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Estimating Rates of Denitrification Enzyme Activity in Wetland Soils with Direct Simultaneous Quantification of Nitrogen and Nitrous Oxide by Membrane Inlet Mass Spectrometry

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Abstract

The microbial mediated process of denitrification is a major pathway for the removal of reactive nitrogen a pollutant in natural environments and waste treatment facilities. Denitrification potential, measured as denitrification enzyme activity (DEA), was quantified in novel short-term (4 h) anaerobic assays using a more sensitive and precise technique, membrane inlet mass spectrometry (MIMS) rather than the traditional headspace electron capture gas chromatography (GC-ECD) method. Using MIMS modifications made to the instrument and sample handling allowed for the simultaneous and direct measurement of reaction products nitrous oxide (N_2O), a potent greenhouse gas, and the chemically unreactive dinitrogen (N_2). Rate determinations were made from the slope of a linear curve generated by plotting increasing concentrations of the reaction products with time. Strong evidence for the validity of MIMS measured DEA rates was provided by showing consistent, linear accumulations of N_2O or N_2 and close agreement in rates from replicate reactions. Reactions were performed using wetland soils and cultures of *Pseudomonas aeruginosa* and *P. chloroaphis* that generated denitrification end products of N_2 and N_2O, respectively. Under acetylene inhibition *P. aeruginosa* produced the N_2O end product at a rate equivalent to the rate obtained in the uninhibited reaction that produced N_2. No significant ($p>0.05$) difference was observed between MIMS or headspace with GC-ECD, determined DEA in wetland soil reactions under acetylene inhibition. Because of anoxic conditions in the reaction vessels used with MIMS, detectable rates of N_2O accumulation were only observed in acetylene blocked reactions or in cultures of *P. chloroaphis*. This method has potential applications ranging from near real-time wastewater treatment process measurements to field studies of nitrogen cycling. The continued development and application of these types of methods are needed to improve our understanding of the mechanisms regulating denitrification and its benign, N_2, and harmful, N_2O, end-products.

Keywords: Denitrification Enzyme Activity; Membrane Inlet Mass Spectrometry; nitrous oxide

Introduction

The denitrification enzyme activity (DEA) assay provides an assessment of the multi-enzyme, process of reactive nitrogen removal by the reduction of nitrate to nitrous oxide (N_2O) and/or nitrogen gas (N_2). Measured in soil slurries under non-limiting carbon and nitrate concentrations, this short term (≤ 4 h) anaerobic assay, also defined as denitrification potential [1], yields quantitative measures on potential denitrification rate. Denitrification enzyme activity is related to the magnitude of denitrifying enzymes and yields an index of the denitrifier population in soils reflective of long-term variation in the factors that control denitrification [2]. Denitrification capacity (DC) is defined in this manuscript as the endogenous denitrification rate measured in the absence of added nutrients. Chloramphenicol is added to prevent de novo synthesis of denitrification enzymes so that DEA is reflective of the amount of denitrification enzymes at the time of sampling. Denitrification enzyme activity is commonly measured by the production of N_2O, which accumulates due to acetylene (C_2H_2) inhibition of the enzyme nitrous oxide reductase. Nitrous oxide, found in low concentration in the atmosphere, is readily measured by gas chromatography [3]. In the absence of an acetylene block the small amount of the major end product, N_2, is difficult to measure in the headspace due to the high background concentration of N_2 in the atmosphere [4].

In water, however, the background concentration of N_2 is much lower allowing denitrification to be determined by measuring the accumulation of N_2 in aqueous samples. Kana et al. [5] modified a membrane inlet mass spectrometer to perform precise measurements of dissolved N_2 based on the N_2/Ar ratio. This analytical capability, plus problems encountered with the C_2H_2 method limiting its usefulness [6], prompted Khalil and Richards [7] to develop a method to measure DEA in soil slurries using membrane inlet mass spectrometry (MIMS). These investigators demonstrated DEA by observing decreased N_2 / Ar ratios with increasing soil depth and peaks for the N_2/Ar ratios when the soil slurries were amended with optimal concentrations of NO_3-N and glucose-C for the expression of DEA.

Denitrification is a miserable process to measure as most methods are problematic for a variety of reasons [4]. The MIMS technique gained popularity for denitrification measurements as it is capable of rapidly (< 2 min) measuring small (< 7 ml) samples with high precision [5]. The purpose of this study was to expand the usefulness of MIMS-based techniques by developing a method that provided a quantitative

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measure of DEA plus provide the instrument with a greatly needed capability, namely, the ability to directly measure N_2O. Validation of these techniques was provided through the use of bacterial controls and by comparing our MIMS based approach to the established, acetylene inhibition, headspace GC-EDC method [3].

Driven largely by increases in emissions from agricultural activities N_2O concentrations in the atmosphere are projected to rise [8]. The application of nitrogenous fertilizers promotes release of N_2O via denitrification and nitrification [9]. Although many chemical and physical factors increase the proportion of N_2O to N_2 released during denitrification [10], little is known about factors controlling N_2O formation by denitrification in sediments of aqueous systems [11]. Therefore, detecting and quantifying N_2O by MIMS should increase the efficacy of this analytical technique for studying ways to minimize the release of this potent greenhouse gas in the atmosphere.

Presented in our study are changes made to enhance the efficacy of MIMS to measure denitrification and modifications to the protocol of Khalil and Richards [7] needed to quantify DEA. These were reaction vessel design, sample treatment, rate calculations, controls and data quality objectives. Reactions were performed and rates calculated in the presence (DEA) and absence (DC) of added glucose and nitrate. Observing higher DEA than DC in the same soil served as a data quality objective. Specific objectives were to: (i) modify the procedure and instrument, mass spectrophotometer, to allow direct simultaneous measurements of N_2 and N_2O; (ii) develop, using MIMS, a short term (4 h) assay to quantify DEA in wetland soils based on measuring the linear accumulation of N_2 and N_2O over time; (iii) express MIMS-measured DEA as a rate, μg N_2 or N_2O h^{-1} kg dry weight (wt) $soil^{-1}$; (iv) compare DEA in a wetland soil under acetylene inhibition using MIMS and headspace with GC-ECD to measure the N_2O product; (v) assay pure cultures of denitrifying bacteria as controls; and (vi) examine storage stability of dissolved N_2 in samples taken for MIMS analysis.

Materials and Methods

Wetland sites, soil sampling and soil characteristics

Wetland soils were obtained from three locations in the Pensacola Bay watershed identified, using the U.S. Fish and Wildlife Service National Wetlands Inventory [12] as freshwater forested/shrub (PF01/4B). Site names and their locations (latitude/longitude) in this northern Gulf of Mexico area are listed in Table 1. Soil samples, collected during the summer of 2012, were obtained from the top 10 cm of soil, passed through a 2-mm sieve and stored at room temperature until analysis. Denitrification enzyme activity or DC was measured in soil samples within 18 h of collection. A portion of the sample, 30 to 50 g, was weighed before and after drying at 110°C to calculate dry weight (dry wt). The percent organic matter was then calculated by the loss in weight after combustion for 18 h at 440°C [13].

Bacteria and growth conditions

Denitrification end products were also studied from pure cultures of two denitrifying bacteria, *Psuedomonas aeruginosa* (ATCC 15692) and *P. chloroaphis* (ATCC 9446), where they served as controls to compare to the soils samples since the former produces only N_2 [14] and the latter produces only N_2O (15). Bacteria were cultured at 25°C for 48 h without shaking in nitrate broth (Difco, Detroit, MI) contained in completely filled 100 ml screw-caped media bottles (Corning, Corning, NY). Dry weight of cells was determined by filtering 20 ml of culture through pre-weighed 0.40 μm polycarbonate filters. Filters were dried in a desiccator (25°C) to a constant weight and reweighted to calculate dry weight of cells per unit volume.

Reaction conditions when MIMS was used to measure end products of DEA or DC rates

The reaction vessel was a 160 ml glass, serum stoppered bottle equipped with two ports. One port was for removal of sample from the top, and the other to add fresh DEA or DC solution at the bottom of the vessel to restore volume after sample removal. Ports were provided with 8 gauge, BD (Franklin Lakes, NJ) Luer-LocTM needles inserted through the septum. A one-way valve was attached to the Luer-Loc connector. The distal end of the 1-way valve was fitted with either a 22 cm long tube (Tygon®, I.D. 5 mm) for the sample removal or a 10 ml syringe for DEA or DC addition (Figure 1A).

Approximately 50 g wet wt of soil or 20 ml of a bacterial culture was added to the reaction vessels followed by a sufficient volume of helium (He) flushed DEA or DC solution to enable the vessels to be sealed, without air entrapment, with a rubber stopper secured by a crimped aluminum cap. When used to block the reduction of N_2O to N_2, 10 ml of atomic absorption grade, 99.6% purity, acetylene (C_2H_2), was added to each vessel using a gas tight syringe. The acetylene was forced to slowly dissolve into the liquid by applying constant pressure to the plunger. The vessels were inverted several times. Two ports were inserted through the rubber septum. The remaining bubbles were removed through one port by displacement with DEA or DC solution added through a second, replenishment port. DEA solution, which made conditions for denitrification non-limiting, contained the following: KNO_3, 1.4 mg ml^{-1}; glucose, 1 mg ml^{-1} and chloramphenicol, 0.025 mg ml^{-1}. The DC solution lacked both nutrients. Reaction vessels were incubated with shaking at 25°C. At approximately 0.5, 1, 2, 3 and 4 h samples were removed via displacement of sample by injecting 9 ml of DEA or DC through the replenishment port. After each sample was removed the vessel was inverted to assure sufficient mixing. Samples were collected into 8 ml ground glass stoppered test tubes containing 50 μl of a saturated solution of sodium hydroxide stopping the reaction and converting CO_2, which interferes with quantification of N_2O, into sodium bicarbonate. The completely filled sample tubes were placed under water in test tube racks held in an appropriate size ice chest. Samples were stored at room temperature until analysis.

Site	Latitude/Longitude	Organic Carbon (%)	Denitrification capacity, no added nutrients[a]	DEA, added glucose and nitrate[b]
			μg N_2 h^{-1} kg dry wt $soil^{-1}$ ± Std Dev[c]	
Nature Reserve	30° 38.335' N/87° 04.525' W	9	4.4 ± 0.3	133 ± 13
Shoreline Park South	30° 21.109' N/87° 10.554' W	20	114 ± 86	525 ± 58
Lake Shore Trail	30° 21.127' N/87° 10.096' W	41	UD[d]	576 ± 98

[a]Denitrification reactions with only endogenous levels of nitrate and glucose. [b]Nutrients for denitrification were made non-limiting by the addition of nitrate and glucose to the reaction vessels. [c]Rates are expressed as the mean of triplicate determinations. Based on the results of t-tests ($p>0.05$), the null hypotheses were rejected. Thus, within each soil the denitrification enzyme activity rates were higher than the denitrification capacity rates. [d]UD, unable to determine (no increase in the N_2 /Ar ratio was observed)

Table 1: Denitrification enzyme activity rates determined in short term (4 h) anaerobic assays in the presence and absence of nutrient (NO_3-N, glucose-C) amendments. The nitrogen product was measured by membrane inlet mass spectrometry.

Analysis of dissolved N_2, N_2O, Ar and O_2 by MIMS

Dissolved gases were measured on a Bay Instruments dissolved gas analyzer (Easton, MD) that used Pfeiffer Vaccuum's Prisma mass spectrometer (Aslar, Germany). A "Shuttle Portable" ultralow freezer, Stirling Ultracold (Athens, OH) provided a -86°C cold trap (Figure 1B). The gas ratio method, employing argon (Ar) as the tracer [5], was used to calculate µg of dissolved N_2, N_2O, and O_2 in the reaction vessels. Calibration factors were used to adjust the N_2/Ar, O_2 /Ar and CO_2/Ar ratios measured in air-equilibrated deionized water (dH_2O, 20°C) to the ratios reported by Colt [16]. Specifically, these ratios were 38.187 for N_2/Ar, 20.393 for O_2/Ar, and 0.879 for CO_2/Ar [16]. The reported CO_2/Ar ratio served as a surrogate to determine a N_2O/Ar correction factor for calibrating the instrument. Carbon dioxide was an appropriate surrogate as both CO_2 and N_2O possess a molecular mass of 44, and the solubility of CO_2 is similar to that of N_2O [17]. Furthermore, a surrogate was necessary as the concentration of N_2O in air saturated water is orders of magnitude less than CO_2 [18]. To calculate the mass (µg) of each gas in the reaction vessel at the time of sampling the corrected ratios were multiplied by µM of Ar in dH_2O at air saturation (13.91 µM, 16). These µM concentrations were multiplied by their respective molecular weights and the volume of the reaction vessel (160 ml) to yield µg of each gas. A correction factor was used to account for loss of product from sampling.

Reaction conditions when GC-ECD was used to measure DEA rates by acetylene inhibition (headspace) method

DEA was measured as the mean of triplicate determinations using a modified version of the headspace with GC-EDC method described by White and Reddy [19] in which denitrification conditions are made non-limiting by the addition of excess nitrate and glucose. To each 160

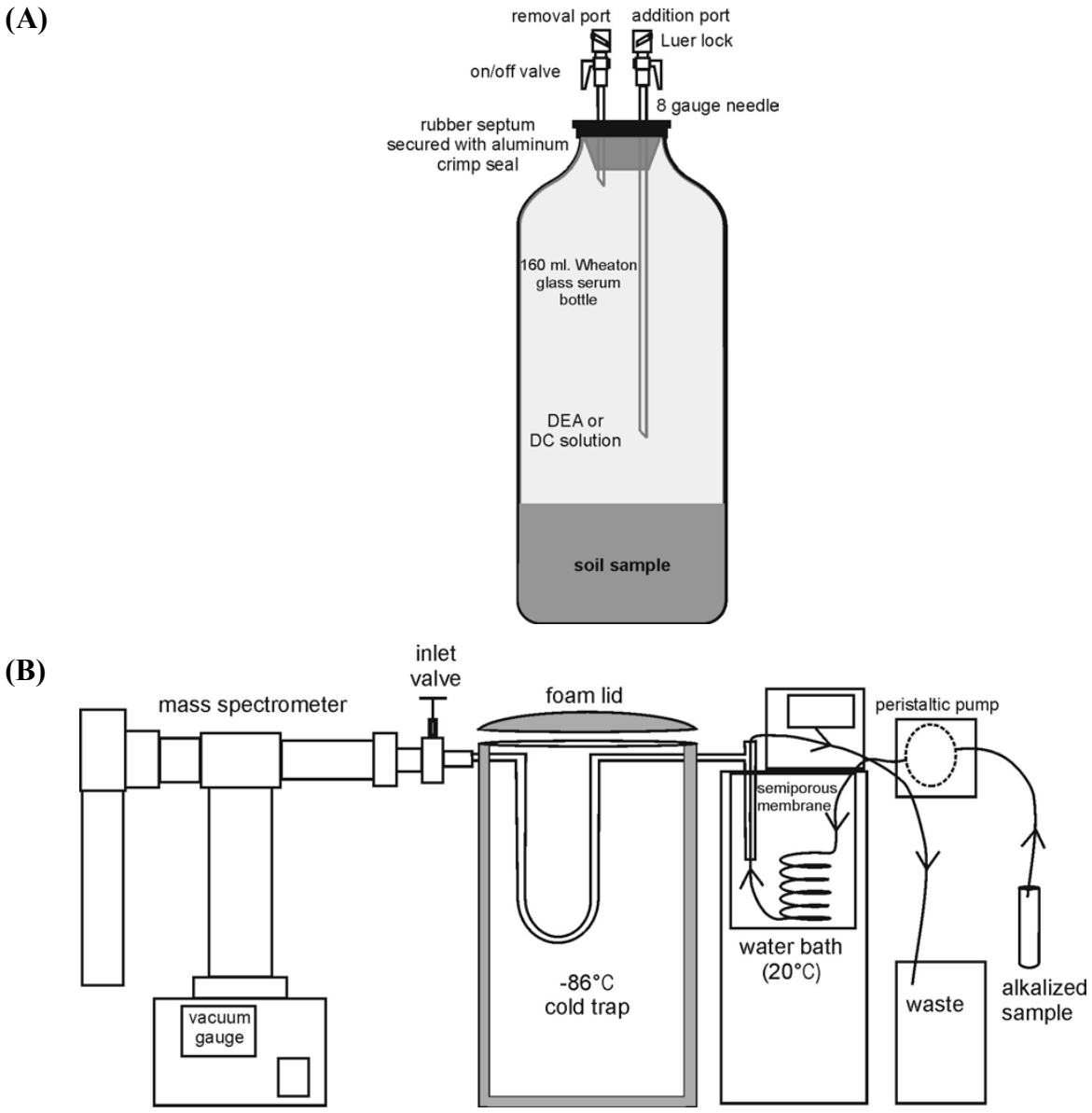


Figure 1: Schematic of reaction vessel (A) and membrane inlet mass spectrometer (B).

ml glass reaction vessel was added approximately 50 g wet wt soil and 50 ml DEA solution. The slurry was made anaerobic by flushing with N_2 gas prior to crimp sealing the vessel. Ten ml of headspace gas was replaced with 12 ml of C_2H_2 (approximately 15% of the headspace). The vessels were incubated with shaking at 25°C. Gas samples, removed at approximately 0.5, 1, 2, 3 and 4 h for N_2O analysis, were immediately injected into a Hewlett-Packard (Palo Alto, CA) 6890 GC with ECD adapted for direct gas injection through an 8-port valve with a 0.25 µL sample loop. Separations were performed using an Agilent (Santa Clara, CA) GS-Q megabore column (30 m X 0.53 mm I.D., porous divinyl homopolymer/durabond) run at 40°C.

Stability of dissolved nitrogen in samples stored for MIMS analysis

To explore the time constrains of performing DEA reactions at a laboratory lacking a membrane inlet mass spectrometer and transporting the samples to a different facility for MIMS analysis, the loss of dissolved N_2 in stored samples was measured over time. A denitrification reaction was set up as follows: soil, 325 g wet wt obtained from the Lake Shore Trail site, was placed in a 1 liter screw cap bottle and filled with DEA solution without air entrapment. Forty-five samples were removed from the reaction vessel after 8 h at 25°C, placed into sample storage tubes and held under water as described previously. After 1, 3, 6, 8 and 10 days nine replicate samples were removed and analyzed by MIMS for loss of dissolved nitrogen.

Statistical analyses

Statistical analyses were performed using SAS for Windows, release 9.2 (SAS Institute, Inc., Cary NC, USA). A two-sample t-test was used to compare the means, at the 95% confidence interval, of DEA and DC activities measured in soils or pure cultures of bacteria. A single factor ANOVA combined with a Tukey's Studentized Range (HSD) test was used to determine the storage time at which the means of the nitrogen concentrations in the samples were significantly ($p<0.05$) lower than the initial concentration.

Results and Discussion

Modifications to allow the direct measurement of nitrous oxide by MIMS

The membrane inlet mass spectrophotometer was reconfigured and samples alkalinized to directly measure N_2O in addition to N_2, Ar and O_2. Overlying waters [20], heavily polluted water [21] and wastewater treatment plants [22] are all aquatic environments where simultaneous fluxes of these gases have been shown to occur. Furthermore, using MIMS to measure denitrification in flow-through systems by only the N_2/Ar ratio may underestimate this reaction if reduction stopped at nitrous oxide rather than continuing to completion [6].

In the current study a mixture of the two end products in the reactions was not observed. This finding was in agreement with the results of Khalil and Richards [7] who, using GC-ECD to measure N_2O gas that had diffused into a helium headspace from aqueous samples taken from their DEA reactions, found that net N_2O production was remarkably lower than N_2 production and could be ignored for estimating DEA or DC.

To allow the direct measurement of N_2O by MIMS, modifications to the instrument and procedure were necessary. Both carbon dioxide (CO_2) and N_2O possess the same atomic mass and, therefore, N_2O cannot be assayed directly at their mass/charge ratio (m/z) of 44. Previously, N_2O was measured in the presence of CO_2 by determining the cracking patterns produced by the mass spectrometer and assaying m/z of 28, 30, and 32 from solutions of defined dissolved gas concentrations [23]. A similar approach was used by Firth and Edwards [24] who distinguished between CO_2 and N_2O in denitrifying cultures of Pseudomonas stutzeri by studying minor ion peaks created from these gasses. However, in the current study, a simpler and more direct measure of N_2O was achieved by removing the CO_2 from the sample. This was accomplished by adding NaOH to the sample to convert the dissolved CO_2 to bicarbonate. The instrument was also equipped with a cold trap maintained at -86°C. This temperature is below the freezing point of CO_2 (-78°C) yet above the freezing point of N_2O (-91°C). If a small ultralow freezer is unavailable for use as a cold trap, a mixture of liquid N_2 and ethyl methyl ketone will attain the same temperature [25].

Validation of MIMS-based protocol for measuring DEA and DC rates in soils

In the following three sections we explain how the MIMS-based protocol for measuring DEA and DC rates was validated namely by: i) observing a linear accumulation of the N_2O or N_2 product; ii) measuring higher DEA than DC rates in soils; iii) assaying two control bacterial cultures that differed in their denitrification end products; and iv) obtaining comparable DEA rates in a wetland soil measured under acetylene inhibition using our MIMS-based and the headspace GC-ECD methods.

Denitrification enzyme activity and DC rates determined in soils using MIMS to measure the N_2 product

The name of each study site, its geographic coordinates and % organic matter in its soils are presented in Table 1; also shown are MIMS-determined DC and DEA rates. A linear accumulation of the N_2O or N_2 product was observed. An R^2 value ≥0.85 for all linear rates was achieved. Standard deviations of triplicate DEA determinations for all wetland soils were <20% of the mean. The Shoreline Park soil showed high variability in its DC. Perhaps non-limiting nutrients conditions in DEA reactions buffered the effects of elevated concentrations of NO_3^- and/or carbon present in very small activity centers in Shoreline Park soil [2]. Within each site DEA activities were significantly higher than DC activities. Results of a two-sample t-test performed between means of DC and DEA activities for Shoreline Park soils provided evidence to reject the null hypothesis ($p=0.002$, t-value=6.85, df=4). Likewise results of a two-sample t-test performed between means of DC and DEA activities for the nature reserve soils also provided evidence to reject the null hypothesis ($p=0.0005$, t-value=44, df=2). These findings not only contributed to validating the method but also suggested nutrient limitation(s) (Table 1). A nitrogen limitation was probably affecting DC activities in all soils assayed as it is frequently limited in wetlands [26]. It is unlikely that carbon was limited in histosols (20% or greater organic matter by weight) of the Shoreline Park and Lake Shore Trail sites. The strongest indication of nitrogen limitation was in Lake Shore Trail where the DEA activity was very high yet the DC activity was below detection (Table 1).

Use of bacterial cultures for validating the MIMS-based DEA method

Pure cultures of bacteria provided control and validated our MIMS-based methods (Table 2, Figure 2). The dynamics of a typical *P. aeruginosa* DEA reaction in the presence and absence of C_2H_2 was illustrated by plotting changes in N_2 /Ar, N_2O/Ar and O_2/Ar over

time (Figure 2A,B). In both reactions the accumulation of either denitrification end product was accompanied by a concomitant decrease in the O_2/Ar ratios over the 4 h reaction period. This decrease in oxygen, the preferred electron acceptor, was most rapid during the first hour of the reaction (Figure 2A, B). Under these highly reduced conditions in the absence of C_2H_2, a complete reduction of NO_2 to N_2 and no increase in the N_2O/Ar ratios was observed (Figure 2A, Table 2). In this reaction a linear increase in the N_2 /Ar ratios (R2 = 0.98) was observed.

In the presence of C_2H_2, which blocks nitrous oxide reductase [27], *P. aeruginosa* produced only N_2O as the sole end product (Figure 2B). Thus, DEA was measured as an increase in N_2O over time (Table 2). However, in the absence of C_2H_2 in the *P. aeruginosa* culture DEA was measured as an increase in N_2 over time. To use a two sample t-test for determining whether the mean DEAs were significantly different in the presence or absence of C_2H_2, these rates needed to be expressed in the same units. Therefore, DEA, measured in the absence of C_2H_2, was also expressed in terms of N_2O produced (Table 2). This was accomplished by multiplying the measured N_2 rate by 1.57 (44 MW of N_2O/28 MW of N_2). Results of an unequal variance t-test (p=0.38, t-value=0.96, df=6) provided evidence to accept the null hypothesis of equal means.

Pseudomonas chloroaphis (ATCC 9446), lacking nitrous oxide reductase, only produced N_2O as the gaseous end product of denitrification (Table 2). Therefore, a DEA rate could only be directly measured as the accumulation of N_2O over time. These reactions were run in the presence and absence of C_2H_2 and mean DEA rates were determined. Results of an equal variance t-test (p=0.51, t-value=0.72, df=4) provided evidence to accept the null hypothesis of equal means. This indicated that C_2H_2 did not affect the rate of denitrification. Significantly higher mean DEA rates were measured in *P. aeruginosa* than in *P. chloroaphis* cultures (Table 2).

Comparison of DEAs in soil determined under acetylene inhibition using the MIMS-based method versus the headspace with GC-ECD method to measure the N_2O product

DEA rates were measured in soil from the Shoreline Park site by the, widely used, acetylene inhibition (headspace) method that employs GC-EDC to measure the N_2O product and the MIMS-based method developed in the present study. Acetylene was used with the MIMS-based method to allow direct comparisons with a common end product, N_2O. Mean DEA rates of 853 ± 302 µg N_2O h^{-1} Kg dry wt $soil^{-1}$ and 896 ± 431 µg N_2O h^{-1} Kg dry wt $soil^{-1}$ were measured with the MIMS-based and headspace with GC-ECD based methods, respectively. DEAs were expressed as the mean of triplicate determinations. Within methods, the headspace with GC-ECD method showed the highest variation; the standard deviation was 48% of the mean rate; whereas with the MIMS-based method the standard deviation was only 35% of the mean rate. Perhaps different reaction vessel conditions, one completely filled with soil slurry and the other possessing a headspace, was partially responsible for the differences in variability. A significant difference was not observed between means of the rates measured by either method.

Bacterial culture	Acetylene	Rate	
		µg N_2 h^{-1} mg dry wt $cells^{-1}$	µg N2O h^{-1} mg dry wt $cells^{-1}$
Pseudomonas aeruginosa	No	22.6 ± 6.8[a,b]	35.5 ± 10.7 [a,b,c]
Pseudomonas aeruginosa	Yes	UD[d]	25.8 ± 3.3[a,b]
Pseudomonas chloroaphis	No	UD[d]	2.9 ± 1.0[b,e]
Pseudomonas chloroaphis	Yes	UD[d]	2.3 ± 1.1[b,e]

[a]mean of five determinations; [b]Based on the results of t-tests (p > 0.05), we failed to reject the null hypothesis that the means of the rates obtained the presence and absence of acetylene with either *P. aeruginosa* (single underline) and *P. chloroaphis* (double underline) were the same; [c]Value was not measured directly as N_2O was not produced. The rate was obtained by converting the rate, measured by the production of N_2, to N_2O h^{-1} mg dry wt $cells^{-1}$; [d]UD, unable to determine as N_2 was not produced. [e]mean of triplicate determination

Table 2: Denitrification enzyme activity rate (± Std Dev) of bacterial cultures determined by measuring accumulation of the nitrogen or nitrous oxide end product by membrane inlet mass spectrometry.

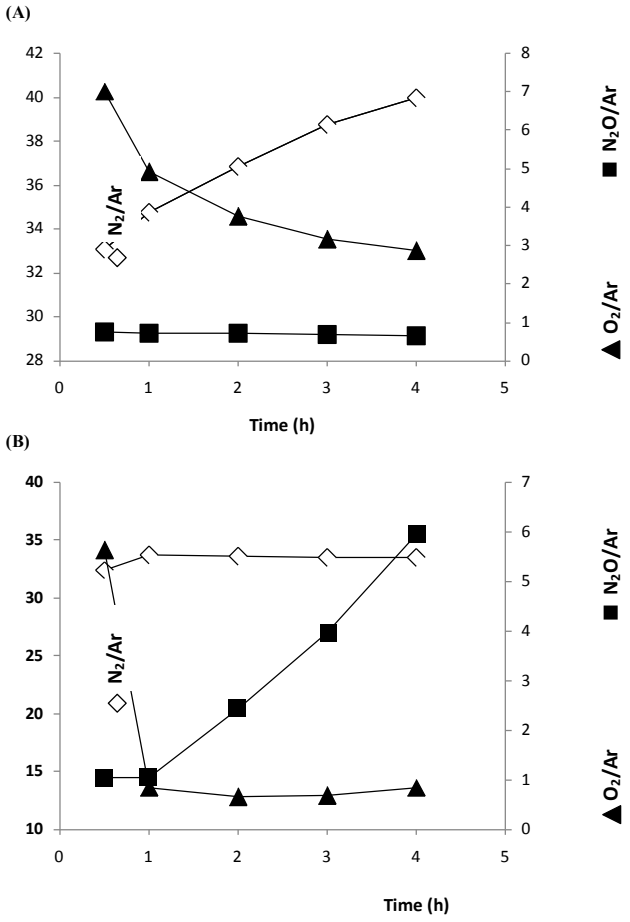


Figure 2: Membrane inlet mass spectrometry measured N_2/Ar; ◇ N_2O/Ar and ■ ▲O_2 /Ar ratios in *Pseudomonas aeruginosa* DEA reactions with under normal (A) and acetylene inhibited (B) conditions.

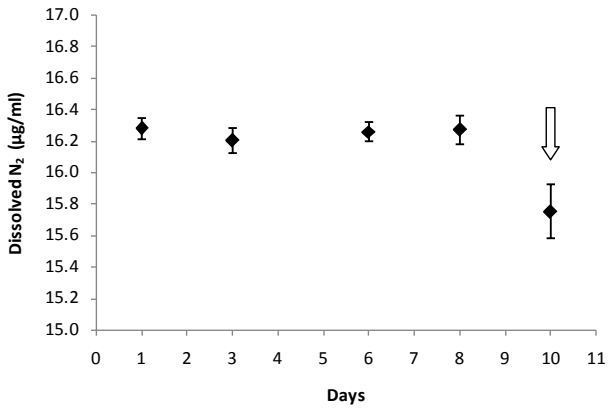
Sensitivity of measuring N_2O by MIMS

An analysis of the sensitivity of N_2O measurement by MIMS was warranted as it may be of interest when applying this measurement to low flux systems. As the concentration of N_2O in air saturated water is extremely low and reliable standards are unavailable, instrument noise was used to gauge sensitivity with the premise that N_2O/Ar ratios higher than instrument noise could provide a sensitivity assessment. Instrument noise was calculated as the mean (± Std Dev) of 15 N_2O/Ar ratios measured in alkalized dH_2O (200 C, pH 14) after instrument stabilization at approximately 4 minute intervals over a one hour time period. Three standard deviations above the mean N_2O/Ar was selected as the level of sensitivity as there is only a 0.3 % probability that

normally distributed ratios lie above this value [28]. A mean N_2O/Ar ratio (± Std Dev) of 0.097 ± 0.011 was measured as instrument noise. An instrument calibration factor of 1.97 for the N_2O/Ar ratio was obtained as described in the Materials and Methods section. Multiplying by this factor yielded a corrected mean N_2O/Ar ratio (± Std Dev) of 0.192 ± 0.022. At three standard deviations above this mean, a ratio of 0.258 or greater would have at least a 99.7% probability of representing a true increase in N_2O above instrument noise. Multiplying this ratio by the concentration of Ar in air saturated dH_2O at 200 C (13.91 µM, 16) suggested that a minimum N_2O concentration of 3.59 µM could be measured with the aforementioned degree of confidence. Furthermore, three times the standard deviation of the corrected N_2O/Ar ratio is 0.066. This value again multiplied by the concentration of Ar in air saturated dH_2O at 200 C (13.91 µM, 16) suggested that a 0.92 µM increase in the dissolved N_2O concentration of above 3.59 µM could be measured with the same degree of confidence.

Stability of dissolved N_2 in samples stored for MIMS analysis

Work at laboratories not possessing a membrane inlet mass spectrometer may require sample transport and/or storage before analysis. To explore the time constrains of performing DEA reactions at a laboratory lacking a membrane inlet mass spectrometer and transporting the samples to a different facility for MIMS analysis, the loss of dissolved N_2 in stored samples was measured over time. Samples held for MIMS analysis were examined for loss of dissolved N_2 after 1, 3, 6, 8 and 10 days of storage (Figure 3). Replicate samples, supersaturated with N_2, were removed from a denitrification reaction and stored underwater in ground glass-stopper test tubes as described previously. At each time period nine samples were analyzed for loss of N_2 by leakage from the storage tubes. No significant loss of N_2 was measured in the sample storage tubes after eight days. On day 10 the mean N_2 concentration in the storage tubes decreased. A single factor ANOVA showed that this average N_2 concentration was significantly lower than the average N_2 concentration in the storage tubes held for all shorter time periods. Results demonstrated that samples could be stored under water in the ground glass stopper collection tubes for a sufficient length of time before analysis to allow flexibility for MIMS-based denitrification studies to be conducted at remote locations (Figure 3).



♦, mean of nine replicate determinations; error bars, standard deviation.

⇩, mean N_2 concentration was significantly lower than all previous measured concentrations.

Figure 3: Stability of dissolved nitrogen in aqueous samples stored in completely filled, 7 ml ground glass stoppered test tubes submerged in water at 25°C.

Conclusions

The membrane inlet mass spectrometer was reconfigured and samples alkalized to allow the direct measurement of N_2O. Using MIMS DEA and DC were measured short term assays as rates. These protocols were validated by i) observing a linear increase in end products; ii) observing close agreement between replicates; iii) measuring higher DEA than DC capacity in the same soil; iv) obtaining comparable DEA rates in a soil measured under acetylene inhibition using our MIMS-based and the headspace with GC-ECD method; v) using pure cultures of bacteria that differed in their denitrification end products. Furthermore, an approach to assess the sensitivity of measuring N_2O by our MIMS-based method in the absence of reliable standards suggested that a minimum N_2O concentration of 3.59 µM could be measured with a 99.7% probability of confidence. Finally, to determine the time constrain imposed by transporting samples to a different facility for MIMS analysis revealed that samples could be stored submerged in water for 8 days without a significant loss of N_2.

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References

1. Jordan TE, Andrews MP, Szuch RP, Whigham DF, Weller DE, et al. (2007) Comparing functional assessments of wetlands to measurement of soil characteristics and nitrogen processing. Wetlands 27: 479-497.
2. Groffman PM, Holland E, Myrold DD, Robertson GP, Zou X (1999) Denitrification, in: Robertson GP, Bledsoe CS, Coleman DC, Sollins P (Eds.) Standard Soil Methods for Long Term Ecological Research. Oxford University Press, New York 272-288.
3. Tiedje JM, Simkins S, Groffman PM (1989) Perspectives on measurement of denitrification in the field including recommended protocols for acetylene based methods. Plant and Soil 115: 261–284.
4. Groffman PM, Altabet MA, Bohlke JK, Butterbach-Bahl K, David MB, et al. (2006) Methods for measuring denitrification: Diverse approaches to a difficult problem. Ecological Applications 16: 2091-2122.
5. Kana TM, Darkangelo C, Hunt MD, Oldham JB, Bennett GE, et al. (1994) A membrane inlet mass spectrometer for rapid high precision determination of N_2, O_2, and Ar in environmental water samples. Anal Chem 66: 4166–4170.
6. Bernot MJ, Dodds WK, Gardner WS, McCarthy MJ, Sobolev, D, et al. (2003) Comparing denitrification estimates for a Texas Estuary by using acetylene inhibition and membrane inlet mass spectrometry. Appl Environ Microbiol 69: 5950-5956.
7. Khalil MI, Richards KG (2011) Denitrification enzyme activity and potential of subsoils under grazed grasslands assayed by membrane inlet mass spectrometer. Soil Biol Biochem 43: 1787-1797.
8. U.S. Department of State (2007) Fourth Climate Action Report to the UN Framework Convention on Climate Change: Projected Greenhouse Gas Emissions. U.S. Department of State, Washington, D.C.
9. Poth M, Focht DD (1985) 15N kinetic analysis of N_2O production by Nitrosomonas europaea: an examination of nitrifier denitrification. Appl Environ Microbiol 49: 1134-1141.
10. Reddy KR, DeLaune RD (2008) Biogeochemistry of Wetlands: science and applications. Taylor & Francis CRC Press.
11. Master Y, Shavit U, Shaviv A (2005) Modified isotope pairing technique to study

N transformations in polluted aquatic systems: theory. Environ Sci Technol 39: 1749-1756.

12. U.S. Fish and Wildlife Service (2012) National Wetlands Inventory website. U.S. Department of Interior, Fish and Wildlife Service, Washington, D.C.
13. ASTM (2000) Standard test methods for moisture, ash, and organic matter of peat and other organic soils. Method D 2974-00. American Society for Testing and Materials. West Conshohocken, PA.
14. Drysdale G, Kasan H, Bux F (1999) Denitrification by heterotrophic bacteria during activated sludge treatment. Water SA. 25: 357-362.
15. Greenberg EP, Becker GE (1977) Nitrous oxide as end product of denitrification by strains of fluorescent pseudomonads. Can J Microbiol 23: 903-907.
16. Colt J (2012) Dissolved Gas Concentration in Water. (2nd edn), Elsevier, Amsterdam, Netherlands.
17. Budavari S (Ed.) (1996). The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals (12th ed.). NJ: Merck.
18. Pidwirny M (2007) "Atmospheric composition". Encyclopedia of Earth. Cleveland CJ (ed) Environmental Information Coalition (EIC) of the National Council for Science and the Environment (NCSE), Washington D.C., USA.
19. White JR, Reddy KR (1999) Influence of nitrate and phosphorous loading on denitrifying enzyme activity in Everglades wetland soils. Soil Sci Soc Am J 63: 1945–1954.
20. Kieskamp WM, Lohse L, Epping E, Helder W (1991) Seasonal variation in denitrification rates and nitrous oxide fluxes in intertidal sediments of the western Wadden Sea. Marine Ecology Series 72: 145-151.
21. García-Ruiz R, Pattinson SN, Whitton BA (1998) Denitrification and nitrous oxide production in sediments of the Wiske, a lowland eutrophic river. Sci Total Environ 210: 307-320.
22. Czepiel PM, Crill PM, Harris RC (1995) Nitrous oxide emissions from municipal wastewater treatment. Environ Sci and Technol 29: 2352–2356.
23. Cartaxana P, Lloyd D (1999) N_2, N_2O and O_2 profiles in a Tagus estuary salt marsh. Estuarine Coastal Shelf Sci 48: 751-756.
24. Firth JR, Edwards C (1999) Effects of cultural conditions on denitrification by Pseudomonas stutzeri measured by membrane inlet mass spectrometry. J Appl Microbiol 87: 353-358.
25. Rondeau RE (1966) Slush baths. J Chem Eng Data 11: 124.
26. Mitsch WJ, Gosselink JG (2000) Wetlands (3rd edn) John Wiley and Sons, New York.
27. Yoshinari T, Knowles R (1976). Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem Biophys Res Commun 69: 705-710.
28. Hoel PG (1966) Elementary Statistics. John Wiley and Sons, Inc. New York 96-99.

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