



Perturbation-free measurement of *in situ* di-nitrogen emissions from denitrification in nitrate-rich aquatic ecosystems



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ARTICLE INFO

Article history:

Received 28 June 2016

Received in revised form

20 October 2016

Accepted 10 November 2016

Available online 12 November 2016

Keywords:

Denitrification
Isotopic fractionation
Reactive nitrogen
Nitrogen loss
N₂O reduction

ABSTRACT

Increased production of reactive nitrogen (Nr) from atmospheric di-nitrogen (N₂) has greatly contributed to increased food production. However, enriching the biosphere with Nr has also caused a series of negative effects on global ecosystems, especially aquatic ecosystems. The main pathway converting Nr back into the atmospheric N₂ pool is the last step in the denitrification process. Despite several attempts, there is still a need for perturbation-free methods for measuring *in situ* N₂ fluxes from denitrification in aquatic ecosystems at the field scale. Such a method is needed to comprehensively quantify the N₂ fluxes from aquatic ecosystems. Here we observed linear relationships between the δ¹⁵N-N₂O signatures and the logarithmically transformed N₂O/(N₂+N₂O) emission ratios. Through independent measurements, we verified that the perturbation-free N₂ flux from denitrification in nitrate-rich aquatic ecosystems can be inferred from these linear relationships. Our method allowed the determination of field-scale *in situ* N₂ fluxes from nitrate-rich aquatic ecosystems both with and without overlaying water. The perturbation-free *in situ* N₂ fluxes observed by the new method were almost one order of magnitude higher than those by the sediment core method. The ability of aquatic ecosystems to remove Nr may previously have been severely underestimated.

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1. Introduction

Globally, the creation of reactive nitrogen (Nr) via anthropogenic activities has accelerated from 15.3 Tg N yr⁻¹ in 1910 to 187 Tg N yr⁻¹ in 2005, and further to 210 Tg N yr⁻¹ in 2010 (Galloway et al., 2008; Cui et al., 2013; Fowler et al., 2013). Excess Nr threatens the quality of air, soil and water (Sutton et al., 2011). Some Nr species, especially nitrate, will be converted back into inert dinitrogen (N₂) through denitrification (Seitzinger, 2008). However, there is a large uncertainty associated with estimating the amount of Nr denitrified back into inert N₂ since robust methods are still not readily available for perturbation-free *in situ* N₂ fluxes

determinations. This is especially true for nitrate-rich aquatic ecosystems (Seitzinger, 2008). The available methods, e.g., the ¹⁵N enrichment method, acetylene inhibition technique and the sediment core method, perturb the ecosystems under study and consequently cause large uncertainties in N₂ fluxes (Devol, 1991; Piña-Ochoa and Álvarez-Cobelas, 2006; Schlesinger, 2009; Duncan et al., 2013; Li et al., 2013). An integrated, whole-ecosystem approach based on membrane inlet mass spectrometry has been previously developed to quantify *in situ* N₂ emission from rivers (Laursen and Seitzinger, 2002). This is a powerful method for perturbation-free N₂ flux determination but applicable only with overlaying water because the N₂ fluxes are calculated using the measurements of dissolved N₂ concentrations in the overlaying waters (Laursen and Seitzinger, 2005). Wetland sediments are periodically exposed to atmosphere due to the variation in water

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levels, during which significant N₂ emissions into atmosphere may occur (Kern et al., 1996). Additionally, the aquatic-terrestrial transition zones (riparian buffer zones) have been reported to remove large amounts of Nr from wetland ecosystems (Hefting et al., 2003). There remains a need to develop new methods to determine *in situ* N₂ fluxes from sediments both with and without overlaying water (Davidson and Seitzinger, 2006; Groffman et al., 2006). Development of such methods will help to comprehensively evaluate the N₂ fluxes from wetland ecosystems.

Both nitrous oxide (N₂O) and N₂ are simultaneously emitted from nitrate-rich sediments following microbially-mediated denitrification. Denitrifiers preferentially reduce N₂O molecules that contain the lighter ¹⁴N stable isotope, which leads to increasing ¹⁵N enrichment of the remaining N₂O (Ostrom et al., 2007; Vieten et al., 2007), a phenomenon known as isotopic fractionation (Ostrom et al., 2007). Theoretically, the larger the reduction of N₂O by microorganisms, the higher the δ¹⁵N-N₂O signature of N₂O emitted into the atmosphere. In this study, we firstly verified two assumptions, i.e., i) the dynamics in the δ¹⁵N-N₂O signature of the emitted N₂O from nitrate-rich sediments are predominantly controlled by reduction; and ii) the δ¹⁵N-N₂O signature of N₂O emitted into the atmosphere is related to the N₂O/(N₂+N₂O) emission ratios. After verifying these assumptions, we hypothesized that the perturbation-free N₂ fluxes from a nitrate-rich river sediment could be inferred from the relationships between the δ¹⁵N-N₂O signatures and the N₂O/(N₂+N₂O) emission ratios. We assumed equilibrium between the gas and solute phases for both N₂O and N₂.

2. Materials and methods

2.1. Sediment sampling

Intact sediment cores were sampled from the Hutuo River in Hebei Province, China, using a hollow PVC cylinder (220 mm in inner diameter, 160 mm in length). In order to comprehensively test the proposed assumptions and hypothesis, we collected four sediments (no. 1, 2, 3 and 4) with different clay contents along the Hutuo River. The locations of the sampling sites are shown in Fig. 1. The sampling times for each location are shown in Table 1. For each location, three intact sediment cores were collected for each sampling time. At the same time, four additional small sediment cores (3.2 cm in diameter, 140 mm in depth) were collected to test the proposed assumptions. The collected soil cores were transported to the laboratory within 2 h and stored at 4 °C. All of the analyses were finished within one week.

2.2. Verification of assumption i)

Assumption i) was verified by the acetylene (C₂H₂) addition experiment. The procedure was as follows: equivalents of 10 g (oven dry weight) of either sediment no. 1 or 2 were added into 24 serum flasks (120 ml). Then 20 ml of 1 mM glucose solution and a magnetic stirring bar were added into each flask. The flasks were sealed with an air-tight butyl rubber septa and aluminum cap, and brought to vacuum and filled with an artificial N₂-free atmosphere (99.999% helium) five times until the N₂ concentration was below 30 ppmv. A preliminary experiment indicated that repeatedly exposing the soil to a vacuum (0.1 k Pa) had no statistically significant effects on soil respiration, suggesting that the microbial activity was not negatively affected (Qin et al., 2012). For each flask, 10 ml of C₂H₂ was injected into the flask with an air-tight syringe after removing an equal amount of headspace gas from the flask. The final C₂H₂ concentration in the headspace of the flask was 10% (v/v). Finally, the flasks were incubated in a thermostatically

controlled water bath at 125 rev min⁻¹ and 25 °C. Non-C₂H₂ control samples were identically treated except for the C₂H₂ addition. Four flasks were used to monitor N₂O and N₂ emissions. The other 20 flasks were used to monitor the δ¹⁵N-N₂O signatures. The dynamics of N₂O and N₂ concentrations in the headspace were monitored using a robotized sampling and analyzing system, similar to that described by Molstad et al. (2007). This robotized system had the advantage of minimizing N₂ leaks during sampling. Briefly, the system consisted of a gas chromatograph (GC, Agilent 7890) coupled to a peristaltic pump, an auto-sampler and a thermostatically controlled water bath. The δ¹⁵N-N₂O signatures in the headspace were determined by an isotope ratio mass spectrometer. Before δ¹⁵N-N₂O signature measuring, the gas samples were washed with 0.1% potassium permanganate solution to remove C₂H₂ to avoid the C₂H₂ interference with the mass spectrometer. Preliminary experiments showed that washing the gas samples by 0.1% potassium permanganate solution slightly decreased the N₂O concentration but did not significantly affected the δ¹⁵N-N₂O signatures (data not shown).

2.3. Verification of assumption ii)

Assumption ii) was verified by the glucose/acetate addition experiments. We used these two carbon sources to test whether different carbon sources yielded similar functions between the logarithmically transformed N₂O/(N₂+N₂O) emission ratios and the δ¹⁵N-N₂O signatures.

2.3.1. Glucose addition experiment

Glucose was added into the sediment to obtain a range of N₂O/(N₂+N₂O) emission ratios (Raut et al., 2012). Again the equivalents of 10 g sediment (oven dry weight) were added into 120 ml serum flasks. Then 20 ml of solution (1 mM glucose) and a magnetic stirring bar were added into each flask. The flasks were sealed with air-tight butyl rubber septa and aluminum caps, and brought to vacuum (0.1 k Pa) and filled with an artificial N₂-free gas (79% He and 21% O₂) five times. The flasks were incubated in a thermostatically controlled water bath at 25 °C. Before incubation, the slurries were homogenized by magnetic stirring at 125 rev min⁻¹ for 30 s. The N₂O and N₂ concentrations and δ¹⁵N-N₂O values in the headspace were measured as noted in the C₂H₂ addition experiment. The functions between the logarithmically transformed N₂O/(N₂+N₂O) emission ratios and the δ¹⁵N-N₂O signatures were fitted using the Sigma-Plot software, version 12.5 (Systat Software, San Jose, CA, USA).

2.3.2. Sodium acetate addition experiment

A sodium acetate addition experiment was performed identical to the glucose addition experiment, except that 1 mM glucose was replaced by 1 mM sodium acetate.

2.4. Hypothesis testing

The hypothesis was tested by the sediment core method. In this method, the N₂ surrounding and within the sediment cores was substituted by an artificial N₂-free gas (79% He and 21% O₂) to determine the trace N₂ emissions. A simple cylinder-in-cylinder setup was developed to lower inward N₂ leakage from the atmosphere and consequently to directly determine N₂ emissions from the sediment cores. This setup consisted of an inner and an outer cylinder. The interlayer between the two cylinders acts as a low-N₂ buffer against N₂ leakage from atmosphere. The details of the construction and the operation of the cylinder-in-cylinder setup are described in the Supplemental Information. The procedure was as follows: fresh sediment cores were put into the inner cylinder

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