

Modified Isotope Pairing Technique to Study N Transformations in Polluted Aquatic Systems: Theory

Y. MASTER,* U. SHAVIT, AND A. SHAVIV

The Faculty of Civil and Environmental Engineering,
The Lowdermilk Division of Agricultural Engineering,
Technion-IIT, Haifa 32000, Israel

Denitrification of nitrate in sediments of polluted aquatic systems has the potential to release considerable nitrogen amounts into the atmosphere in the form of dinitrogen (N_2) and/or nitrous oxide (N_2O). Nitrate may diffuse into the anoxic sediment layer either from the oxic sediment layer (after being produced there by nitrifiers) or directly from the overlying water. Currently, the most common technique that explicitly distinguishes between the two main nitrate sources is the Isotope Pairing Technique (IPT). ^{15}N -labeled nitrate is added to the water column, and formation of ^{15}N labeled N_2 molecules is subsequently monitored. The main shortcoming of the IPT is that the formation of N_2O is ignored, thus resulting in an underestimation of sediment denitrification. Another limitation is the inability to account for a possible influx of nitrate through the hyporheic zone (e.g., nitrate-polluted groundwater) into the anoxic sediment layer. We have further developed and analyzed the theoretical basis of the original IPT. The two important factors that we took into account are the isotopic composition of N_2O and the input of an external source of nitrate. We also examined the option of adding ^{15}N -labeled ammonium to the water column to specifically adjust the technique to field studies. The presented modified technique allows us to (i) improve the estimation of sediment denitrification capacity, (ii) gain an insight into the N_2O formation mechanism(s) and fluxes, and (iii) assess inputs of nitrate-polluted water through the hyporheic zone.

Introduction

Denitrification is the main microbially mediated nitrogen (N) process through which N is lost to the atmosphere from aquatic systems. In systems that suffer from anthropogenic pollution (e.g., treated or untreated sewage, agricultural return flows, aquaculture discharge, etc.), denitrification has the capability to remove surplus nitrate (NO_3^-) by converting it into gaseous N species and consequently assisting in the recovery of the water body. It has long been established that the sediment, rather than the water itself, is the main site where major N transformations take place (e.g., refs 1 and 2). While aerobic processes, such as nitrification and mineralization, occur in the oxic sediment layer (i.e., the upper 1.5 to 5.5 mm (1)), denitrification takes place below the oxic-anoxic interface. The two main sources of NO_3^- deemed to be responsible for denitrification in the anoxic

layer are (i) NO_3^- diffusing directly from the overlying water and (ii) NO_3^- being produced by nitrification in the oxic layer (2). Denitrification of the latter NO_3^- source is called coupled nitrification-denitrification (CND) and is controlled by a variety of factors, such as levels of O_2 , organic matter (OM), and various N species both in the sediment oxic layer and the overlying water. Thirty and up to 70% of the total denitrification was attributed to CND in a shallow, hyper-eutrophic lake (3) and in coastal and estuarine sediments (4), respectively. In polluted water bodies CND is likely to be significant due to the high and continuous loading of OM and N species to the sediments.

Although dinitrogen (N_2) has generally been considered the main product of denitrification in aquatic systems, recent studies provide evidence that the production of nitrous oxide (N_2O) cannot be discounted, especially in heavily polluted water bodies. Garcia-Ruiz et al. (5) found that N_2O accounted for up to 100% (with an average of 42%) of the total N gas production in the sediments of the highly polluted Wiske River. The amounts of N_2O emitted from the highly polluted South Platte River were comparable to the amounts emitted from all primary municipal wastewater plants in the USA (6). Another major process responsible for N_2O formation is nitrification (7). Little is known about this pathway of N_2O formation in sediments of aqueous systems. Prevention of N_2O emissions to the atmosphere and predictions of a polluted water body self-recovery are based on our understanding of the interrelations between the environmental conditions prevalent in a water body and the dominant N_2O -forming processes.

Nitrate may also reach the anoxic sediment layers through hyporheic transport processes (8). When the hydraulic head of a NO_3^- polluted aquifer is high, the discharge into the gaining water body may also contain NO_3^- (9). Segal Rozenhaimer et al. (10), for example, have recently shown that a significant mass of NO_3^- is being added to the Lower Jordan River through such groundwater influx. This contribution of NO_3^- may predominate over the other sources when NO_3^- levels in the groundwater are high enough.

The ^{15}N natural abundance techniques (11) are among the most common used to study N transformations and to track N contamination sources on a field scale. These techniques are based on measuring the minute differences between the ^{15}N content of the substrate and the product that occur as a result of isotopic fractionation. However, due to the enormous complexity of the N processes within the watershed and associated water bodies, most of the natural abundance studies to date provide only general trends and qualitative assessment of the processes, which are responsible for the changes in natural abundance of various N species (e.g., ref 12).

The main techniques that have been used to directly measure denitrification are the acetylene block technique, N_2 flux method, and isotope pairing technique (IPT). The main shortcomings of the acetylene block technique are that acetylene inhibits nitrification and thus an underestimation of denitrification occurs (CND is not accounted for) (13). The impeded diffusion of acetylene into the deeper layers of the sediment may result in partial inhibition of N_2O reductase and subsequently in an additional underestimation of denitrification. The N_2 flux method (direct measurement of N_2 production) is very sensitive to contamination by the background N_2 and thus requires special care in order to avoid leakage from and into the experimental setup (3, 14). Determination of CND using this technique involves inhibition of the nitrification process with a nitrification inhibitor

* Corresponding author phone: 972 4 8293329; fax: 972 4 8295696; e-mail: master@tx.technion.ac.il.

and measurement of N_2 production from the NO_3^- in the water column. This method may face diffusional constraints similar to those encountered in the acetylene block technique. The only technique that explicitly takes the coupled process into account is the IPT (2). In the IPT, ^{15}N -labeled NO_3^- is added to the water column and the formation of N_2 molecules with masses of 28, 29, and 30 is subsequently monitored. This $^{15}NO_3^-$ addition allows distinguishing between the denitrification intensities of the two main sources of NO_3^- (i.e., diffusion from the overlying water and nitrification in the oxic layer). The use of the IPT in conjunction with other techniques (e.g., ^{15}N dilution of the NO_3^- and NH_4^+ species) provides a powerful tool to evaluate, in the same experimental setup, the rate of not only denitrification, but also processes such as nitrification, mineralization, and dissimilatory NO_3^- reduction to NH_4^+ (e.g., ref 15). The IPT has been successfully implemented in a variety of aquatic systems, including streams (e.g., ref 2), lakes (e.g., refs 16 and 17), estuaries (e.g., refs 15 and 18), and coastal sediments (e.g., refs 4 and 19).

The original version of the IPT focuses on N_2 formation and neglects the formation of N_2O . As a result, the total denitrification capacity is underestimated and valuable information about N_2O fluxes and formation mechanisms is disregarded. In addition, the technique does not account for the possible influx of NO_3^- -polluted groundwater into the anoxic layer, thus resulting in a possible overestimation of the CND . In this paper we further develop and analyze the theoretical basis of the original IPT to specifically adapt it to systems with high nitrogenous and organic loads. The two important factors that we take into account are the isotopic composition of N_2O and the influx of NO_3^- -polluted water through a hyporheic zone (referred to hereafter as "HZ- NO_3^- "). This new modified technique will enable: (i) a more accurate estimation of sediment denitrification capacity by considering the production of N_2O , (ii) an identification of the N_2O sources (i.e., nitrification or denitrification), and (iii) an estimation of the contribution of HZ- NO_3^- to sediment denitrification capacity. Some critical points relevant for the examination of the modified techniques both on the laboratory and field scales are raised in the paper.

Theory of the Original Isotope Pairing Technique

As our modifications are based on the assumptions and equations of the original IPT, it is essential to present the original derivation at this stage (after ref 20). The IPT (2) is based on the addition of $^{15}NO_3^-$ to the overlying water and a subsequent measurement of the production rates of N_2 molecules with masses of 28, 29, and 30 (see Figure 1).

The full lines in Figure 1 correspond to the original scheme (which appears in 20), while the dashed lines refer to our modification (to be addressed later). The production rates p_{28} , p_{29} , and p_{30} are then used to distinguish between denitrification rate of the NO_3^- diffusing from the overlying water column into the sediment (i.e., D_w), and denitrification of NO_3^- produced within the sediment by nitrification (i.e., D_n).

The inherent assumption of the IPT is a complete and uniform mixing of the added $^{15}NO_3^-$ with the endogenous sources of $^{14}NO_3^-$ (the $^{14}NO_3^-$ initially present in the water column, and the $^{14}NO_3^-$ formed in the oxic layer via nitrification), while diffusing into the anoxic sediment layer. A subsequent assumption is that the formed N_2 species follow an ideal binomial distribution (2, 14). The denitrification rate of $^{15}NO_3^-$ (i.e., D_{15}) is calculated by adding the production rates of ^{15}N atoms in the N_2 molecules:

$$D_{15} = p_{29} + 2p_{30} \quad (1)$$

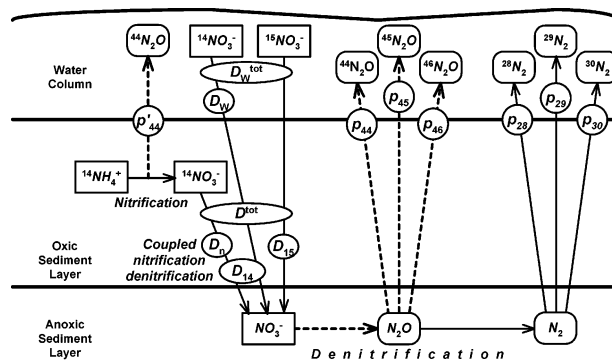


FIGURE 1. A schematic representation of the various N transformation rates, as described in the original isotope pairing technique (IPT, after ref 20) and the modified technique that includes emission of nitrous oxide- IPT- N_2O (marked by dashed lines). Denitrification rates of various N species are designated by " D_x^y " (a detailed explanation of the subscripts and superscripts is given in the text) and the production rates of gaseous N species with a molecular weight z are designated by " p_z ". Note: the dimensions of all denitrification (i.e., D_x^y) and production (p_z and N_z) rates of gaseous N species are moles (NO_3^- for denitrification and N_2 and/or N_2O for gaseous N species) per sediment surface area per time unit (e.g., $\mu\text{mol m}^{-2} \text{h}^{-1}$).

The binomial distribution of the N_2 species means that the $^{29}N_2$ and the $^{30}N_2$ molecules are formed with frequencies proportional to $2D_{14}D_{15}$ and $D_{15}D_{15}$, respectively (2), where D_{14} is the denitrification rate of $^{14}NO_3^-$. This assumption allows the calculation of D_{14} :

$$D_{14} = D_{15} \frac{p_{29}}{2 \cdot p_{30}} \quad (2)$$

The total denitrification rate of the sediment, D^{tot} , is the sum of D_{14} and D_{15} :

$$D^{tot} = D_{15} + D_{14} \quad (3)$$

As shown in Figure 1, D_w^{tot} is the denitrification of the water column nitrate ($^{14}NO_3^-$ and $^{15}NO_3^-$). It is calculated using the value of D_{15} and the ^{15}N atom fraction of NO_3^- in the overlying water (ϵ):

$$D_w^{tot} = \frac{D_{15}}{\epsilon} \quad (4)$$

The CND (i.e., D_n) is then calculated by the difference:

$$D_n = D^{tot} - D_w^{tot} \quad (5)$$

Assuming a linear relationship between D_w^{tot} and the concentrations of NO_3^- in the water column (e.g., refs 17 and 18), we obtain the "natural" denitrification rate D_w (i.e., the denitrification rate of $^{14}NO_3^-$ diffusing from the water column):

$$D_w = D_w^{tot} - D_{15} \quad (6)$$

All terms in Equations (5) and (6) can now be expressed in terms of p_{29} , p_{30} and ϵ . The following explicit expressions for D_w and D_n are, therefore, obtained:

$$D_w = (p_{29} + 2p_{30}) \left(\frac{1}{\epsilon} - 1 \right) \quad (7)$$

$$D_n = (p_{29} + 2p_{30}) \left(1 - \frac{1}{\epsilon} + \frac{p_{29}}{2p_{30}} \right) \quad (8)$$

rates pertaining to IPT- $^{15}\text{NO}_3^-$ are hereafter designated by the superscript * in order to distinguish between the rates obtained in this setup and those obtained in IPT- N_2O . This added notation (p_{28}^* , p_{29}^* , p_{30}^* , p_{44}^* , p_{45}^* , and p_{46}^*) is necessary because the distributions of the gaseous N species in the two setups are different (except the N_2O formed by nitrification, i.e., p_{44}^*).

The IPT- $^{15}\text{NO}_3^-$ setup is based on the following three assumptions:

i. All sources of NO_3^- (including HZ- NO_3^-) are completely mixed within the anoxic layer before denitrification takes place and N_2O and N_2 are produced.

ii. HZ- NO_3^- is completely denitrified in the anoxic layer, i.e., no HZ- NO_3^- reaches the overlying water (as mentioned, we exclude advection and consider HZ- NO_3^- transport by diffusion only).

iii. The flux of HZ- NO_3^- into the anoxic layer does not affect other denitrification rates (i.e., D_{14}^* , D_{15}^* , D_W^* , D_W^{*tot} , and D_n^*) nor the production rate of N_2O via nitrification (i.e., p_{44}^*).

The basic assumptions of the original IPT and those of the IPT- N_2O are assumed to remain valid in this setup. The denitrification rate of ^{15}N , D_{15}^* , does not change and denitrification of ^{14}N is now equal to $[D_{14}^* + D'_{14}]$. Hence, we can rewrite Equations (9) and (10) to obtain:

$$D_{15}^* = p_{29}^* + 2 \cdot p_{30}^* + p_{45}^* + 2 \cdot p_{46}^* \quad (15)$$

and

$$D_{14}^* + D'_{14} = D_{15}^* \cdot \frac{p_{29}^*}{2 \cdot p_{30}^*} = D_{15}^* \cdot \frac{p_{45}^*}{2 \cdot p_{46}^*} \quad (16)$$

Since

$$D_{14}^* = D_n^* + D_W^* \quad (17)$$

Equation 16 yields:

$$D'_{14} = D_{15}^* \cdot \frac{p_{29}^*}{2 \cdot p_{30}^*} - (D_n^* + D_W^*) \quad (18)$$

The value of D_W^* is calculated similar to eq 11.

$$D_W^* = (p_{29}^* + 2p_{30}^* + p_{45}^* + 2p_{46}^*) \cdot \left(\frac{1}{\epsilon} - 1\right) \quad (19)$$

Finally, eq 14 is used to calculate the rate of N_2O formation via nitrification:

$$p_{44}^* = [p_{44}^* + p_{44}^{*IRMS}] - p_{45}^* \cdot \frac{p_{28}^*}{p_{29}^*} \quad (20)$$

Although D_{15}^* , D_W^* , and p_{44}^* were resolved, the derivation is still incomplete since D_n^* is unknown and eq 18 cannot be used to calculate the effect of HZ- NO_3^- . Under real field conditions it is impossible to “disconnect” the input of HZ- NO_3^- and distinguish between this and other sources of $^{14}\text{NO}_3^-$, therefore the assessment of D'_{14} cannot be obtained by using NO_3^- labeling alone. To isolate the contribution of HZ- NO_3^- , it is necessary to evaluate the intensity of CND (D_n^* in eq 18). We, therefore, examine an additional scenario in which $^{15}\text{NH}_4^+$ is added to the water column.

2.2. Isotope Pairing Technique Considering Nitrate Input from Hyporheic Sources Using Labeled Ammonium- IPT- $^{15}\text{NH}_4^+$. The IPT- $^{15}\text{NH}_4^+$ setup is similar to that of the IPT- $^{15}\text{NO}_3^-$, only here the labeled species to be applied to the water column is $^{15}\text{NH}_4^+$. Figure 3 schematically depicts the transformation rates in IPT- $^{15}\text{NH}_4^+$. All the denitrification

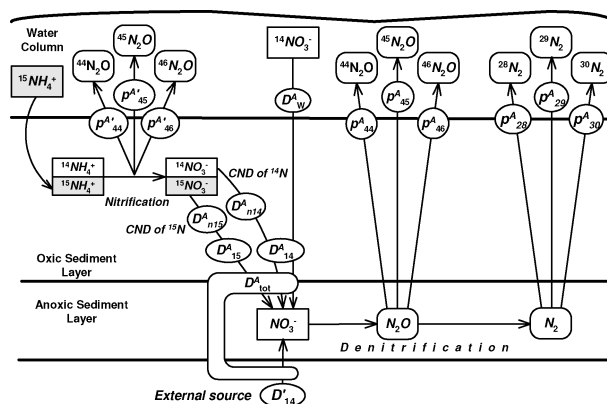


FIGURE 3. A schematic representation of the various N transformation rates in the IPT- $^{15}\text{NH}_4^+$ experimental setup. The impact of NO_3^- input from the hyporheic zone is analyzed using ^{15}N -labeled ammonium. Denitrification and production rates of various N species pertaining to this setup are marked by the superscript * . Note: all denitrification and production rates of various N species pertaining to this figure are marked by the superscript * . The meaning of p_{28}^* , p_{29}^* , p_{30}^* , p_{44}^* , p_{45}^* , p_{46}^* , D_W^* , D_{14}^* , D_{15}^* , and D'_{14} remains similar to that in Figure 1. The symbols N_{tot} , N_{44} , N_{45} , N_{46} , q , x , and d appear only in the text.

and production rates pertaining to this setup are hereafter designated by the superscript * . The addition of ^{15}N -labeled NH_4^+ provides a new data set, which will be used to evaluate the CND of the native NH_4^+ pool and eventually assess D'_{14} according to eq 18.

The first two assumptions of the IPT- $^{15}\text{NO}_3^-$ (a complete mixing of all sources of NO_3^- and a complete denitrification of HZ- NO_3^- within the anoxic sediment layer) are also assumed to be valid in the IPT- $^{15}\text{NH}_4^+$ set up. The specific assumptions of IPT- $^{15}\text{NH}_4^+$ are that (i) the added $^{15}\text{NH}_4^+$ homogeneously mixes with the $^{14}\text{NH}_4^+$ initially present in the oxic layer and that (ii) this addition does not interfere with the indigenous processes. The first assumption means that the addition of $^{15}\text{NH}_4^+$ results in a formation of uniformly labeled NH_4^+ pool with a certain ^{15}N atom fraction. Ignoring isotopic fractionation (a common assumption in tracer studies that involve addition of highly labeled N species, e.g., ref 4), nitrification of this NH_4^+ mixture produces a NO_3^- pool with a similar ^{15}N atom fraction. This is the only available source, which continuously adds $^{15}\text{NO}_3^-$ into the anoxic layer NO_3^- pool. The CND rate of these $^{15}\text{NO}_3^-$ molecules (D_{n15}^* ; see Figure 3) is thus equal to the denitrification rate of ^{15}N (i.e., D_{15}^*). The latter was left in the scheme for the sake of consistency with the methodology of the previous schemes. Also, nitrification of the labeled NH_4^+ pool now produces ^{15}N labeled N_2O molecules that are assumed to be binomially distributed (unlike in IPT- $^{15}\text{NO}_3^-$, where only $^{44}\text{N}_2\text{O}$ molecules are formed). The second assumption means that the CND of the $^{14}\text{NO}_3^-$ pool in the oxic layer (i.e., D_{n14}^*) must be equal to the value of D_n^* in IPT- $^{15}\text{NO}_3^-$ (Figure 2; eq 18), because it represents the CND of the native NH_4^+ pool.

As in IPT- $^{15}\text{NO}_3^-$ (eq 15), D_{n15}^* (or D_{15}^*) is calculated adding the production rates of ^{15}N atoms in the N_2 and N_2O molecules formed by denitrification:

$$D_{n15}^* = D_{15}^* = p_{29}^* + 2 \cdot p_{30}^* + p_{45}^* + 2 \cdot p_{46}^* \quad (21)$$

Here, unlike in the previous setups, the calculation of D_{15}^* is not straightforward, because the values of p_{45}^* and p_{46}^* are not directly measured by IRMS. The IRMS measures the sum of the N_2O molecules formed by both nitrification and denitrification, i.e., $[p_{44}^* + p_{44}^{*IRMS}]$, $[p_{45}^* + p_{45}^{*IRMS}]$, and $[p_{46}^* + p_{46}^{*IRMS}]$ (see Figure 3). The distributions of the N_2O species produced by nitrification in the oxic layer (i.e., p_{44}^* , p_{45}^* ,

and $p^{A_{46}}$) and of those produced by denitrification in the anoxic layer (i.e., $p^{A_{44}}$, $p^{A_{45}}$, and $p^{A_{46}}$) are not the same, since they originate from two pools with different ^{15}N atom fractions. To distinguish between production rates of the N_2O species formed by either process, it is necessary to assess (i) the ^{15}N atom fraction of each pool and (ii) the relative amounts of N_2O molecules produced from each pool.

We begin by defining q as the ^{15}N atom fraction of the NO_3^- pool within the anoxic layer (with $1-q$ being the ^{14}N atom fraction of the NO_3^- pool). Similarly, we define x as the ^{15}N atom fraction of the NH_4^+ pool within the oxic layer (with $1-x$ being the ^{14}N atom fraction of the NH_4^+ pool). The assumption that the N_2O molecules formed by either process possess binomial distribution holds in this case too. As in IPT- $^{15}\text{NO}_3^-$, the distributions of the N_2 and N_2O molecules evolved from the NO_3^- pool of the anoxic layer are assumed to be similar. It means that the $^{44}\text{N}_2\text{O}$ (and $^{28}\text{N}_2$), $^{45}\text{N}_2\text{O}$ (and $^{29}\text{N}_2$), and $^{46}\text{N}_2\text{O}$ (and $^{30}\text{N}_2$) molecules are formed by denitrifiers with probabilities $(1-q)^2$, $2q(1-q)$, and q^2 , respectively. These probabilities are used to calculate the ratio between the production rates of the $^{29}\text{N}_2$ and the $^{30}\text{N}_2$

$$\frac{p_{29}^A}{p_{30}^A} = \frac{2q(1-q)}{q^2} \quad (22)$$

and therefore the ^{15}N fraction of the NO_3^- pool within the anoxic layer is equal to

$$q = \frac{2}{2 + (p_{29}^A/p_{30}^A)} \quad (23)$$

Next, we derive an expression for x . The $^{44}\text{N}_2\text{O}$, $^{45}\text{N}_2\text{O}$, and $^{46}\text{N}_2\text{O}$ molecules are formed by nitrifiers with probabilities $(1-x)^2$, $2x(1-x)$, and x^2 , respectively. The value of x could have been evaluated by direct measurement of the $^{15}\text{NH}_4^+$ content within the oxic layer. This, however, is problematic due to the difficulty in determining exact vertical profiles of the ^{15}N enrichment of NH_4^+ in the active site of nitrification (4). Alternatively, it is suggested to back-evaluate the ^{15}N content of the NH_4^+ pool from the isotopic composition of the N_2O species formed by nitrification (i.e., $p^{A_{44}}$, $p^{A_{45}}$, and $p^{A_{46}}$).

Let N_{tot} indicate the total production rate of N_2O molecules by both nitrification and denitrification ($N_{\text{tot}} = p^{A_{44}} + p^{A_{44}} + p^{A_{45}} + p^{A_{45}} + p^{A_{46}} + p^{A_{46}}$). We further define N_{44} , N_{45} , and N_{46} as the production rates of N_2O molecules with masses of 44, 45, and 46, respectively, formed by both processes ($N_{44} = p^{A_{44}} + p^{A_{44}}$, $N_{45} = p^{A_{45}} + p^{A_{45}}$, $N_{46} = p^{A_{46}} + p^{A_{46}}$). Finally, d will represent the fraction of the N_2O molecules produced by denitrification (with $1-d$ being the fraction of the molecules produced by nitrification). The following expressions can be then written:

$$N_{44} = (1-q)^2 \cdot N_{\text{tot}} \cdot d + (1-x)^2 \cdot N_{\text{tot}} \cdot (1-d) \quad (24)$$

$$N_{45} = 2 \cdot q \cdot (1-q) \cdot N_{\text{tot}} \cdot d + 2 \cdot x \cdot (1-x) \cdot N_{\text{tot}} \cdot (1-d) \quad (25)$$

$$N_{46} = q^2 \cdot N_{\text{tot}} \cdot d + x^2 \cdot N_{\text{tot}} \cdot (1-d) \quad (26)$$

This is a set of 3 nonlinear equations with 2 unknowns- x and d . Any 2 equations can be solved independently to obtain the values of x and d . Equations (24) and (25) yield

$$d = \frac{N_{\text{tot}} \cdot N_{44} - 0.25 \cdot N_{45}^2 - N_{44} \cdot N_{45} - N_{44}^2}{N_{\text{tot}} \cdot (N_{\text{tot}} - N_{44} - N_{45} - 2 \cdot q \cdot N_{\text{tot}} + 2 \cdot q \cdot N_{44} + q \cdot N_{45} + q^2 \cdot N_{\text{tot}})} \quad (27)$$

and

$$x = \frac{N_{44} + N_{45} + q \cdot N_{\text{tot}} - q \cdot N_{44} - 0.5 \cdot q \cdot N_{45} - N_{\text{tot}}}{N_{44} + q \cdot N_{\text{tot}} + 0.5 \cdot N_{45} - N_{\text{tot}}} \quad (28)$$

Now, that the values of q , x , and d are resolved, it is possible to estimate the value of D_{n15}^A and D_{n14}^A (Figure 3). We apply first Equations (25) and (26) and calculate the values of $p^{A_{45}}$ and $p^{A_{46}}$

$$p_{45}^A = 2 \cdot q \cdot (1-q) \cdot N_{\text{tot}} \cdot d \quad (29)$$

and

$$p_{46}^A = q^2 \cdot N_{\text{tot}} \cdot d \quad (30)$$

These values are plugged into eq 21 to obtain an explicit expression for D_{n15}^A . Assuming there is no preferential exploitation of $^{14}\text{NO}_3^-$ relative to $^{15}\text{NO}_3^-$ (i.e., no fractionation), the values of D_{n15}^A and D_{n14}^A are proportional to the size of their predecessors in the oxic layer, i.e.

$$\frac{D_{n15}^A}{D_{n14}^A} = \frac{(^{15}\text{NO}_3^-)_{\text{oxic}}}{(^{14}\text{NO}_3^-)_{\text{oxic}}} \quad (31)$$

As has been mentioned before, our assumption is that nitrification of the NH_4^+ mixture (with x being its ^{15}N atom fraction) in the oxic layer produces a NO_3^- pool with a similar ^{15}N atom fraction (i.e., also x). The ratio between $^{15}\text{NO}_3^-$ and $^{14}\text{NO}_3^-$ in the oxic layer may be expressed in terms of x , since

$$x = \frac{(^{15}\text{NO}_3^-)_{\text{oxic}}}{(^{15}\text{NO}_3^-)_{\text{oxic}} + (^{14}\text{NO}_3^-)_{\text{oxic}}} \quad (32)$$

and therefore

$$D_{n14}^A = D_{n15}^A \cdot \left(\frac{1}{x} - 1 \right) \quad (33)$$

Equation 33 provides an estimation of the CND rate of the native NH_4^+ pool, which should be equal to the value of D_n^* in IPT- $^{15}\text{NO}_3^-$ (Figure 2). Finally, substituting D_n^* with D_{n14}^A and rewriting eq 18 allows calculating the contribution of HZ- NO_3^- to sediment denitrification

$$D_{14}^A = D_{15}^* \cdot \frac{P_{29}^*}{2P_{30}^*} - (D_{n14}^A + D_W^*) \quad (34)$$

where D_{15}^* and D_W^* are calculated using Equations (15) and (19), respectively.

In summary, the assessment of the contribution of HZ- NO_3^- to sediment denitrification in field studies requires two parallel experiments using two setups. The first setup involves the addition of ^{15}N -labeled NO_3^- to the water column (IPT- $^{15}\text{NO}_3^-$; Figure 2) and the second setup involves the addition of ^{15}N -labeled NH_4^+ (IPT- $^{15}\text{NH}_4^+$; Figure 3).

Measurement of production rates of N_2 and N_2O species in IPT- $^{15}\text{NO}_3^-$ enables calculation of: (i) natural denitrification rate of the $^{14}\text{NO}_3^-$ diffusing from the water column (i.e., D_W^* ; eq 19), (ii) total denitrification rate of the sediment $^{14}\text{NO}_3^-$ pool (i.e., $[D_{14}^* + D_{14}^A]$; eq 16), and (iii) rate of N_2O formation via nitrification (i.e., p_{44}^* ; eq 20). Measurement of production rates of N_2 and N_2O species in IPT- $^{15}\text{NH}_4^+$ enables calculation of the: (i) ^{15}N atom fraction of the NO_3^- pool of the anoxic layer (i.e., q ; eq 23), (ii) ^{15}N atom fraction of the NH_4^+ (and NO_3^-) pool in the oxic layer (i.e., x ; eq 28), (iii) relative contribution of denitrification to the total flux of N_2O evolved from the sediments (i.e., d ; eq 27), and finally, (iv) the CND rate of the $^{14}\text{NO}_3^-$ in the oxic layer (i.e., D_{n14}^A ; eq 33). The value of D_{n14}^A , in conjunction with the data

obtained in the IPT- $^{15}\text{NO}_3^-$ setup (D_{15}^* and D_w^*) allows estimating the contribution of HZ- NO_3^- to sediment denitrification (i.e., D_{14}^* ; eq 34).

Discussion

The extraction of additional and valuable information offered by the modified IPT depends on the validity of the assumptions (specific to each setup) and correctness of the theoretically developed new/modified expressions. These are critically discussed below, highlighting issues and points to be considered when implementing the modified theory. Suitable experimental setups may be constructed on the basis of this theory.

Our basic assumption is that nitrification and denitrification are the two major N_2O -producing processes. Nitrous oxide, however, may be also produced by chemodenitrification and dissimilatory NO_3^- reduction to NH_4^+ (DNRA). Chemodenitrification (a chemical reaction of nitrite with the sediment OM and a subsequent production of N_2O) may theoretically interfere with this basic assumption. This phenomenon, however, was found to be significant only under extreme conditions, such as in soils with enormous amounts of OM (e.g., ref 23) or in very acidic environments (e.g., ref 24). It is unlikely that this process be significant in the sediments of most water bodies, however it should be taken into account when dealing with acidic environments with a very high OM content. The most favorable conditions to DNRA are strictly anaerobic, high pH environments conditions with significant quantities of readily fermentable C and low NO_3^- concentrations. Deep and continuously anoxic sediments will usually support obligate anaerobes, such as DNRA reducers, while the more O_2 -rich environments (i.e., the upper few cm of the sediment) will support denitrifiers (25). The relative contributions of DNRA and denitrification to N_2O production are impossible to quantify using ^{15}N alone and would require bacterial identification and enumeration (26). To date, assessment of the relative importance of either process utilizes mostly indirect methods. For instance, based on the positive correlation between NO_2^- and NO_3^- and an increase in NH_4^+ concentrations, Kelso et al. (25) concluded that DNRA was the dominant NO_3^- reduction mechanism in six rivers in Northern Ireland. The enrichment of the NH_4^+ pool and the relationship between N_2O flux and soil NO_2^- concentrations led Stevens et al. (26) to believe that N_2O formation via DNRA is enhanced at high pH values. On the other hand, both Binnerup et al. (27) and Rysgaard et al. (15) concluded that DNRA was an insignificant process (up to 5% of the total NO_3^- uptake) in the studied sediments. They based their estimates on the difference between the isotopic composition of the NH_4^+ in the inflow and outflow and on the acetylene inhibition of nitrification. They also claimed that in earlier investigations NO_3^- assimilatory uptake by benthic microphytes might have been misinterpreted as DNRA. Although no direct evidence has been found that N_2O production via DNRA is a significant process in sediments, it is advisable to track the changes in the ^{15}N content of NH_4^+ during the experiments in order to rule out that possibility.

Another basic assumption of the original IPT is that the N_2 species are produced solely by denitrification. In some systems (mainly in marine sediments), anammox (anaerobic oxidation of NH_4^+ coupled to NO_3^- reduction) may also contribute to N_2 production (28) and an overestimation of denitrification may occur (29). The theoretical analysis of Risgaard-Petersen et al. (29) of estuarine sediments, where anammox and denitrification coexist, however, suggests that the IPT does not seriously overestimate N_2 production, because anammox accounts for <6% of N_2 production in such sediments. They do suggest, however, setting up parallel experiments with different levels of $^{15}\text{NO}_3^-$ to test for the

sediment anammox potential. A positive correlation between the concentration of added $^{15}\text{NO}_3^-$ and D_{14} (eq 2) would be indicative of the occurrence of anammox.

The basic assumption of the original IPT (a complete and uniform mixing of all sources of NO_3^- within the anoxic sediment layer and a subsequent ideal binomial distribution of the formed N_2 species) must be presumed in all setups. A breakdown in this assumption might lead to an underestimation of the total denitrification (2, 14). Despite that, Risgaard-Petersen et al. (3) found that the rate of denitrification in their setup had been underestimated by a maximum 3–7% due to nonideal binomial distribution. Higher concentrations of $^{15}\text{NO}_3^-$ added to the water column are likely to minimize the error introduced by inhomogeneous mixing (2). Additional assumptions of the IPT, namely, a linear relationship between D_w^{tot} and the concentrations of NO_3^- in the water column and the independency of D_n and D_w on the added amounts of $^{15}\text{NO}_3^-$, have been shown to be valid in numerous studies (e.g., refs 17 and 18).

The specific assumption postulated for the IPT- N_2O setup was that N_2 and N_2O from denitrification evolve from the same uniformly mixed NO_3^- pool and possess a similar binomial distribution (e.g., refs 21 and 22). Only if this assumption holds are we able to partition between various sources of N_2O , i.e., to calculate p'_{44} and p_{44} . This means that the ratios p_{29}/p_{30} and p_{45}/p_{46} measured by the IRMS must be similar. If this is not the case, a more thorough investigation should be pursued, e.g., by active inhibition of the CND and a subsequent examination of the isotopic compositions of the N_2 and N_2O species.

The assumption of a complete mixing between all NO_3^- sources in the anoxic layer in the IPT- $^{15}\text{NO}_3^-$ setup, is similar to the assumptions made for the original technique. The assumption that there is a complete denitrification of HZ- NO_3^- in the anoxic layer is new, but could be validated experimentally using a range of HZ- NO_3^- fluxes. If the magnitude of HZ- NO_3^- flux will not affect the outflow concentrations and enrichments of NO_3^- , then this assumption is likely to be valid. A variety of HZ- NO_3^- fluxes need to be examined to determine the optimal range of D_{14} that can be assessed using this method. A comparison with a parallel setup with no contribution of HZ- NO_3^- (as in IPT- N_2O) may be useful for demonstrating the insensitivity of rates, such as D_w^* , D_n^* and p_{44}^* to the introduction of HZ- NO_3^- .

The added $^{15}\text{NH}_4^+$ in the IPT- $^{15}\text{NH}_4^+$ setup increases the total concentration of NH_4^+ in the oxic layer. The higher proportion between the added $^{15}\text{NH}_4^+$ and the indigenous $^{14}\text{NH}_4^+$ is likely to result in a better mixing of the NH_4^+ pool. This is likely to be the case in studies that simulate conditions in polluted systems that usually suffer from high loads of nutrients, including NH_4^+ . A range of $^{15}\text{NH}_4^+$ concentrations, added to the water column, will provide the means to test the accuracy of the D_{n14}^A (or D_n^*) estimation. Should the value of D_{n14}^A not be affected by the addition of $^{15}\text{NH}_4^+$, the estimation is likely to be correct.

Theoretically, the value of x (and subsequently the value of q) may decrease during the course of experiment. This decrease is due to the dilution of the NH_4^+ pool in the oxic layer by $^{14}\text{NH}_4^+$ produced by processes such as mineralization of the organic N or dissimilatory NO_3^- reduction to NH_4^+ . This, however, should not affect the calculations because instantaneous values of x and q are always used to calculate the rates. Although it has been mentioned that vertical profiles of the ^{15}N enrichment of NH_4^+ in the active site of nitrification are difficult to determine, it may be possible to sample the upper several millimeters to get some rough estimates of the average ^{15}N enrichment in the oxic layer.

Crosschecking is needed when employing two parallel setups. For example, the value of p_{44}^* in the IPT- $^{15}\text{NO}_3^-$ setup (eq 20) should be equal to the sum [$p_{44}^A + 0.5p_{45}^A$] in IPT-

$^{15}\text{NH}_4^+$ (these values are derived from Equations (24) and (25), respectively). Additional crosschecking may be gained via comparison of the known value of D_W^* in IPT- $^{15}\text{NO}_3^-$ (eq 19) with the value of D_W^A in IPT- $^{15}\text{NH}_4^+$, assuming that the diffusion of NO_3^- from the overlying water is not affected by the addition of $^{15}\text{NH}_4^+$. The value of D_W^A can be calculated using the previously estimated values of D_{n14}^A , D_{n15}^A , and D_{14}^A , since

$$D_{n14}^A + D_W^A + D_{14}^A = D_{n15}^A \cdot \frac{p_{29}^A}{2 \cdot p_{30}^A} \quad (35)$$

In the field, the reliability of the results might be affected by the spatial and temporal heterogeneities of the sediments and the HZ- NO_3^- intensity. It is, therefore, most important that the two setups are carried out simultaneously in adjacent locations, and the number of replications be high enough.

Nomenclature

IPT- N_2O	Isotope pairing technique including emission of Nitrous Oxide
IPT- $^{15}\text{NO}_3^-$	Isotope pairing technique considering nitrate input from the hyporheic zone using addition of labeled nitrate
IPT- $^{15}\text{NH}_4^+$	Isotope pairing technique considering nitrate input from the hyporheic zone using addition of labeled ammonium
HZ- NO_3^-	nitrate entering the stream through a hyporheic zone (e.g., via influx of NO_3^- -polluted groundwater)
p_{28} , p_{29} , and p_{30}	production rates of N_2 molecules with masses 28, 29, and 30, respectively, via denitrification
p_{44} , p_{45} , and p_{46}	production rates of N_2O molecules with masses 44, 45, and 46, respectively, via denitrification
p'_{44}	production rate of N_2O molecules with mass 44 via nitrification
D_W	denitrification rate of the $^{14}\text{NO}_3^-$ diffusing from the water column into the sediment anoxic layer
D_W^{tot}	denitrification rate of the [$^{14}\text{NO}_3^- + ^{15}\text{NO}_3^-$] mixture diffusing from the water column into the sediment anoxic layer
D_n	denitrification rate of the $^{14}\text{NO}_3^-$ produced within the sediment oxic layer via nitrification
D_{14}	denitrification rate of all $^{14}\text{NO}_3^-$ molecules (i.e., the sum of D_W and D_n)
D_{15}	denitrification rate of the $^{15}\text{NO}_3^-$ molecules
D^{tot}	total denitrification rate of the sediment (i.e., the sum of D_{14} and D_{15})
D_{14}^A	denitrification rate of HZ- NO_3^-
p_{44}^A , p_{45}^A , and p_{46}^A	production rates of N_2O molecules with masses 44, 45, and 46, respectively, via nitrification of the [$^{14}\text{NH}_4^+ + ^{15}\text{NH}_4^+$] mixture formed in the sediment oxic layer

N_{tot}	the total production rate of N_2O molecules by nitrification and denitrification (equal to the sum of [$p_{44}^A + p_{44}^A + p_{45}^A + p_{45}^A + p_{46}^A + p_{46}^A$])
N_{44} , N_{45} , and N_{46}	the production rates of N_2O molecules with masses of 44, 45, and 46, respectively, formed by nitrification and denitrification (equal to [$p_{44}^A + p_{44}^A$], [$p_{45}^A + p_{45}^A$], and [$p_{46}^A + p_{46}^A$], respectively)
D_{n14}^A	denitrification of the ^{14}N fraction of the NO_3^- pool produced within the sediment oxic layer via nitrification of the $^{14}\text{NH}_4^+$
D_{n15}^A	denitrification of the ^{15}N fraction of the NO_3^- pool produced within the sediment oxic layer via nitrification of the $^{15}\text{NH}_4^+$
q [-]	the ^{15}N fraction of the NO_3^- pool of the anoxic layer
x [-]	the ^{15}N fraction of the NH_4^+ (and NO_3^-) pool of the oxic layer
d [-]	the fraction of the total amount of the N_2O molecules attributable to denitrification

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