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Comparison of Isotope Pairing and N_2:Ar Methods for Measuring Sediment Denitrification—Assumptions, Modifications, and Implications

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ABSTRACT: Denitrification has been measured during the last few years using two different methods in particular: isotope pairing measured on a triple-collector isotopic ratio mass spectrometer and N_2:Ar ratios measured on a membrane inlet mass spectrometer (MIMS). This study compares these two techniques in short-term batch experiments. Rates obtained using the original N_2:Ar method were up to 3 to 4 times higher than rates obtained using the isotope pairing technique due to O_2 reacting with the N_2 during MIMS analysis. Oxygen combines with N_2 within the mass spectrometer ion source forming NO^+ which reduces the N_2 concentration. The decrease in N_2 is least at lower O_2 concentrations and since oxygen is typically consumed during incubations of sediment cores, the result is often a pseudo-increase in N_2 concentration being interpreted as denitrification activity. The magnitude of this oxygen effect may be instrument specific. The reaction of O_2 with N_2 and the subsequent decrease in N_2 was only partly corrected using an O_2 correction curve for the relationship between N_2 and O_2 concentrations. The O_2 corrected N_2:Ar denitrification rates were lower, but still did not match the isotope pairing rates and the variability between replicates was much higher. Using a copper reduction column heated to 600°C to remove all of the O_2 from the sample before MIMS analysis resulted in comparable rates (slightly lower), and comparable variability between replicates, to the isotope pairing technique. The N_2:Ar technique determines the net N_2 production as the difference between N_2 production by denitrification and N_2 consumption by N-fixation, while N-fixation has little effect on the isotope pairing technique which determines a rate very close to the gross N_2 production. When the two different techniques were applied on the same sediment, the small difference in rates obtained by the two methods seemed to reflect N-fixation as also supported from measurements of ethylene production in acetylene enriched sediment cores. The N_2:Ar and isotope pairing techniques may be combined to provide simultaneous measurements of denitrification and N-fixation. Both techniques have several assumptions that must be met to achieve accurate rates; a number of tests are outlined that can be applied to demonstrate that these assumptions are being meet.

Introduction

Denitrification is an ecologically important nitrogen (N) cycling pathway because it permanently removes fixed nitrogen from ecosystems and, as such, is one of the few natural processes that is capable of counteracting the process of nutrient enrichment. Direct measurement of sediment denitrification rates are difficult due to high background concentrations of dissolved N_2 in natural waters against which small changes in N_2 must be measured. To overcome these difficulties a number of direct and indirect methods for measuring sediment denitrification have been developed including acetylene blockage (Sørensen 1987), stoichiometry (Nixon 1981; Berelson et al. 1998), direct N_2 fluxes after background removal (Seitzinger 1987; Nowicki 1994), direct N_2 fluxes measured using high precision gas chromatography (Devol 1991; LaMontagne and Valiela 1995), nitrate profiles measured with a nitrate microsensor (Larsen et al. 1996), direct N_2 fluxes determined from changes in N_2:Ar ratios (Kana et al. 1994), and ^{15}N isotope pairing (Nielsen 1992). Major limitations associated with the first five methods (see Cornwell et al. 1999 for a review of these limitations) has made N_2:Ar and isotope pairing the preferred techniques for the measurement of sediment denitrification rates.

The N_2:Ar and isotope pairing techniques are conceptually very different and both techniques rely on a number of different assumptions. The isotope pairing technique has been used in numerous studies, mainly in Western Europe and the Arctic (Nedwell and Trimmer 1996; Rysgaard et al. 1998; Tuominen et al. 1998; Dong et al. 2000; Sundbäck and Miles 2000). The N_2:Ar technique although not currently as widely published (Kana et al. 1994, 1998; An and Joye 1997; Cornwell et

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al. 1999; Heggie et al. 1999; Smith et al. 2000; An et al. 2001; Eyre and Ferguson 2002; Laursen and Seitzinger 2002), is being used by a number of laboratories across North America and Australia (Cornwell personal communication). A comparison of denitrification rates at a recent American Society of Limnology and Oceanography meeting in Copenhagen suggested higher rates were commonly reported in North American coastal systems compared to Western Europe (Christensen personal communication). It is important for cross-system comparisons and global nitrogen budgets that these two widely used denitrification techniques (N_2:Ar and isotope pairing method) give comparable rates and that the differences recorded between systems are ecological and not due to methodological differences.

Direct N_2 fluxes and isotope pairing have previously been compared (Risgaard-Petersen et al. 1998), but this was under strict laboratory conditions using a continuous flow-through system and long incubation times (> 30 d), which are not typical of most studies. The N_2 analysis was undertaken on a GC-MS which required background removal which is unnecessary with the N_2:Ar technique. The purpose of this study was to compare the N_2:Ar and isotope pairing techniques in short-term batch experiments, which is typical to measure sediment denitrification rates.

Material and Methods

Study Sites, Core Collection, and Pre-incubation

Undisturbed sediment cores (core liners made of Plexiglas (polymethyl metacrylate), 5.2 or 8.1 cm i.d.) were collected on three occasions from Åarhus Bay (October and November) and Norsminde Fjord (November), Denmark. Bottom water temperatures during sample collection were 14°C, 10°C, and 10°C at Åarhus Bay (October), Åarhus Bay (November), and Norsminde Fjord, respectively. Sediment was collected by divers at St. 6 in Åarhus Bay (56°09′10″N, 10°19′20″E) at a water depth of 16 m. Åarhus Bay covers an area of 320 km² and has a mean water depth of 15 m. The sediment consisted of 21% sand, 23% silt, and 56% clay and had a porosity of ~ 0.8 and 2.1–3.7% organic carbon. Further descriptions of the Bay can be found in Jørgensen (1996). Sediment was also collected by hand in Nordsminde Fjord at a water depth of 0.5 m. Nordsminde Fjord is only 1.9 km² and has a mean water depth of 0.6 m. The sediment consisted mostly of fine to medium-sized sand with a porosity of ~ 0.2 and 1% organic carbon. Further descriptions of Nordsminde can be found in Nielsen et al. (1995).

The cores were returned to the laboratory within 4 h and placed uncapped and submerged in a thermostatically regulated reservoir of water from the sampling station at in situ temperature. Teflon-coated magnets were suspended in the water column of each core, 5 cm above the sediment surface, and driven by an external rotating magnet (60 rpm). The cores were pre-incubated in darkness for 24 to 48 h to ensure steady-state concentration profiles. All lids, stoppers, replacement water lines, and other applied materials were also carefully pre-incubated in the water reservoir to avoid the introduction of any new surfaces for argon and N_2 absorption and desorption.

N_2:Ar Method

N_2:Ar fluxes were measured in 8.1 (i.d.) × 30 cm cores with a 15 cm water column. At the start of the incubation the cores were sealed air-tight by a top Plexiglas plate with a sample port. The cores were incubated in the dark for 8 to 10 h which allowed a 20% decrease in the dissolved oxygen concentrations. Samples for N_2:Ar were collected in triplicate at the start and every 2 to 4 h (i.e., 3 to 5 point time series). One set of triplicate samples for each of the three analytical techniques described below was obtained. To minimize the introduction of bubbles, N_2:Ar samples were collected by allowing water to flow, driven by a gravity-feed reservoir head, directly into 7 ml gas tight glass vials with glass stoppers filled to overflowing. The reservoir water was kept at the same temperature as the sediment cores. N_2 samples were poisoned with 20 μl of 5% $HgCl_2$ and stored submerged at in situ temperature. One to three extra core liners with only filtered water (0.2 μm; blanks) were pre-incubated, incubated, and sampled as above.

N_2:Ar ratios were measured using three different approaches. In the first case, the method and instrumentation of Kana et al. (1994) was used with the following modifications. Gases were detected with a Balzers QMS422 quadrupole mass spectrometer and a water bath (± 0.01°C) was used to stabilize sample temperature in the water-line upstream of the membrane. All analyses were undertaken in a constant environment room at 10°C to avoid degassing of cold samples in the inlet line. This approach was used for Åarhus Bay samples in October and November.

The method described above was also used with the following modifications. The effect of O_2 in the sample on the N_2 signal measured by the MIMS was corrected by making a standard curve of O_2 concentration against N_2:Ar ratios using water standards made from the incubation water equilibrated with the atmosphere at constant temperature. This was done in two ways. Oxygen concentrations in

the sample water were lowered to different degrees, without changing the dinitrogen concentration, by adding varying amounts of sodium dithionite ($Na_2S_2O_4$) to a series of 7-ml glass vials with glass stoppers. The amount of oxygen actually entering the mass spectrometer was also varied by directing the gas through a copper reduction column located between the membrane inlet and the mass spectrometer. The column consisted of 200 mm of copper oxide granules held inside a 9-mm quartz glass tube with glass wool at either end. The temperature of the copper reduction column was controlled by a muffle furnace and was varied between 0°C and 600°C to produce a range of oxygen amounts actually entering the mass spectrometer. Oxygen concentrations were determined using standards as outlined in Kana et al. (1994). From the relation obtained between the O_2 and N_2 signal all N_2:Ar ratios were corrected back to the O_2 concentration at the start of the incubation so that N_2 concentrations were comparable over the course of the incubation. This approach was used for Åarhus Bay samples in October and November.

The third approach was as outlined above except that all oxygen was removed from the sample gas, before it entered the mass spectrometer, using a copper reduction column heated to 600°C as described above. This approach was used for Norsminde Fjord samples and Åarhus Bay samples in November.

N_2 fluxes across the sediment-water interface were calculated by linear regression of the concentration data, corrected for the addition of replaced water and changes in the blank, as a function of incubation time, core water volume, and surface area. Only the linear portions of the concentration versus incubation time curve were used in the flux calculations.

Isotope Pairing Method

Following the N_2:Ar flux incubations the cores were uncapped and pre-incubated for 12 h before the start of the isotope pairing experiments. The isotope pairing experiments were performed on all the N_2:Ar flux cores as well as additional cores. The rate of denitrification was determined using the isotope pairing technique (Nielsen 1992) as described by Risgaard-Petersen and Rysgaard (1995) and Rysgaard et al. (1995). A total of nine sediment cores were incubated and the sediment cores were processed at different time intervals during the ~ 12 h incubation period.

At each time period one core was sacrificed for sampling after incubation and samples of the water column and sediment porewater were collected for analysis of the ^{15}N-labelling of N_2 and NO_3^-. Samples for the ^{15}N-content of N_2 and NO_3^- in the water column were taken immediately upon removal of the stopper. The sediment and water column were then carefully mixed with a Plexiglas rod. A sample for the ^{15}N-N_2 content of the resultant slurry was taken by syringe. The samples for the ^{15}N-abundance in NO_3^- were frozen (−18°C) until later analysis and samples for ^{15}N-N_2 analysis were preserved in glass vials (Exetainer, Labco, High Wycombe, UK) containing 2% (vol) of a $ZnCl_2$ solution (50% w/v).

A test incubation was performed on Åarhus Bay sediment in order to find the optimal $^{15}NO_3^-$ range for the denitrification measurements and to test the hypothesis of the isotope pairing technique (Nielsen 1992). Five different concentrations of $^{15}NO_3^-$ in the overlying water (5, 10, 25, 45, and 100 μM) were selected and 7 intact sediment cores (5.2 cm i.d., 11 cm sediment, and 18 cm water) were incubated for each concentration and sampled as described above.

The concentration of $NO_3^- + NO_2^-$ was analyzed by chemiluminescense after reduction to NO (Braman and Hendrix 1989). The abundance and concentration of $^{14}N^{15}N$ and $^{15}N^{15}N$ was analyzed on a gas chromatograph coupled to a triple-collector isotopic ratio mass spectrometer (GC-MS, RoboPrep-G$^+$ in line with TracerMass, Europa Scientific, Crewe, UK) as described by Risgaard-Petersen and Rysgaard (1995). The ^{15}N isotopic distribution in the NO_3^- pool was likewise analyzed by mass spectrometry after reduction of NO_3^- to N_2 using a denitrifying bacterial culture (Risgaard-Petersen et al. 1993).

The production rate of the isotopes p($^{14}N^{15}N$ or $^{15}N^{15}N$) was calculated as follows:

$$p(^{15}N^{14}N \text{ or } ^{15}N^{15}N) = \frac{[V_1(C_{water} - C_{ini})] + [(C_{slurry} - C_{ini})V_2]}{A \times t} \quad (1)$$

where C_{water} and C_{slurry} are the concentrations of the isotope in the water column and the sediment slurry, respectively, C_{ini} is the initial concentration of the isotope, V_1 is the volume of the sampled water before mixing water column and sediment, V_2 is the volume of porewater plus the remaining water column after the initial sampling, A is the area, and t the incubation time.

Denitrification rates were estimated from the production of ^{15}N isotopes (Nielsen 1992):

$$D_{15} = p(^{14}N^{15}N) + 2p(^{15}N^{15}N) \quad (2)$$

$$D_{14} = \frac{p(^{14}N^{15}N)}{2p(^{15}N^{15}N)} \times D_{15} \quad (3)$$

where D_{15} and D_{14} are the rates of denitrification based on $^{15}NO_3^-$ and $^{14}NO_3^-$, respectively, and

$p(^{14}N^{15}N)$ and $p(^{15}N^{15}N)$ are the rates of production of the two labelled N_2 species ($^{14}N^{15}N$ and $^{15}N^{15}N$, respectively, calculated from Eq. 1). While D_{15} expresses denitrification activity of added $^{15}NO_3^-$, D_{14} expresses the total in situ denitrification activity.

The proportion of D_{14} that is based on NO_3^- from the water phase (D_w) was calculated from D_{15} and the ^{14}N:^{15}N ratio of water column NO_3^-:

$$D_w = \frac{[^{14}NO_3^-]_w}{[^{15}NO_3^-]_w} \times D_{15} \quad (4)$$

where $[^{14}NO_3^-]_w$ is the concentration of $^{14}NO_3^-$ and $[^{15}NO_3^-]_w$ the concentration of $^{15}NO_3^-$ in the water column. In situ denitrification of NO_3^- produced by nitrification (D_n) was calculated as:

$$D_n = D_{14} - D_w \quad (5)$$

To estimate D_w and D_n as shown above, it was necessary to measure the ^{15}N labeling of the water column NO_3^- as described above.

Results and Discussion

Denitrification activities measured in Åarhus Bay by the original N_2:Ar method described by Kana et al. (1994) gave significantly higher (3–4 times) and considerably more variable values (140 ± 43 and 92 ± 13 μmol N m^{-2} h^{-1} in October and November, respectively) than rates obtained by the isotope pairing technique (34 ± 5 and 32 ± 2 μmol N m^{-2} h^{-1}, respectively; Fig. 1a,b).

Influence of O_2 on N_2:Ar Measurements

One of the prerequisites of the N_2:Ar method and the use of the MIMS is that there are no gasses in the samples which interfere with the N_2 and argon signal. After diffusing through the membrane, gases pass through a liquid nitrogen trap removing water vapor and CO_2 which may interfere with N_2 analysis (Kana et al. 1994). This type of trap, however, allows O_2 to pass freely into the mass spectrometer. We observed that the O_2 concentration of the sample significantly affected the measured N_2:Ar ratios, an effect not reported by Kana et al. (1994).

Measured NO concentrations (based on m/z = 30) in standard water increased with increasing O_2 concentrations (Fig. 2) due to O_2 combining with N_2 within the mass spectrometer ion source forming NO^+ (Jensen et al. 1996). The result is reduced N_2 concentrations at higher O_2 concentrations and vice versa (Fig. 2). Over the course of a typical sediment incubation, O_2 concentrations will decrease in the overlying water. This will result in an increased N_2 signal and thus an increased N_2:Ar ratio. This increase in the N_2 signal will appear as an apparent increase in the benthic N_2 flux

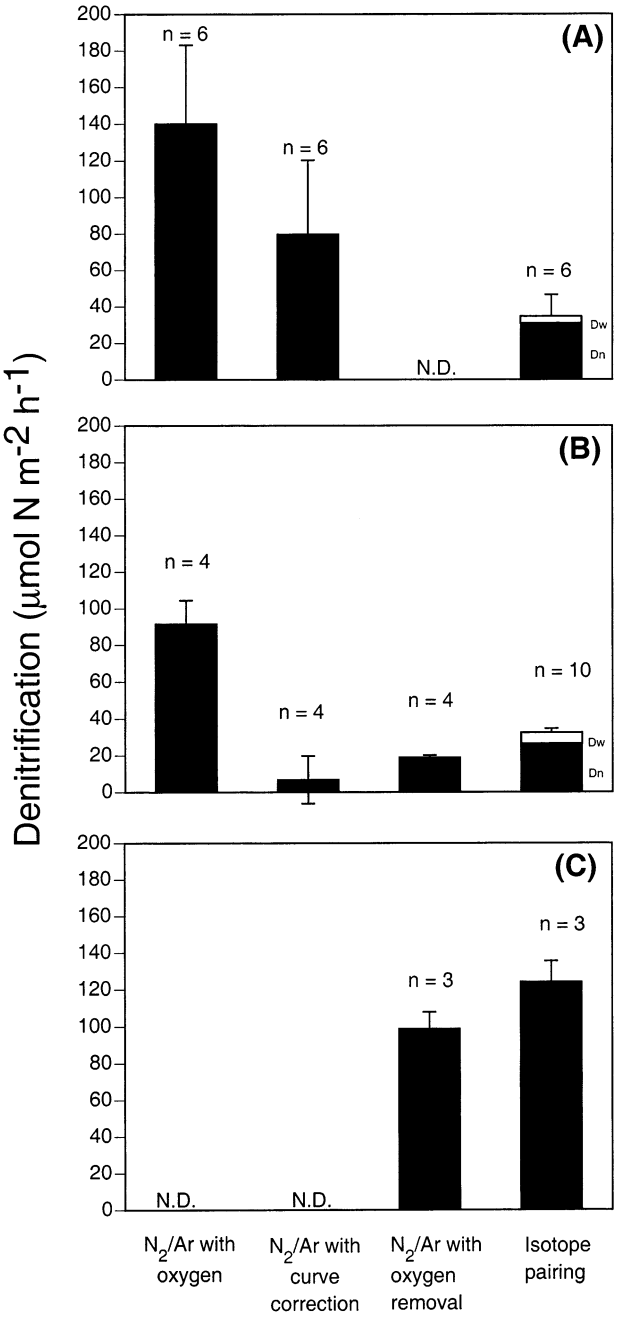

Fig. 1. Denitrification rates measured in Åarhus Bay in October (A), Åarhus Bay in November (B), and Norsminde Fjord in November (C). The first bar from the left shows the rates obtained by the original N_2:Ar method without any correction for oxygen in the samples. The second bar shows data obtained by the N_2:Ar method with correction for the effect of oxygen using a curve of the relationship between N_2 and O_2 concentrations. The third bar shows data obtained by the N_2:Ar method after oxygen was removed from the samples. The fourth bar shows rates obtained by the isotope pairing technique, where the open part of the bar is the coupled nitrification-denitrification (D_n) and the filled bar is denitrification of nitrate from the water column (D_w). ND = not determined.

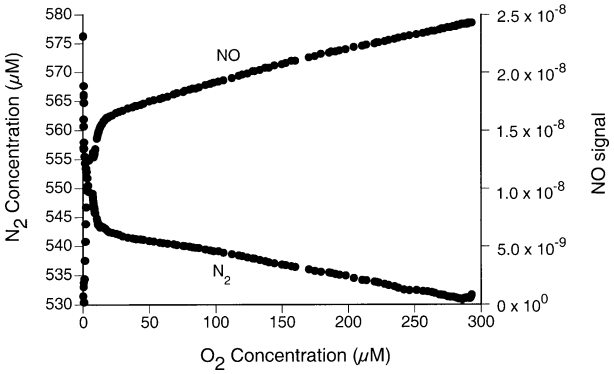

Fig. 2. N_2 concentration and NO signal versus O_2 concentrations in the MIMS. Air saturated seawater with a salinity of 26 and a temperature of 10.9°C was measured on a modified MIMS (i.e., including the copper reduction column and furnace). As the furnace was switched on and allowed to heat to 600°C, gradually more O_2 was removed from the samples reaching the detector. In parallel to decreasing O_2 concentrations, the NO signal also decreased due to O_2 combining with N_2 within the mass spectrometer ion source forming NO^+ (Jensen et al. 1996). The result is a higher N_2 signal at lower O_2 concentration. Note the very rapid and non-linear change in the N_2 signal associated with only a small change in O_2 concentration at low O_2 concentration.

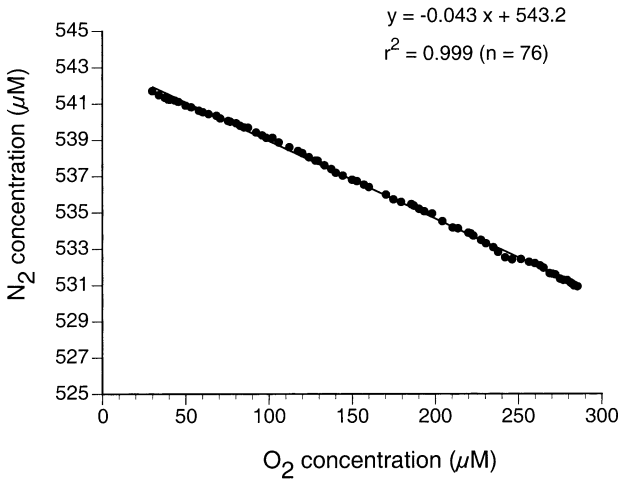

Fig. 3. Linear regression between N_2 concentration and O_2 concentration for incubation water from Åarhus Bay in November (subset of data from Fig. 2).

which was reflected in the higher, and much more variable, N_2:Ar determined denitrification rate in Åarhus Bay compared to the rate obtained from isotope pairing (Fig. 1). Shallow water sediments incubated in light often increase the water column O_2 concentration due to photosynthesis by benthic microalgae. In such situations, the reaction of O_2 with N_2 would cause the opposite error. The increasing O_2 concentrations alone would result in a reduced N_2:Ar ratio that would be interpreted as a reduced denitrification or nitrogen fixation. From Fig. 2 it is further evident that severe problems in obtaining any useful N_2:Ar ratios will occur at low oxygen concentrations, at least with the particular mass spectrometer used during this study. Heggie et al. (1999) also found a large change in N_2 concentration (> 10 μM) over the course of a 24 h incubation using the N_2:Ar method. Similarly, Cornwell et al. (1999) observed a large change in N_2:Ar ratios (0.40) over a 7 h incubation. The high N_2 production rate may only be due to a pseudo-denitrification caused by the reaction of O_2 with N_2 in the samples during analysis.

Clearly the reaction of O_2 with N_2 in the MIMS must be corrected to give N_2:Ar ratios expressing the correct denitrification rates. Oxygen correction curves were therefore developed for incubation water from Åarhus Bay using both addition of increasing amounts of sodium dithionite and the introduction of a copper reduction column operated at different temperatures (0°C to 600°C). The copper reduction column was much easier to use to get a large number of data points (data not shown) than the sodium dithionite additions and as such, was used to correct the denitrification rates for Åarhus Bay. Despite an excellent relationship between measured O_2 and N_2 concentrations in the standard water (r^2 = 0.999; n = 76; Fig. 3) the corrected denitrification rates were much more variable and still very different from the rates obtained from isotope pairing. For example, the corrected denitrification rate from Åarhus Bay in October (80 ± 41 μmol N m^{-2} h^{-1}) was still more than twice as high, and three times more variable, than the rate obtained using isotope pairing (34 ± 5 μmol N m^{-2} h^{-1}; Fig. 1a), while the corrected denitrification rates in November were only one quarter of the isotope pairing rates, and 30 times more variable (Fig. 1b). To correct for the pseudo-production of N_2 within the mass spectrometer, two high signals (much higher than the signal from bacterial denitrification) were subtracted, which most likely causes the observed high variability. We found it difficult to reproduce the slope of the correction curve with sufficiently high precision and thus the use of correction curves is not recommended.

To avoid having to correct for O_2 reacting with N_2, O_2 was removed from the samples from Norsminde Fjord and Åarhus Bay (November), before they entered the mass spectrometer. A copper reduction column heated to 600°C removed all O_2 within the samples. Once O_2 was removed, the N_2:Ar based denitrification rates in Norsminde Fjord and Åarhus Bay (November; 99 ± 12 and 19 ± 1 μmol N m^{-2} h^{-1}, respectively) agreed very well with the denitrification rates obtained by the isotope pairing technique (124 ± 19 and 32 ± 2 μmol N

TABLE 1. Assumption of the N_2:Ar and isotope pairing techniques and how to test/insure the assumptions are being met.

N_2:Ar Method		Isotope Pairing Technique	
Assumptions	How to Test/Insure Assumptions are Being Met	Assumptions	How to Test/Insure Assumptions are Being Met
Ar concentrations remain constant during incubation	Avoid temperate changes during incubation; incubation chamber must be sealed; Use glass incubation chambers or a long (24 h) pre-incubation period and blanks; Avoid bubble formation; Replacement water must be kept at same temperature as incubation chamber.	The addition of tracer ($^{15}NO_3^-$) may not alter in situ denitrification rate.	A test incubation with different additions of $^{15}NO_3^-$ will reveal the optimal addition of tracer for a correct estimation of the in-situ denitrification rate.
Precision (%CV) of 0.01%	Collect triplicate samples and determine average %CV over incubation.	A uniform mixing of the added $^{15}NO_3^-$ with the endogenous source of $^{14}NO_3^-$ must be present.	A test incubation with different additions of $^{15}NO_3^-$ will also reveal if isotopes are uniformly mixed.
N-fixation rates are low compared to denitrification rates	Run isotope pairing and N_2:Ar techniques simultaneously to check N-fixation rates.	A stable NO_3^- concentration gradient must be established within a short time of $^{15}NO_3^-$ addition relative to the total incubation period.	A test incubation where cores are processed at different time intervals will show if the nitrate gradient is stable.
N_2 gradient is in steady state	Long pre-incubation; linear changes in N_2.	N_2 production by combined NO_3^- reduction and NH_4^+ oxidation should be low.	A test incubation with anoxic sediment slurries amended with $^{15}NO_3^-$ should be performed.

m^{-2} h^{-1}, respectively; Fig. 1b,c). Furthermore, the precision of the estimates were almost the same. In both cases the N_2:Ar rate was slightly lower than the isotope pairing rate. Because the N_2:Ar method measures net N_2 fluxes (denitrification minus N-fixation), the difference between the two methods most likely reflects N-fixation.

The significant reduction in variability between rates obtained from samples with O_2 being removed before processing reflects, to some degree, the improvement in precision of the N_2:Ar analysis. Coefficients of variation of N_2:Ar in triplicate samples ($n = 42$) without O_2 removal ranged from 0.111% to 0.014% and averaged 0.037%. In contrast, when O_2 was removed coefficients of variation of N_2:Ar in triplicate samples ($n = 21$) ranged from 0.032% to 0.003% and averaged 0.010%. The removal of oxygen also decreased the time it took for the signal to stabilize between samples, as the N_2 signal was no longer dependent on variations in the O_2 concentration. This in-turn increased the through-put of samples. The magnitude of oxygen effect may be instrument specific because the geometry and electronic characteristics of the ionization source will vary between instruments. There is little disadvantage in removing oxygen from the MIMS analysis as it can be very easily measured by other equally good techniques (e.g., electrochemical probe, Winkler Titration).

Implications and Assumptions—N_2:Ar Technique

This study has shown that denitrification rates using N_2:Ar ratios in batch experiments with O_2 removal were significantly lower than rates obtained using the original method of Kana et al. (1994), at least when using a Balzers QMS422 mass spectrometer. Lower denitrification rates have a number of important implications regarding the assumptions of the N_2:Ar method (Table 1).

Measuring Small Changes in N_2 Concentrations

In our experiment with a surface area to water volume fairly typical of batch experiments, a denitrification rate of 19 μmol N m^{-2} h^{-1} (Aarhus Bay, November) resulted in only a 0.65 μM change in N_2 concentration over a 10 h incubation (Fig. 4). Against a background of 535 μM N_2, an analytical precision (coefficient of variation) of $< 0.03\%$ was required (0.008% was achieved) to see a statistically significant change (ANOVA, $p = 0.05$). For denitrification rates of < 5 μmol N m^{-2} h^{-1}, which are routinely measured using isotope pairing (e.g., Rysgaard et al. 1995; Dong et al. 2000; Sundbäck and Miles 2000), an analytical precision (coefficient of variation) of $< 0.01\%$ would be required using our experimental set-up. A precision of

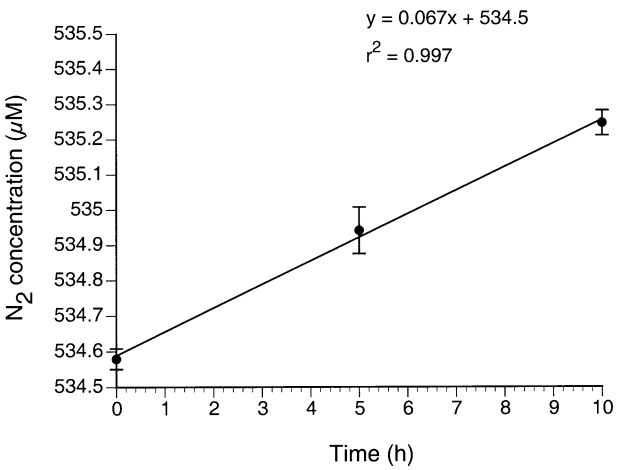

Fig. 4. Change of N_2 concentration versus time in incubated cores from Åarhus Bay, November. Analyses were undertaken on a modified MIMS where oxygen was removed from the samples using a copper reduction column at 600°C. Error bars are ± two times the standard deviation of triplicate samples.

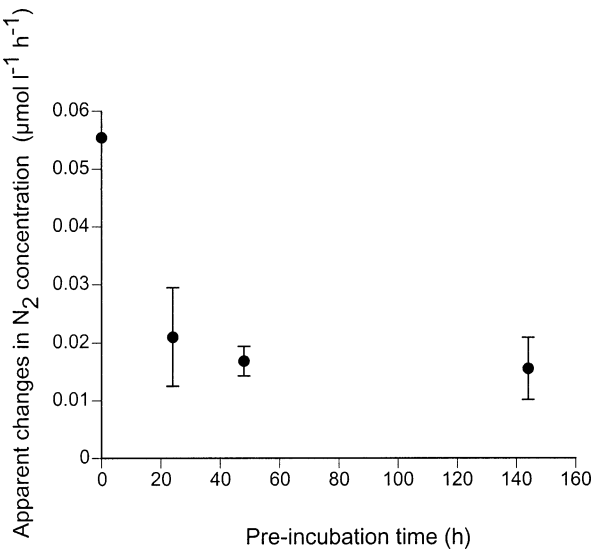

Fig. 5. Apparent changes in N_2 concentrations in core tubes filled with filtered sea water as a function of the pre-incubation period. The N_2 concentration is determined from the N_2:Ar ratio which changed due to different permeabilities of N_2 and argon in Plexiglas cores.

0.01% is five times better than the precision reported in the original method of Kana et al. (1994). Although we were able to achieve an average precision of 0.01% with our modified MIMS set up, it required meticulous attention to instrument set-up. Most importantly an extended warm-up period to minimize drift (a drift of less than 0.01 in the N_2:Ar ratio per hour was achieved) and frequent re-tuning of the ion source to maximize signal stability.

The detection of the very low N_2 concentration changes (i.e., 0.65 µM N_2 production over a 10 h incubation in Åarhus Bay, November; Fig. 4), requires a MIMS set-up with in-line O_2 removal, as even small changes in oxygen concentrations would otherwise obscure the real changes in N_2 concentrations. A reduction of only 15 µM in the O_2 concentration during the 10 h incubation alone would give an apparent change in N_2 concentration of 0.65 µM due to the effect of O_2 on the N_2 signal (Fig. 3). O_2 correction curves could have been applied, but this would result in a lower precision. Denitrification would then be determined as the difference between two relative large numbers (apparent N_2 concentration change minus N_2 concentration change due to the O_2 effect) and the end result would be highly variable.

Constant Argon Concentrations

Another major assumption of the N_2:Ar method is that argon concentrations remain constant over the course of an incubation (Table 1). This is of critical importance if small changes in N_2 (< 1 µM) associated with denitrification are to be detected against a large N_2 background (300 to 500 µM). The change in argon concentration needed to give a substantial change in N_2:Ar ratio is very small. During a typical incubation of the Åarhus Bay sediment, the N_2:Ar ratio changed from 36.3790 to 36.4267 resulting in a calculated increased N_2 concentration of 0.7011 µM from a starting concentration of 534.5623 µM (corresponding to an increase of 0.13%). If on the other hand it is assumed that the N_2 concentration remained constant, an equivalent change in the N_2:Ar ratio could have been obtained by a reduction in the argon concentration of only 0.0192 µM (from a starting concentration of 14.6942 µM). Using the N_2:Ar method, it is therefore absolutely essential that the argon concentration remains totally constant during the entire incubation. The argon concentration may change due to the type of material used in core construction, introduction of gas bubbles, and introduction of replacement water of a different temperature. It is necessary to minimize these sources of error.

The Type of Material. Blank cores incubated with filtered seawater (0.2 µm) showed a pseudo-change in N_2 concentration due to the different permeabilities and solubilities of N_2 and argon in Plexiglas (Fig. 5) which affect the measured N_2:Ar ratios. The magnitude of this pseudo-change was dependent on how long the cores were pre-incubated (Fig. 5). In our experimental set-up, blank cores taken from room temperature (23°C) and incubated with filtered sea water at 14°C with no pre-incubation period, produced a pseudo-denitri-

fication rate of 7 μmol N m^{-2} h^{-1}. The effect decreased rapidly with pre-incubation and showed little change beyond 48 h (Fig. 5) equalling a pseudo-denitrification rate of about 2 μmol N m^{-2} h^{-1}. This effect will vary depending on the composition of the cores but it is expected that all plastic type cores will experience a similar problem. We recommend either glass cores which will not effect the N_2:Ar ratio or long pre-incubation periods (minimum 24 h) with plastic type cores and associated blank corrections, although the latter may still give problems for sediments with very low denitrification rates.

Another error of this type can be made when storing water samples for N_2:Ar ratio measurements in vials with rubber septa. Due to the different solubilities and permeabilities of N_2 and argon in rubber the N_2:Ar ratio in the sample water changes. The measured N_2:Ar ratios would not reflect the ratios in the water phase of the sediment core at the time of sampling.

Introduction of Gas Bubbles. Bubbles introduced from the atmosphere (e.g., leaking cores; replacement water) or by benthic oxygen production also changes the argon concentrations due to the different solubility of argon and N_2 in bubbles which affect the measured N_2:Ar ratios. For example, blank cores incubated with filtered seawater (0.2 μm) into which about 30–50 bubbles of 1 mm diameter were introduced showed a significant pseudo-decrease in the N_2 concentration due to the higher solubility of N_2 compared to argon in the bubbles (data not shown). If denitrification rates are to be measured using N_2:Ar ratios in light incubations, where there is likely to be bubble production, the partial pressure and concentration of O_2 must first be lowered to avoid bubble formation. This can be easily achieved by running a dark incubation prior to the light incubation (Eyre and Ferguson 2002).

Changing Temperatures. Dissolved argon concentrations change with temperature (Weiss 1970). Once a core is sealed it is assumed that small changes in temperature will not affect dissolved argon concentrations. In incubations where the water is replaced when a sample is withdrawn, a small change in the temperature of the replacement water (± 1°C) is sufficient to change the argon concentration in the core and produce a pseudo-denitrification rate. The replacement water must be keep at exactly the same temperature as the core or benthic chamber or be withdrawn from a sealed container (e.g., glass syringe, collapsible bag) kept at the same temperature as at the start of the incubation.

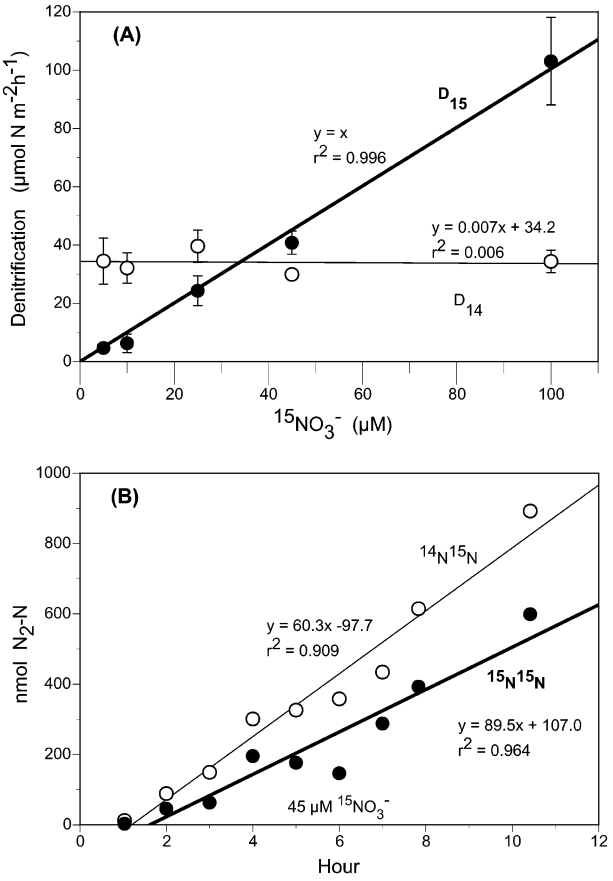

Fig. 6. Test incubation from Åarhus Bay. A) Denitrification of added $^{15}NO_3^-$ (D_{15}) and of in situ NO_3^- (D_{14}) as a function of water column $^{15}NO_3^-$ concentrations. Error Bars indicate SE (n = 9). B) Production of $^{29}N_2$ and $^{30}N_2$ as a function of time after addition of $^{15}NO_3^-$. The latter time series represents the 45 μM $^{15}NO_3^-$ concentration in (A).

Implications and Assumptions—Isotope Pairing Technique

Correct determination of in situ denitrification using the isotope pairing technique requires four important assumptions to be fulfilled (Table 1):

Addition of $^{15}NO_3^-$

Additions of $^{15}NO_3^-$ must not alter the rate of denitrification of in situ NO_3^-. This assumption was tested in detail for the Åarhus Bay sediment. Measurement of in situ denitrification (D_{14}) was not affected by addition of different concentrations (5–100 μM) of water phase $^{15}NO_3^-$ (Fig. 6a), and the first assumption was fulfilled. A larger standard error of the mean D_{14} was observed at the lower $^{15}NO_3^-$ additions, and if routine measurements are to be continued at this site we recommend the addition of ~ 50 μM $^{15}NO_3^-$.

Uniform Mixing of $^{15}NO_3^-$

The added $^{15}NO_3^-$ must mix uniformly with the NO_3^- already present in the water column and in the sediment, and the ratio of $^{15}NO_3^-$ to $^{14}NO_3^-$ must be constant throughout the denitrification zone. Heterogeneous topography, bioturbation, inhomogeneous nitrification activity, etc., may cause local variations in the transport of $^{14}NO_3^-$ and $^{15}NO_3^-$ to the anoxic denitrification zone. This may underestimate in situ denitrification activity (D_{14}) since $^{14}N^{15}N$ production would then be less than that predicted from the assumptions of homogeneity (Broast et al. 1988). As demonstrated by several authors (Nielsen 1992; Rysgaard et al. 1995; Dong et al. 2000) this possible underestimation can also be analyzed by incubating the sediment cores at different $^{15}NO_3^-$ concentrations. At increasing $^{15}NO_3^-$ concentrations, an increased denitrification of $^{14}NO_3^-$ will be detected directly as $^{14}N^{15}N$ on the mass spectrometer, thereby lowering the possible underestimation of D_{14}. The estimate of D_{14} would consequently increase with increased $^{15}NO_3^-$ addition until a constant level where the optimal addition of $^{15}NO_3^-$ is found. As demonstrated in the optimization experiment, D_{14} was independent of the water phase NO_3^- concentration at concentrations higher than 5 μM, indicating uniform mixing of the added $^{15}NO_3^-$ (Fig. 6a). Hence, the second assumption was fulfilled.

Stable NO_3^- Concentration Gradient

A stable NO_3^- concentration gradient must be established in the surface layer of the sediment within a short time after $^{15}NO_3^-$ addition relative to the duration of incubation. If not, denitrification activity will be underestimated since the added $^{15}NO_3^-$ will not be immediately available to the denitrifying bacteria in the anoxic zone of the sediment. The time needed to establish a stable NO_3^- gradient depends on the O_2 penetration depth. During summer, when oxygen typically penetrates only a few millimeters down into coastal sediments, the 90% equilibration time is approximately 5 min (Nielsen 1992). During winter, when the O_2 penetration is deeper, the establishment of a new NO_3^- gradient takes longer. Time series are recommended where different sediment cores are processed at different time intervals making it possible to check that a linear production rate is occurring. The optimization experiment showed a linear ^{15}N-dinitrogen production after ~ 1 h (Fig. 6b). Stable NO_3^- profiles were established within less than 1 h, a short period compared to the total incubation time of 11 h. The third assumption was also fulfilled for this sediment.

Interference by Anammox

The fundamental limitation of the isotope pairing method is the demand for a uniform mixing of the added $^{15}NO_3^-$ with the endogenous source of $^{14}NO_3^-$. The bacterial process anammox, oxidizing NH_4^+ with NO_3^-, has recently been demonstrated in marine sediments (Thamdrup and Dalsgaard 2002). The process may interfere with the assumption of uniform mixing because added $^{15}NO_3^-$ may react with $^{14}NH_4^+$ in the anoxic zone of denitrification with the formation of $^{29}N_2$. Anammox occurring at high rates will interfere with the mathematics of the isotope pairing and to various degrees overestimate denitrification. It is relatively easy to test if anammox is occurring by incubating sediment in anoxic slurries with added $^{15}NH_4^+$ and $^{14}NO_3^-$ and looking for the formation of $^{29}N_2$ which would indicate that the anammox process occurs (Thamdrup and Dalsgaard 2002). An anoxic slurry incubation with $^{15}NO_3^-$ added would in addition give both the denitrification and the anammox rates and the relative importance of the two processes can be evaluated. Those test incubations were carried out on the Åarhus Bay sediment and the anammox process was found to be of minor importance in this sediment (responsible for $< 2\%$ of total N_2 production). Thus the fourth assumption was also fulfilled.

Isotope Pairing on the Membrane Inlet Mass Spectrometer

The addition of a copper reduction column heated to 600°C to the MIMS and the subsequent decrease in the formation NO^+ which interferes at $m/z = 30$ allowed the analysis of $^{29}N_2$ and $^{30}N_2$. Duplicate samples for $^{29}N_2$ and $^{30}N_2$ analysis were collected from the Åarhus Bay (November) isotope pairing experiments ($n = 10$) and run on both the modified MIMS and the GC-MS. On the MIMS, the sediment slurry was introduced directly from 7-ml glass vials into the membrane inlet after the larger particles had settled out. A linear regression between the two sets of data showed very little difference between the two instruments (Fig. 7). The slope is slightly, but significantly ($p = 0.001$), different from one (1.082) which most likely reflects the different instrument response factors for the different sensitivities at $m/z = 29$ and $m/z = 30$ (Jensen et al. 1996). The correlation between the two instruments could be improved by determining, and correcting for, these instrument response factors. These small differences are of little ecological significance because the denitrification rates calculated from the isotope production using the modified MIMS (32 ± 3 μmol N m^{-2} h^{-1}) and GC-MS (35 ± 2 μmol N m^{-2} h^{-1}) are not significantly

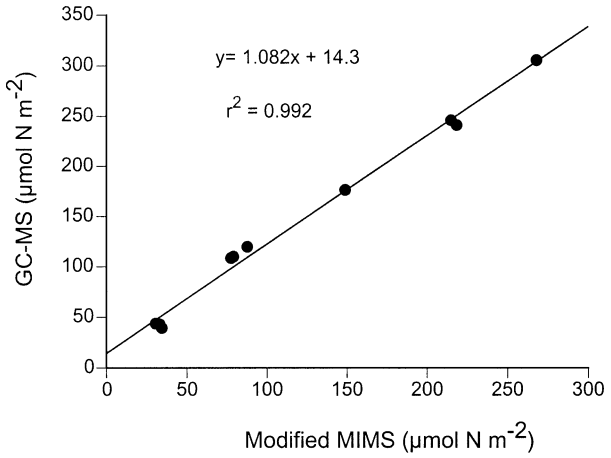

Fig. 7. Linear regression between denitrification data estimated by isotope measurements on the modified MIMS and GC-MS, respectively.

different. We also tried the approaches where the effect of NO^+ formation is corrected for using a regression between the products of signals at m/z = 32 and m/z = 28 and the signal at m/z = 30 (Jensen et al. 1996) or the square root of the products of signals at m/z = 32 and m/z = 28 and the signal at m/z = 30 (An et al. 2001). The m/z = 30 signal was consistently too high and variable (data not shown) and not suitable for use in the isotope pairing calculations.

Measuring N_2 Fixation Combining the Two Techniques

The N_2:Ar method measures net N_2 fluxes resulting from denitrification minus N-fixation, whereas the isotope pairing technique measures a rate very close to the gross N_2 production (the real denitrification). If N-fixation is significant, the N_2:Ar method will underestimate denitrification activity. If both methods are applied, a good estimate of N-fixation may be obtained. An et al. (2001) have previously estimated N-fixation and denitrification rates by simultaneously measuring $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ gas species on a MIMS and applying a series of formulas. Our approach is more straightforward (simple subtraction) and does not suffer from the problems associated with removing the effect of NO^+ formation using a regression between the square root of the products of signals at m/z = 32 and m/z = 28 and the signal at m/z = 30 (as discussed above).

When including the copper furnace to remove all oxygen from the samples, the N_2 production rate measured with the N_2:Ar method was slightly lower than the isotope pairing technique. The differences between the two assays were 13 and 25 µmol N m^{-2} h^{-1} for Åarhus Bay and Norsminde Fjord, respectively (Fig. 1b,c). The potential for N-fixation in the Åarhus Bay sediment was estimated by applying the acetylene reduction technique (Capone 1993) which gave rates of 6 µmol N m^{-2} h^{-1} (assuming a 3:1 ratio between ethylene production and N-fixation; data not shown) which is in the same order of magnitude as the difference between the isotope pairing estimate and the N_2:Ar estimate. It is well known that the conversion factor to relate ethylene production to N_2 reduction may vary significantly (Seitzinger and Garber 1987), and it should not be expected that the N-fixation estimate would exactly match the difference between the two assays. It seems promising to combine the N_2:Ar method and the isotope pairing technique to obtain new information on the relative importance of N-fixation in coastal sediments. This approach has the advantage of being based on direct measurements of N_2 fluxes providing denitrification and N-fixation rates simultaneously.

Literature Cited

An, S., W. Gardner, and T. Kana. 2001. Simultaneous measurement of denitrification and nitrogen fixation using isotope pairing with membrane inlet mass spectrometry analysis. *Applied and Environmental Microbiology* 67:1171–1178.

An, S. and S. B. Joye. 1997. An improved gas chromatographic method for measuring nitrogen, oxygen, argon and methane in gas or liquid samples. *Marine Chemistry* 59:63–70.

Berelson, W. M., D. Heggie, A. Longmore, T. Kilgore, G. Nickolson, and G. Skyring. 1998. Benthic nutrient recycling in Port Phillip Bay, Australia. *Estuarine, Coastal and Shelf Science* 46:917–934.

Braman, R. S. and S. A. Hendrix. 1989. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection. *Analytical Chemistry* 61:2715–2718.

Broast, C. W., R. L. Mulvaney, and P. Baveye. 1988. Evaluation of the nitrogen-15 tracer techniques for direct measurements of denitrification in soil: 1 Theory. *Soil Science Society of America Journal* 52:1317–1322.

Capone, D. G. 1993. Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure, p. 621–631. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (eds.). Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers, Boca Raton, Florida.

Cornwell, J. C., W. M. Kemp, and T. M. Kana. 1999. Denitrification in coastal ecosystems: Methods, environmental controls and ecosystem level controls, a review. *Aquatic Ecology* 33:41–54.

Devol, A. H. 1991. Direct measurement of nitrogen gas fluxes from continental shelf sediments. *Nature* 349:319–321.

Dong, L. F., D. C. O. Thornton, D. B. Nedwell, and G. J. C. Underwood. 2000. Denitrification in sediments of the river Colne estuary, England. *Marine Ecology Progress Series* 203:109–122.

Eyre, B. D. and A. J. P. Ferguson. 2002. Comparison of carbon production and decomposition, benthic nutrient fluxes and denitrification in seagrass, phytoplankton, benthic microalgal and macroalgal dominated warm temperate Australian lagoons. *Marine Ecology Progress Series* 229:43–59.

Heggie, D. T., G. W. Skyring, J. Orchardo, A. R. Longmore, G. J. Nicholson, and W. M. Berelson. 1999. Denitrification and denitrifying efficiencies in sediments of Port Phillip Bay:

Direct determinations of biogenic N_2 and N-metabolites fluxes with implications for water quality. *Marine and Freshwater Research* 50:589–596.

JENSEN, K. M., M. H. JENSEN, AND R. P. COX. 1996. Membrane inlet mass spectrometric analysis of N-isotope labelling for aquatic denitrification studies. *FEMS Microbiology and Ecology* 20:101–109.

JØRGENSEN, B. B. 1996. Case study—Aarhus Bay, p. 137–154. *In* B. B. Jørgensen and K. Richardson (eds.). Coastal and Estuarine Studies. American Geophysical Union, Washington, D.C.

KANA, T. M., C. DARKANGELO, M. D. HUNT, J. B. OLDAM, G. E. BENNETT, AND J. C. CORNWELL. 1994. Membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2 and Ar in environmental samples. *Analytical Chemistry* 66: 4166–4170.

KANA, T. M., M. B. SULLIVAN, J. C. CORNWELL, AND K. GROSZKOWSKI. 1998. Denitrification in estuarine sediments determined by membrane inlet mass spectrometry. *Limnology and Oceanography* 43:334–339.

LAMONTAGNE, M. G. AND I. VALIELA. 1995. Denitrification measured by a direct N_2 flux method in sediments of Waquoit Bay, Massachusetts. *Biogeochemistry* 31:63–83.

LARSEN, L. H., N. P. REVSBECH, AND S. J. BINNERUP. 1996. A microsensor for nitrate based on immobilised denitrifying bacteria. *Applied and Environmental Microbiology* 62:1248–1251.

LAURSEN, A. E. AND S. P. SEITZINGER. 2002. The role of denitrification in nitrogen removal and carbon mineralisation in Mid-Atlantic Bight sediments. *Continental Shelf Sediments* 22: 1397–1416.

NEDWELL, D. B. AND M. TRIMMER. 1996. Nitrogen fluxes through the upper estuary of the great Ouse, England: The role of the bottom sediments. *Marine Ecology Progress Series* 142:273–286.

NIELSEN, L. P. 1992. Denitrification in sediment determined from nitrogen isotope pairing. *FEMS Microbiology and Ecology* 86:357–362.

NIELSEN, K., L. P. NIELSEN, AND P. RASMUSSEN. 1995. Estuarine nitrogen retention independently estimated by the denitrification rate and mass balance methods: A study of Nordsminde Fjord, Denmark. *Marine Ecology Progress Series* 119:275–283.

NIXON, S. W. 1981. Remineralization and nutrient cycling in coastal marine ecosystems, p. 111–113. *In* B. J. Nielson and L. E. J. Cronin (eds.). Estuaries and Nutrients. Humana, Clifton, New Jersey.

NOWICKI, B. L. 1994. The effect of temperature, oxygen, salinity and nutrient enrichment on estuarine denitrification rates measured with a modified nitrogen gas flux technique. *Estuarine, Coastal and Shelf Science* 38:137–156.

RISGAARD-PETERSEN, N., L. P. NIELSEN, AND T. H. BLACKBURN. 1998. Simultaneous measurement of benthic denitrification, with the isotope pairing technique and the N_2 flux method in a continuous flow-through system. *Water Research* 32:3371–3377.

RISGAARD-PETERSEN, N. AND S. RYSGAARD. 1995. Nitrate reduction in sediments and waterlogged soil measured by ^{15}N techniques, p. 287–310. *In* K. Alef and P. Nannipieri (eds.). Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London, U.K.

RISGAARD-PETERSEN, N., S. N. P. RYSGAARD, AND N. P. REVSBECH. 1993. A sensitive assay for determination of $^{14}N/^{15}N$ isotope distribution in NO_3^-. *Journal of Microbial Methods* 17:155–164.

RYSGAARD, S., P. B. CHRISTENSEN, AND L. P. NIELSEN. 1995. Seasonal variation of denitrification and nitrification in a bioturbated and microphyte inhabited estuarine sediment, Kertinge Nor (Denmark). *Marine Ecology Progress Series* 126:111–121.

RYSGAARD, S., B. THAMDRUP, N. RISGAARD-PETERSEN, P. BERG, H. FOSSING, P. B. CHRISTENSEN, AND T. DALSGAARD. 1998. Seasonal carbon and nitrogen mineralization in the sediment of Young Sound, Northeast Greenland. *Marine Ecology Progress Series* 175: 261–276.

SEITZINGER, S. P. 1987. Nitrogen biogeochemistry in an unpolluted estuary: The importance of benthic denitrification. *Marine Ecology Progress Series* 41:177–186.

SEITZINGER, S. P. AND J. H. GARBER. 1987. Nitrogen fixation and $^{15}N_2$ calibration of acetylene reduction assay in coastal marine sediments. *Marine Ecology Progress Series* 37:65–73.

SMITH, L. K., J. J. SARTORIS, J. S. THULLEN, AND D. C. ANDERSEN. 2000. Investigation of denitrification rates in an ammonia-dominated constructed wastewater treatment wetland. *Wetlands* 20:684–696.

SØRENSEN, J. 1987. Nitrate reduction in marine sediments: Pathways and interactions with iron and sulphur cycling. *Geomicrobiology Journal* 5:401–421.

SUNDBÄCK, K. AND A. MILES. 2000. Balance between denitrification and microalgal incorporation of nitrogen in microtidal sediments, NE Kattegat. *Aquatic Microbial Ecology* 22:291–300.

THAMDRUP, B. AND T. DALSGAARD. 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Applied and Environmental Microbiology* 68:1312–1318.

TUOMINEN, L., A. HEINANEN, J. KUPARINEN, AND L. P. NIELSEN. 1998. Spatial and temporal variability of denitrification in the sediments of the northern Baltic proper. *Marine Ecology Progress Series* 172:13–24.

WEISS, R. F. 1970. The solubility of nitrogen, oxygen and argon in water and sea water. *Deep Sea Research* 17:721–735.

SOURCES OF UNPUBLISHED MATERIALS

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