraction of ^{15}N in NO_x^- undergoing dissimilatory NO_x^- reduction and any other N_2 isotopic species produced via a matching NO_x^- reduction process (see Appendix 1).

2. The mole fraction of ^{15}N in the NO_3^- and NO_2^- pools undergoing dissimilatory reduction is similar. That is, we assume that NO_x^- is supplied to anoxic sediment strata mainly as NO_3^- and then reduced further to N_2 with NO_2^- as a free intermediate. Direct transfer of NO_2^- between aerobic ammonia oxidation and anammox/denitrification is thus

assumed to be insignificant. The absence of this shunt is also central in the application of the IPT in sediments without anammox because it will not be traced by $^{15}NO_3^-$ amendment, leading to underestimation of denitrification. The good agreement between estimates obtained by the isotope pairing, N_2 -flux, and N_2/Ar methods (Risgaard-Petersen et al. 1998; Eyre et al. 2002), when applied in identical experimental setups, suggests that direct NO_2^- transfer between aerobic ammonia oxidation and anammox/denitrification is insignificant compared with the supply of NO_3^- . Porewater profiles of NO_2^- measured in biofilms with NO_2^- microsensors (de Beer 2000) in freshwater sediments using fine-scale porewater extraction methods and microsensors (Stief et al. 2002) and in marine sediments using biosensors (Meyer and Risgaard-Petersen in prep. unref.) indicate that net NO_2^- production takes place mainly in the anoxic zone of the sediment, as a result of NO_3^- reduction. The data from Stief et al. (2002) and Meyer and Risgaard-Petersen (in prep. unref.) also suggest a very efficient capacity for aerobic NO_2^- oxidation, leading to no net transport of NO_2^- to the anaerobic sediment strata. In addition, Stief et al. (2002) observed an increased anaerobic NO_2^- production after addition of NO_3^- to the overlying water. Thus, there is good support for the assumption that $^{15}NO_3^-$ addition leads to the formation of similar mole fractions of ^{15}N in both the NO_3^- and NO_2^- pools.

However, if the concentration of NO_2^- in the water column is sufficiently high, it may penetrate the oxic zone, thus leading to anaerobic gas formation in the anoxic zone (Dong et al. 2002). If $^{15}NO_3^-$ is used as a tracer in such systems the mole fraction of ^{15}N in the NO_3^- and NO_2^- pools in the anoxic sediment strata will not be similar.

3. Anammox and denitrification are both limited by the supply of NO_3^- from above the oxic–anoxic interface. In this case, addition of $^{15}NO_x^-$ will result in an increase in both anammox and denitrification activity and will neither affect

^{14}N - N_2 production via denitrification (i.e., $2 \cdot ^{28}\text{N}_2 + ^{29}\text{N}_2$) nor $^{28}\text{N}_2$ production via anammox. This assumption is confirmed with respect to denitrification in a variety of environments where anammox probably plays an insignificant role by the demonstration of a linear response of ^{15}N - N_2 production rates to $^{15}\text{NO}_x^-$ amendment and constant ^{14}N - N_2 production rates (Nielsen and Glud 1996; Rysgaard et al. 1995; Dong et al. 2000; Eyre et al. 2002). In situ control of anammox and denitrification in sediments exhibiting significant anammox activity is poorly described, and no data are available at present on NO_x^- dependency of the processes in intact sediments. However, porewater profiles of NH_4^+ measured in earlier studies of Skagerrak sediments (Canfield et al. 1993) where a significant capacity for anammox has been reported (Thamdrup and Dalsgaard 2002) do not indicate that NH_4^+ supply is limiting to the anammox process, because NH_4^+ was found in the oxic zone and in the NO_x^- reduction zone in concentrations between 10 and 20 μM . The K_m -value for NH_4^+ in anammox bacteria has been reported to be less than 5 μM (Strous et al. 1999). Because addition of NO_x^- will expand the NO_x^- reduction zone as shown in microsensors studies (Christensen et al. 1989), bacteria with anammox capacity situated in deeper and more NH_4^+ rich strata (Thamdrup and Dalsgaard 2002) will be exposed, resulting in an increase of overall depth-integrated activity. We assume in this argument that the uptake kinetics for denitrifiers and bacteria with capacity for anammox are similar, and thereby that NO_3^- addition will not change the proportion between anammox and denitrification. The data from Dalsgaard and Thamdrup (2002) indicate that this presumption is valid. In a series of slurry experiments, these authors observed that K_m for NO_2^- reduction in sediments was $<3 \mu\text{M}$. Nitrite reduction in these sediments was due to both anammox and denitrification, and according to Dalsgaard and Thamdrup (2002), the proportion between the processes was constant from the beginning of their experiment to the end, where NO_2^- was depleted.

4. Nitrification is not affected by the addition of $^{15}\text{NO}_x^-$. In sediments with efficient anammox, it is possible that an increase in anammox activity in response to NO_3^- addition (see above) will reduce the flux of NH_4^+ into the oxic zone. This may lead to NH_4^+ limitation of nitrification and thereby to a reduced N_2 production coupled with nitrification. However, moderate NO_3^- additions will probably only have a minor impact on the NH_4^+ flux into the oxic zone and thus will not seriously affect the nitrification process. According to data of Dalsgaard and Thamdrup (2002), the anammox process does not deplete NH_4^+ in slurry incubations. In earlier studies of these sediments (Station S9, Skagerrak), Canfield et al. (1993) observed that C-oxidation and consequently NH_4^+ production was mainly the result of Mn reduction, suggesting that NH_4^+ for anammox and nitrification is mainly supplied from below the O_2 and NO_x^- reduction zones. This may indicate that NH_4^+ is in excess and that a stimulation of the rate of anammox will have only a minor impact on the flux of NH_4^+ into the oxic zone.

The error imposed on the IPT in determination of sediment N_2 production—In this section, we present a more formal discussion of the consequences of anammox for the estimate of N_2 production obtained by the IPT. Here we investigate the difference between the true rate and the estimate of N_2 production obtained by the IPT.

According to Nielsen (1992), the isotope pairing technique estimates genuine N_2 production (p_{14}) from the production rates of $^{29}\text{N}_2$ ($p^{29}\text{N}_2$) and $^{30}\text{N}_2$ ($p^{30}\text{N}_2$):

$$p_{14IPT} = \frac{(D_{29} + A_{29})}{2 \cdot D_{30}} \cdot (2 \cdot D_{30} + D_{29} + A_{29}) \quad (\text{see Eq. 1 and Appendix 1}).$$

If anammox is present, the measured production of $^{29}\text{N}_2$ will integrate the $^{29}\text{N}_2$ production from both denitrification and anammox, whereas the measured $^{30}\text{N}_2$ production only represents denitrification. Thus, in these situations $p^{29}\text{N}_2$ is the sum of $^{29}\text{N}_2$ production from anammox (A_{29}) and denitrification (D_{29}) whereas $p^{30}\text{N}_2$ is the production of $^{30}\text{N}_2$ from denitrification alone (D_{30}) (Fig. 1). Eq. 1 then becomes

$$\begin{aligned} p_{14IPT} &= \frac{(D_{29} + A_{29})}{2 \cdot D_{30}} \cdot (2 \cdot D_{30} + D_{29} + A_{29}) \\ &= \frac{D_{29}}{2 \cdot D_{30}} \cdot (2 \cdot D_{30} + D_{29}) + \left[\frac{A_{29} \cdot (2 \cdot D_{30} + A_{29} + 2 \cdot D_{29})}{2 \cdot D_{30}} \right]. \end{aligned} \quad (2)$$

Assuming that it was practically possible to distinguish between the N_2 production rates resulting from anammox and denitrification, the correct estimate of p_{14} would be the sum of ^{14}N - N_2 production from denitrification and anammox, respectively. This production corresponds to twice the $^{28}\text{N}_2$ formed via denitrification (D_{28}) plus the $^{29}\text{N}_2$ production resulting from denitrification (D_{29}) plus twice the $^{28}\text{N}_2$ production from anammox (A_{28}). As mentioned above, $^{29}\text{N}_2$ production from anammox must be excluded as this production represents induced oxidation of $^{14}\text{NH}_4^+$ caused by $^{15}\text{NO}_x^-$ addition. The correct procedure for determination of genuine N_2 production is therefore

$$p_{14} = 2 \cdot D_{28} + D_{29} + 2 \cdot A_{28}. \quad (3)$$

According to assumption 1, the denitrification term $2 \cdot D_{28} + D_{29}$ equals $\frac{D_{29}}{2D_{30}} \cdot (2 \cdot D_{30} + D_{29})$, i.e., the classical IPT term. According to assumptions 1 and 2, the anammox term $2 \cdot A_{28}$ equals $2 \cdot A_{29} \cdot r_{14}$, where r_{14} is the ratio between $^{14}\text{NO}_x^-$ and $^{15}\text{NO}_x^-$ ($^{14}\text{NO}_x^- : ^{15}\text{NO}_x^-$) in the NO_x^- reduction zone (see Appendix 1).

By substitution, Eq. 3 becomes:

$$p_{14} = \frac{D_{29}}{2 \cdot D_{30}} \cdot (2 \cdot D_{30} + D_{29}) + (2A_{29} \cdot r_{14}). \quad (4)$$

The difference between the correctly calculated ^{14}N - N_2 production and the production estimated by the IPT is thus the difference between Eqs. 2 and 4:

$$\begin{aligned} \text{Error} = p_{14IPT} - p_{14} &= \left[\frac{A_{29} \cdot (2 \cdot D_{30} + A_{29} + 2 \cdot D_{29})}{2 \cdot D_{30}} \right] - (2 \cdot A_{29} \cdot r_{14}) \\ &= A_{29} \cdot \left(\frac{2 \cdot D_{30} + A_{29} + 2 \cdot D_{29}}{2 \cdot D_{30}} - 2 \cdot r_{14} \right). \end{aligned} \quad (5)$$

This corresponds to the error that arises from use of the isotope pairing technique.

We wish to express the error in terms that are easier to interpret, and further rewriting is therefore necessary. Our strategy is to first express all terms in Eq. 5 as functions of the genuine ^{14}N - N_2 production (p_{14}), the contribution of anammox to N_2 production (ra) and the $^{14}\text{NO}_x^- : ^{15}\text{NO}_x^-$ ratio in the NO_x^- reduction zone (r_{14}), then substitute these new terms into Eq. 5.

First we consider the denitrification terms, D_{29} and D_{30} . The production of ^{14}N - N_2 formed via denitrification can be expressed as follows:

$$p_{14} \cdot (1 - ra) = D_{29} + 2 \cdot D_{28}. \quad (6)$$

The production of $^{28}\text{N}_2$ formed via denitrification can be expressed as a function of the production of $^{29}\text{N}_2$ via this process, a consequence of assumption 1 (see Appendix 1).

$$D_{28} = D_{29} \cdot \frac{r_{14}}{2}. \quad (7)$$

Hence Eq. 6 is equivalent to

$$p_{14} \cdot (1 - ra) = D_{29} + 2 \cdot D_{29} \cdot \frac{r_{14}}{2}, \quad (8)$$

and D_{29} is isolated as follows:

$$D_{29} = \frac{p_{14} \cdot (1 - ra)}{(1 + r_{14})}. \quad (9)$$

The production of $^{30}\text{N}_2$ via denitrification can also be expressed as a function of the production of $^{29}\text{N}_2$ via this process (see Appendix 1).

$$D_{30} = D_{29} \cdot \frac{1}{2 \cdot r_{14}} \quad (10)$$

D_{29} is substituted with Eq. 9, and D_{30} becomes

$$D_{30} = \frac{p_{14} \cdot (1 - ra)}{2 \cdot r_{14} \cdot (1 + r_{14})}. \quad (11)$$

Likewise, we can express A_{29} in terms of p_{14} , ra , and r_{14} . The genuine ^{14}N - N_2 production via anammox can be expressed as follows:

$$p_{14} \cdot ra = 2 \cdot A_{28} \quad (12)$$

Because $A_{28} = r_{14} \cdot A_{29}$ (see Appendix 1), A_{29} can be expressed as follows:

$$A_{29} = \frac{p_{14} \cdot ra}{2 \cdot r_{14}}. \quad (13)$$

All terms in Eq. 5 can now be expressed as functions of p_{14} , ra , and r_{14} , and by substitution, Eq. 5 becomes

$$\begin{aligned} \text{Error} &= \frac{p_{14} \cdot ra}{2 \cdot r_{14}} \cdot \left[\frac{p_{14} \cdot (1 - ra) + p_{14} \cdot ra + 2p_{14}(1 - ra)}{r_{14}(1 + r_{14})} - \frac{2 \cdot p_{14}(1 - ra)}{p_{14} \cdot (1 - ra)} - 2 \cdot r_{14} \right] \\ &= \frac{ra \cdot [2 + ra \cdot (r_{14} - 1)]}{4 \cdot r_{14} \cdot (1 - ra)} \cdot p_{14}. \end{aligned} \quad (14)$$

The magnitude of the error caused by the IPT calculation procedure relative to the amount of ^{14}N - N_2 being produced according to the revised calculation procedure is thus:

$$\text{Error}_{\text{rel}} = \frac{ra \cdot [2 + ra \cdot (r_{14} - 1)]}{4 \cdot r_{14} \cdot (1 - ra)}. \quad (15)$$

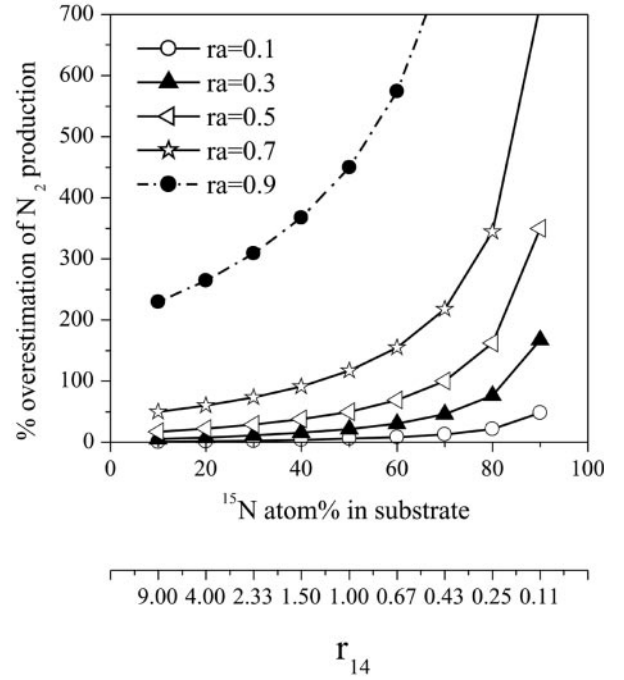


Fig. 2. Overestimation of N_2 production as a function of ^{15}N in the NO_x^- pool undergoing dissimilatory NO_x^- reduction (upper X-axis) and r_{14} (lower X-axis). The lines represent scenarios with different values of ra , the contribution of anammox to total N_2 production.

Eq. 15 is a nonlinear increasing function of ra and a decreasing function of r_{14} , the $^{14}\text{N} : ^{15}\text{N}$ ratio of the NO_x^- substrate undergoing dissimilatory reduction (Fig. 2). Presence of anammox will therefore lead to overestimation of N_2 production when estimated by the IPT, and this overestimation is positively correlated with the concentration of $^{15}\text{NO}_x^-$ added to the system.

This is not surprising because the IPT includes $^{29}\text{N}_2$ production from anammox in the calculation of ^{14}N - N_2 production, and this production represents an additional oxidation of $^{14}\text{NH}_4^+$, which would not have taken place in the absence of $^{15}\text{NO}_x^-$ addition. In a previous study, it has been proposed that presence of anammox will not affect the genuine N_2 production as estimated by the IPT, but only complicate identification of the processes involved (Ogilvie et al. 1997). As shown in the present study, this is not correct.

Estimating ^{14}N - N_2 production correctly: an alternative approach—In the previous section, we have focused on the problems associated with application of the IPT in sediments where anammox and denitrification coexist. In the following, we propose two alternative calculation procedures for calculation of genuine N_2 production (p_{14}). One of these relies on data from at least two sets of intact sediment cores incubated with different $^{15}\text{NO}_x^-$ amendments. The other procedure relies on a single $^{15}\text{NO}_x^-$ incubation and measurements of ra , the contribution of anammox to N_2 production.

Both procedures are based on calculations of p_{14} from measured production rates of ^{15}N -labeled N_2 and estimates of r_{14} —

Table 2. Equations used for calculating genuine N_2 production

Parameter	Equation
Estimate of N_2 production	$p_{14} = 2r_{14}[p^{29}N_2 + p^{30}N_2(1-r_{14})]$
Estimate of r_{14} if ra is known	$r_{14} = \frac{(1-ra) \cdot R^{29} - ra}{(2-ra)}$ R^{29} is the ratio between $^{29}N_2$ and $^{30}N_2$ production, ra is the contribution of anammox to N_2 production.
Estimate of r_{14} from incubation with two different $^{15}NO_x^-$ concentrations	$r_{14}^{(1)} = \frac{p^{29}N_2^{(1)} - V \cdot p^{30}N_2^{(2)}}{p^{30}N_2^{(1)} - V^2 \cdot p^{30}N_2^{(2)}}$ $p^{29}N_2^{(1)}$ and $p^{30}N_2^{(1)}$ are production rates of $^{29}N_2$ and $^{30}N_2$ in incubation 1 and 2, and $p^{29}N_2^{(2)}$ and $p^{30}N_2^{(2)}$ are production rates of $^{29}N_2$ and $^{30}N_2$ in incubation 2 — a parallel incubation with a different $^{15}NO_3^-$ concentration in the water column. V is the ratio between $^{15}NO_x^-$ concentration in the water column in incubations 1 and 2. V can also be estimated from: $V = \frac{p^{29}N_2^{(1)} + 2 \cdot p^{30}N_2^{(1)}}{p^{29}N_2^{(2)} + 2 \cdot p^{30}N_2^{(2)}}$

the ratio between $^{14}NO_x^-$ and $^{15}NO_x^-$ in the NO_x^- reduction zone—and both procedures estimate this ratio. The equations of interest are given in Table 2. Below we present the rationale. First we will develop the general formula, in which the only unknown term is r_{14} , and then we will show how to estimate r_{14} with the two procedures.

Expressing p_{14} from measurable parameters: $p^{29}N_2$, $p^{30}N_2$, and r_{14} —The correct estimate of genuine N_2 production is given by Eq. 3. In the previous section, we expressed D_{30} as a function of D_{29} and r_{14} (Eq. 10). Because only denitrification produces $^{30}N_2$ (Fig. 1), D_{30} equals $p^{30}N_2$ and D_{29} is therefore equivalent to

$$D_{29} = 2r_{14} \cdot p^{30}N_2. \quad (16)$$

In the previous section, we also expressed D_{28} as a function of D_{29} and r_{14} (see Eq. 7). Using Eq. 16, D_{28} can be expressed as follows:

$$D_{28} = \frac{r_{14}}{2} \cdot D_{29} = r_{14}^2 \cdot p^{30}N_2. \quad (17)$$

The production of $^{28}N_2$ via anammox (A_{28}) can be expressed as a function of the production of A_{29} and r_{14} :

$$A_{28} = r_{14} \cdot A_{29}. \quad (18)$$

The $^{29}N_2$ production via anammox corresponds to the difference between total $^{29}N_2$ production ($p^{29}N_2$) and the production of $^{29}N_2$ via denitrification. Hence A_{29} equals

$$A_{29} = p^{29}N_2 - 2 \cdot r_{14} \cdot p^{30}N_2. \quad (19)$$

Using this relationship we can express A_{28} as follows:

$$A_{28} = r_{14} \cdot (p^{29}N_2 - 2 \cdot r_{14} \cdot p^{30}N_2). \quad (20)$$

All terms in Eq. 3 are now expressed as functions of $p^{29}N_2$, $p^{30}N_2$, and r_{14} , and Eq. 3 becomes

$$p_{14} = 2 \cdot r_{14}^2 \cdot p^{30}N_2 + 2 \cdot r_{14} \cdot p^{30}N_2 + 2 \cdot r_{14} \cdot (p^{29}N_2 - 2 \cdot r_{14} \cdot p^{30}N_2) \\ = 2r_{14}[p^{29}N_2 + p^{30}N_2(1-r_{14})]. \quad (21)$$

In Eq. 21, all parameters can be directly measured, except for r_{14} for which calculation procedures will be described in the following section.

Estimation of r_{14} , method 1: determination of the contribution of anammox to total N_2 production—It is possible to estimate r_{14} and thereby p_{14} from production rates of $^{29}N_2$ and $^{30}N_2$ if ra , the contribution of anammox to N_2 production, is known. At present, published techniques enable simultaneous quantification of the potential for anammox and denitrification in anoxic jars with sediment from defined sediment strata. From such data it is possible to estimate the contribution of anammox to N_2 production (Thamdrup and Dalsgaard 2002). Such an estimate can be considered as a measurable proxy for ra .

We can express the contribution of anammox to N_2 production (ra) according to Eq. 12:

$$ra = \frac{2A_{28}}{p_{14}}. \quad (22)$$

By combining Eqs. 20 and 21 with Eq. 22, we can express r_{14} in terms of measurable parameters:

$$ra = \frac{2 \cdot r_{14} \cdot (p^{29}N_2 - 2 \cdot r_{14} \cdot p^{30}N_2)}{2 \cdot r_{14} [p^{29}N_2 + p^{30}N_2(1-r_{14})]} \\ = \frac{p^{29}N_2 - 2 \cdot r_{14} \cdot p^{30}N_2}{p^{29}N_2 + p^{30}N_2 \cdot (1-r_{14})}. \quad (23)$$

Solving for r_{14} gives

$$r_{14} = \frac{(1-ra) \cdot R_{29} - ra}{(2-ra)} \quad (24)$$

where R_{29} is the ratio between $^{29}N_2$ and $^{30}N_2$ production. Thus, if ra is known, for instance through the technique described by Thamdrup and Dalsgaard (2002), the genuine N_2 production can be estimated by combining Eqs. 21 and 24.

Estimation of r_{14} method 2: multiple $^{15}\text{NO}_x^-$ incubation—It is possible to estimate r_{14} and p_{14} on the basis of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production rates measured in at least two sets of parallel intact cores incubated with different $^{15}\text{NO}_x^-$ concentrations. In this section, we will develop a set of equations for that purpose. In order to do so, we will use an assumption central to our theory: namely that the production of $^{28}\text{N}_2$ via anammox (A_{28}) is independent of $^{15}\text{NO}_x^-$ addition.

Thus, using Eq. 20 we can express A_{28} in each of two sets of sediment cores incubated with different $^{15}\text{NO}_x^-$ additions as follows:

$$A_{28} = r_{14}^{(1)} (p^{29}\text{N}_2^{(1)} - 2 \cdot r_{14}^{(1)} \cdot p^{30}\text{N}_2^{(1)}) \quad (25)$$

$$A_{28} = r_{14}^{(2)} \cdot (p^{29}\text{N}_2^{(2)} - 2 \cdot r_{14}^{(2)} \cdot p^{30}\text{N}_2^{(2)}) \quad (26)$$

where $p^{29}\text{N}_2^{(1)}$ and $p^{30}\text{N}_2^{(1)}$ are production rates of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ resulting from one incubation with $^{15}\text{NO}_x^-$, and $p^{29}\text{N}_2^{(2)}$ and $p^{30}\text{N}_2^{(2)}$ are the production rates of the respective N_2 species from a parallel series of sediment cores incubated with a different concentration of $^{15}\text{NO}_x^-$ in the water column. $r_{14}^{(1)}$ and $r_{14}^{(2)}$ represent the respective $^{14}\text{NO}_x^- : ^{15}\text{NO}_x^-$ ratios in the NO_x^- reduction zone.

In an experimental setup such as this, it is possible to express r_{14} for one incubation series (series 1) as a function of r_{14} in another incubation series (series 2): $r_{14}^{(2)} = V r_{14}^{(1)}$ where V is the ratio between the concentrations of $^{15}\text{NO}_x^-$ in the water column of the two incubations. This approximation is valid if addition of $^{15}\text{NO}_3^-$ only changes the concentration of $^{15}\text{NO}_x^-$ in the NO_x^- reduction zone, whereas the concentration of $^{14}\text{NO}_x^-$ in that zone remains constant (an implicit consequence of assumption 3). If there is a linear relationship between the concentration of added $^{15}\text{NO}_x^-$ and the amount of $^{15}\text{N}-\text{N}_2$ produced (assumption 3) V equals

$$V = \frac{\left[\begin{matrix} ^{15}\text{NO}_3^- \\ \end{matrix} \right]_{(1)}}{\left[\begin{matrix} ^{15}\text{NO}_3^- \\ \end{matrix} \right]_{(2)}} = \frac{p^{29}\text{N}_2^{(1)} + 2 \cdot p^{30}\text{N}_2^{(1)}}{p^{29}\text{N}_2^{(2)} + 2 \cdot p^{30}\text{N}_2^{(2)}}, \quad (27)$$

Eqs. 25 and 26 can then be expressed as follows:

$$A_{28} = r_{14}^{(1)} (p^{29}\text{N}_2^{(1)} - 2 \cdot r_{14}^{(1)} \cdot p^{30}\text{N}_2^{(1)}) \quad (28)$$

$$A_{28} = r_{14}^{(1)} \cdot V \cdot (p^{29}\text{N}_2^{(2)} - 2 \cdot r_{14}^{(1)} \cdot V \cdot p^{30}\text{N}_2^{(2)}). \quad (29)$$

From this set of equations, r_{14} can be determined from measurable terms:

$$r_{14}^{(1)} = \frac{p^{29}\text{N}_2^{(1)} - V \cdot p^{29}\text{N}_2^{(2)}}{p^{30}\text{N}_2^{(1)} - V^2 \cdot p^{30}\text{N}_2^{(2)}}. \quad (30)$$

With this estimate of r_{14} , the genuine $^{14}\text{N}-\text{N}_2$ production can be calculated from Eq. 21.

Estimation of N_2 production based on nitrate produced in the sediment ($p_{14}n$) or coming from the overlying water ($p_{14}w$)—The classical IPT allows quantification of and distinction between

denitrification of bottom-water NO_3^- and NO_3^- produced by sedimentary nitrification during incubation (Nielsen 1992). The question is whether or not these two sources of denitrification can be estimated in sediments where anammox and denitrification coexist. In the previous section, we showed that it is possible to estimate r_{14} , the ratio between $^{14}\text{NO}_x^-$ and $^{15}\text{NO}_x^-$, in the NO_x^- reduction zone. Using this information, it is a simple task to quantify the dependency between p_{14} and the concentration of NO_x^- in the water column ($p_{14}w$) and the dependency between p_{14} and nitrification ($p_{14}N$):

$$p_{14}w = p_{14} \cdot \frac{r_{14}w}{r_{14}} \quad (31)$$

$$p_{14}n = p_{14} - p_{14}w = p_{14} \cdot \left(1 - \frac{r_{14}w}{r_{14}}\right) \quad (32)$$

where $r_{14}w$ is the ratio between $^{14}\text{NO}_x^-$ and $^{15}\text{NO}_x^-$ in the water column.

Procedures for evaluation of the classical and the updated IPT—Experiments with different $^{15}\text{NO}_3^-$ additions can be used to validate both classical IPT and the alternative procedure for calculation of p_{14} . We recommend that such experiments be performed as a standard procedure when measuring N_2 production with ^{15}N -isotopes. In this context, experiments with multiple (≥ 4) concentrations of $^{15}\text{NO}_3^-$ should be performed and the responses of $^{15}\text{N}_2$ and $^{14}\text{N}-\text{N}_2$ production evaluated using standard techniques such as analysis of variance accompanied by a power analysis to evaluate the experimental design. In addition, the contribution of anammox to N_2 production (ra) should be measured with the method described by Thamdrup and Dalsgaard (2002).

Data from experiments such as these will facilitate interpretation of data from the concentration series experiment.

We will consider two main outcomes of these experiments:

1. There is a positive correlation between $^{14}\text{N}-\text{N}_2$ production estimated by the classical IPT and the amount of $^{15}\text{NO}_x^-$ added. In addition, there is a linear dependency between the production of $^{15}\text{N}-\text{N}_2$ (i.e., $2 \cdot p^{30}\text{N}_2 + p^{29}\text{N}_2$) and the concentration of $^{15}\text{NO}_3^-$ applied.

2. There is no significant correlation between $^{14}\text{N}-\text{N}_2$ production estimated by the classical IPT and the amount of $^{15}\text{NO}_x^-$ added but there is a linear dependency between the production of $^{15}\text{N}-\text{N}_2$ and the concentration of $^{15}\text{NO}_3^-$ applied.

If there is no correlation between $^{15}\text{N}-\text{N}_2$ production and $^{15}\text{NO}_3^-$ addition, N_2 production is not NO_3^- -limited and $^{15}\text{NO}_3^-$ tracer techniques cannot be used to estimate genuine N_2 production.

An experiment resulting in outcome 1 is indicative of unsuccessful application of the classical calculation procedure and may suggest presence of anammox. This is clear from our finding in the section, *The error imposed on the IPT in determination of sediment N_2 production*, where we demonstrated the positive correlation between the fraction of ^{15}N in the NO_x^- undergoing dissimilatory reduction and the degree of overestimation of genuine N_2 production when estimated with the

Table 3. Production rates of $^{29}\text{N}_2$ ($p^{29}\text{N}_2$), $^{30}\text{N}_2$ ($p^{30}\text{N}_2$), the contribution of anammox to total N_2 production as estimated in slurry incubations (ra) and the ratio between $^{14}\text{NO}_x^-$ and $^{15}\text{NO}_x^-$ in the NO_x^- reduction zone estimated with Eq. 22^a

Locality	$p^{29}\text{N}_2$ ($\mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$)	$p^{30}\text{N}_2$ ($\mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$)	ra (%)	r_{14}
Norsminde Fjord (1 m) ^b	128.8 (22.7)	131 (28.6)	0 ^b	0.55 (0.02)
Randers Fjord (1 m) ^b	141.5 (17.4)	44.7 (4.9)	6.2 ^b	1.33 (0.03)
Young Sound (36 m) ^c	7.54 (4.01)	7.72 (3.73)	18.6 ^c	0.3857 (0.14)
S9, Skagerrak (695 m) ^d	1.91 (0.83)	0.191 (0.08)	69.8 ^f	1.79 (0.37)

^aSE of the mean are given in parentheses, $n = 5$.

^bRaw data from Risgaard-Petersen et al. pers. comm. unref.

^cRaw data from Rysgaard et al. (1998).

^dRaw data from Rysgaard et al. (2001).

^eData from Rysgaard et al. unpubl. data. unref.

^fThamdrup and Dalsgaard 2002.

classical IPT (Fig. 2). A similar pattern can however be observed if the assumption of a constant ratio between $^{15}\text{NO}_x^-$ and $^{14}\text{NO}_x^-$ in the NO_x^- reduction zone is violated (Nielsen 1992), but in theory the two error sources are distinguishable. If the assumption is violated, it is reflected in the estimated p_{14} converging to a constant value (Nielsen 1992) and in increasing production of $^{15}\text{N}-\text{N}_2$ with increasing $^{15}\text{NO}_x^-$ addition. If presence of anammox is the cause of the problem, an increase in both the estimated p_{14} and the production of $^{15}\text{N}-\text{N}_2$ will be observed with increasing $^{15}\text{NO}_3^-$ addition. However, whether or not anammox is the problem, it should be evaluated with results from slurry incubations to assess the contribution of anammox to N_2 production (Thamdrup and Dalsgaard 2002).

If capacity for anammox is present and the assumptions are valid, the results of incubations with different $^{15}\text{NO}_x^-$ additions should demonstrate the following: (1) a linear dependency between the production of $^{15}\text{N}-\text{N}_2$ (i.e. $2 \cdot p^{30}\text{N}_2 + p^{29}\text{N}_2$) and the concentration of $^{15}\text{NO}_3^-$ applied and (2) a positive correlation between the applied $^{15}\text{NO}_3^-$ concentration and the production of $^{14}\text{N}-\text{N}_2$ estimated by the classical IPT, but not with the $^{14}\text{N}-\text{N}_2$ production estimated using the new equations.

If both $^{15}\text{NO}_3^-$ concentration experiments and slurry incubations are applied to assess the contribution of anammox to N_2 production, both procedures for estimating genuine N_2 production described in the previous sections should be compared. A full validation of the results requires that the estimate of anammox (or ra) obtained in the concentration experiment is not smaller than the estimate obtained with the alternative method.

An experiment resulting in outcome 2 is indicative of (a) absence of anammox and successful application of the classical IPT, (b) presence of anammox and violation of one to several assumptions of our theory, (c) presence of anammox, but a contribution of anammox to N_2 production too small to cause a significant response in $p_{14\text{IPT}}$ or (d) insufficient number of replicates to account for the spatial heterogeneity of the system being investigated.

Results from slurry incubations assessing the contribution of anammox to N_2 production (Thamdrup and Dalsgaard 2002)

should be used to accept or reject hypothesis *a*. If hypothesis *a* is rejected and significant anammox is present but the expected response to $^{15}\text{NO}_x^-$ addition is not observed, central assumptions may be violated (hypothesis *b*). Unlabeled NO_2^- supplied from nitrification might, for instance, be the main substrate for anammox (i.e., assumption 2 is violated) or nitrification may be reduced by addition of $^{15}\text{NO}_x^-$ (i.e., assumption 4 is violated). Both of these situations can cause lack of correlation between the production of $^{14}\text{N}-\text{N}_2$ estimated with the classical IPT and the concentration of $^{15}\text{NO}_3^-$ applied.

It is also possible that the contribution of anammox to N_2 production is too low to cause significant response in $p_{14\text{IPT}}$ to $^{15}\text{NO}_3^-$ additions (hypothesis *c*). The sensitivity of the experiment can be examined as follows. According to Eq. 24, we have the following relation:

$$R_{29} = \frac{r_{14}(2-ra) + ra}{1-ra} \quad (33)$$

We have shown how r_{14} for one incubation series could be expressed as a function of r_{14} in another incubation series. $r_{14}^{(2)} = V r_{14}^{(1)}$ where V is the ratio between the concentrations of $^{15}\text{NO}_x^-$ in the water column of the two incubations. The difference in R_{29} for two incubation series can then easily be expressed in terms of r_{14} and ra ,

$$R_{29}^{(2)} - R_{29}^{(1)} = \frac{r_{14}^{(1)}[(2-ra)(V-1)]}{1-ra} \quad (34)$$

where $R_{29}^{(1)}$ and $R_{29}^{(2)}$ are the ratios between $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production in incubation series 1 and 2, respectively, $r_{14}^{(1)}$ is the ratio between $^{14}\text{NO}_x^-$ and $^{15}\text{NO}_x^-$ in the NO_x^- reduction zone in incubation series 1, and V is the ratio between the concentrations of $^{15}\text{NO}_x^-$ in the water column of the two incubations. If r_{14} is estimated from Eq. 24 using data from incubation series 1, it is possible to evaluate the response in R_{29} for different values of V and thereby evaluate whether or not the contribution of anammox to N_2 production is high enough to have a significant effect.

Finally, insignificant response of $p_{14\text{IPT}}$ to $^{15}\text{NO}_x^-$ addition might be due to the number of replicates being insufficient to

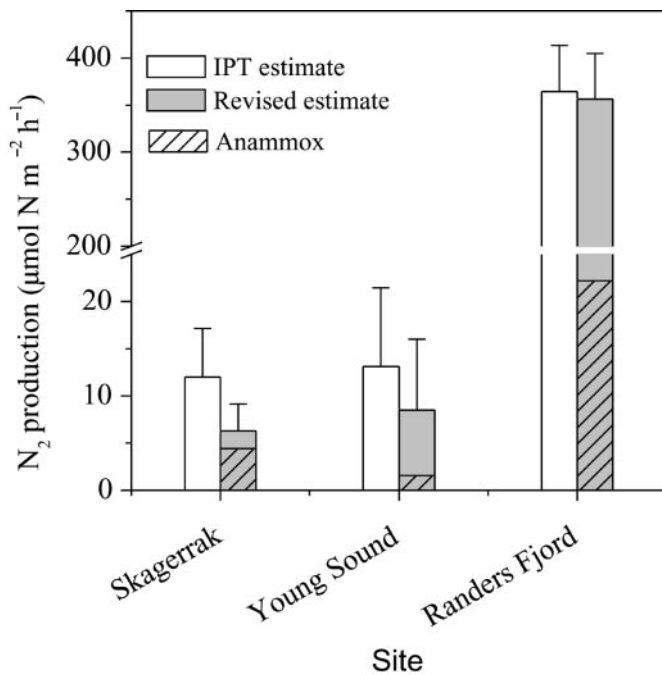


Fig. 3. Estimates of N_2 production calculated from isotope pairing (white columns) and from the procedure outlined in the text (gray columns). The estimated rate of anammox is given as hatched columns. Error bars represent standard error of the mean ($n = 5$).

account for the spatial heterogeneity. The appropriate number of replicates can be assessed by power analysis.

Assessment

We estimated the error imposed by the presence of anammox on calculation of $^{14}N-N_2$ production according to the classical IPT for 4 different sites where anammox has been reported to account for 0% to 69.8% of N_2 production (Table 3). The production rates of $^{29}N_2$ and $^{30}N_2$ measured after addition of $^{15}NO_3^-$ to intact cores were used as values of $p^{29}N_2$ and $p^{30}N_2$. The reported data sets did not include measurements with different $^{15}NO_3^-$ additions, and Eq. 22 was therefore used to estimate r_{14} . The measured contribution of anammox to total N_2 production in anoxic jars incubated with homogenized sediment was used as a proxy for ra (Table 3).

The difference between $^{14}N-N_2$ production estimated by the isotope pairing technique and the revised estimate suggested here was 0%, 2.5%, 31%, and 83% of the revised estimate for Norsminde Fjord, Randers Fjord, Young Sound, and the Skagerrak, respectively (Fig. 3). According to a paired t test based on the individual cores, the apparent overestimation of N_2 production was, however, only significant for the Skagerrak sediment ($P = 0.04$) due to the variation in the recorded activity (Fig. 3). However, it is clear that if the isotope pairing technique is assumed to measure denitrification only, the technique significantly overestimates the process in the Skagerrak

and Young Sound sediments. The true denitrification rate is the difference between the correctly calculated p_{14} and the anammox rate (Fig. 3), and this rate is overestimated by several hundred percent when the classical IPT is used.

The IPT has been applied extensively in temperate estuarine sediments (Nielsen et al. 2001; Risgaard-Petersen et al. 1999; Risgaard-Petersen et al. 1998b; Rysgaard et al. 1995; Rysgaard et al. 1996; Sundback and Miles 2000), and the question is whether these data should be revised given the possible overestimation introduced by the anammox process.

As already pointed out, there is a positive correlation between the concentration of added $^{15}NO_3^-$ and p_{14} estimated from standard IPT equations if anammox is present. With regard to studies where experiments with different $^{15}NO_3^-$ additions have shown independence between the estimated $^{14}N-N_2$ production and the concentration of $^{15}NO_3^-$ and dependency between the production of $^{15}N-N_2$ and $^{15}NO_3^-$ (e.g., Dong et al. 2000; Nielsen and Glud 1996; Rysgaard et al. 1995; Eyre et al. 2002), we find no reason to question the reported denitrification rates. Data from studies where such tests have not been performed might be subject to criticism. However, the still-limited knowledge about the biogeography of anammox suggests that the process accounts for only 0% to 6% of N_2 production in estuarine environments (Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. pers. comm. unref.). According to Eq. 13, this means that the relative error imposed by the IPT is less than 12.5% for $r_{14} > 0.25$ (corresponding to a ^{15}N fraction in a substrate undergoing dissimilatory NO_3^- reduction of 80%), which is not critical. Lower r_{14} values will produce higher relative errors, but a low r_{14} also implies that the availability of $^{14}NO_3^-$ is low. Thereby the production of $^{14}N-N_2$ (p_{14}) is low and even a high relative error will have little quantitative importance in such environments.

Comments and recommendations

In the present communication, we have explored the limitations to application of the IPT as formulated by Nielsen (1992) in sediments where denitrification and anammox coexist. We conclude that the assumptions of the IPT are violated in such systems and that the values of $^{14}N-N_2$ production determined by the technique are overestimated. However, the overestimation of N_2 production in estuarine sediments does not seem to be a serious problem.

We have developed alternative procedures for estimating the production of $^{14}N-N_2$ from measurable variables, namely production rates of $^{15}N-N_2$ and the contribution of anammox to N_2 production (Table 2). These procedures allow quantification of and distinction between anammox and denitrification as well as the dependencies of these processes on NO_3^- supplied from either the water column or nitrification. This improvement of the IPT thus reveals a detailed picture of the N cycle with only minor additional experimental effort.

According to our equations, incorrectness of the IPT induced by anammox can be revealed by a simple test-incubation of

sediment cores with different additions of $^{15}\text{NO}_3^-$. A positive correlation between the estimated production of $^{14}\text{N-N}_2$ and the added concentration of $^{15}\text{NO}_3^-$ is indicative of unsuccessful application of the method. In the same experiment, the true $^{14}\text{N-N}_2$ production calculated by the equations presented here should be independent of the $^{14}\text{N-N}_2$ production and the concentration of $^{15}\text{NO}_3^-$.

The equations are based on several assumptions that seem plausible according to present knowledge of anammox and experience with the IPT. However, we cannot guarantee that the assumptions are valid for all types of sediment. We therefore recommend measuring the potential for anammox by the method of Thamdrup and Dalsgaard (2000) and, furthermore, setting up parallel experiments with different $^{15}\text{NO}_3^-$ concentrations (≥ 4). The results of these very simple additional experiments will expose any problems imposed on the classical IPT and, furthermore, will validate the estimates of N_2 production obtained with the alternative calculation procedure suggested here.

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