Denitrification rate determined by nitrate disappearance is higher than determined by nitrous oxide production with acetylene blockage

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A mixed beech and spruce forest soil was incubated under potential denitrification assay (PDA) condition with 10% acetylene (C2H2) in the headspace of soil slurry bottles. Nitrous oxide (N2O) concentration in the headspace, as well as nitrate, nitrite and ammonium concentrations in the soil slurries were monitored during the incubation. Results show that nitrate disappearance rate was higher than N2O production rate with C2H2 blockage during the incubation. Sum of nitrate, nitrite, and N2O with C2H2 blockage could not recover the original soil nitrate content, showing an N imbalance in such a closed incubation system. Changes in nitrite and ammonium concentration during the incubation could not account for the observed faster nitrate disappearance rate and the N imbalance. Non-determined nitric oxide (NO) and N2 production could be the major cause, and the associated mechanisms could vary for different treatments. Commonly applied PDA measurement likely underestimates the nitrate removal capacity of a system. Incubation time and organic matter/nitrate ratio are the most critical factors to consider using C2H2 inhibition technique to quantify denitrification. By comparing the treatments with and without an antibiotic, the results suggest that microbial N uptake probably played a minor role in N balance, and other denitrifying enzymes but nitrate reductase could be substantially synthesized during the incubation.

1. Introduction

Denitrification can be defined as the reduction of nitrogen (N) oxides – nitrate (NO3−) and nitrite (NO2−) to N gases – nitric oxide (NO), nitrous oxide (N2O), and dinitrogen (N2). Denitrification is the major mechanism that fixed nitrogen in the biosphere returns to the atmosphere, which plays an essential role in global N cycle. Natural N balance has been severely altered by anthropogenic N fixation activity to produce fertilizers mainly for intensifying agriculture. However, excessive N in different ecosystems is becoming a global environmental problem, such as reducing biodiversity (Nordin et al., 2005), acidifying soils and water bodies (Prietzel et al., 2006), changing decomposable N fractions in soils (Scheuner, 2006), degrading water and atmosphere quality (Dickinson and Cicerone, 1986; Sullivan et al., 2005).

On one hand, the capacity of water, soil and sediment to remove excessive N is an important characteristic to maintain system’s sustainability. On the other hand, the intermediate products of gaseous N during the N removal processes (i.e.
Denitrification rate can be determined by nitrate reduction (disappearance) rate (since nitrate is normally in trace amount), or by N gas production rate. Quantifying nitrate disappearance rate is a straightforward mass-balance approach that is normally used for wastewater treatment (Wood et al., 1999; Spieles and Mitsch, 2000; Lansing and Martin, 2006), or in a watershed study (Burns, 1998; Leeds-Harrison et al., 1999; Lane et al., 2003). This technique generally yields little information regarding nitrate removal mechanism, spatial and temporal distribution of the acting processes, and controlling factors. Quantifying N gas production rate, which can provide complementary information, is an indirect approach to determine denitrification rate. Unfortunately, it is very difficult to quantify the dominant end product (N_2) of denitrification because of its large abundance in atmosphere (78% by volume). The most commonly applied method to measure denitrification is C_2H_2 blockage technique (Balderston et al., 1976; Yoshinari and Knowles, 1976). In anaerobic conditions, 10% C_2H_2 in gas phase volume can effectively inhibit N_2O reduction to N_2, making N_2O a major end product of denitrification. Nitrous oxide analysis is relatively easy due to its trace atmospheric abundance and the availability of sensitive detector (commonly by electron capture detector for gas chromatograph).

A standard protocol to characterize soil denitrification process is by potential denitrification assay (PDA). To optimize PDA condition, soil–water slurry is established and oxygen (O_2) is flushed out of the system by pure N_2 or helium (He) gas to ensure an anaerobic incubation environment (Myrold and Tiedje, 1985). Nearly all the PDA conducted has been to determine N_2O production with C_2H_2 blockage of further N_2O reduction to N_2. Keeping N_2O accumulation during the incubation in linear pattern is important for this method being valid. To determine nitrate reduction or disappearance rate is rare in PDA, because of inconvenience of sample handling and replication, and nitrate analysis. In this study, both nitrate disappearance rate and N_2O production rate with C_2H_2 blockage were determined at a typical PDA condition. Special attention was given to (1) if the nitrate disappearance rate agreed with the N_2O production rate with C_2H_2 blockage, and (2) if the sum of N oxides (NO_3^- and NO_2^-) and N_2O produced with C_2H_2 blockage remained constant during the incubation. The result will help ecologists and bioengineers to interpret a system’s N removal function and associated environmental impact.

2. Materials and methods

2.1. Sample soil

Sampling site was located in a mixed beech and spruce forest in Sorø, Denmark (N55 26.42', E11 34.07'). Surface soil (0–20 cm) was sampled after removing surface leaf litter. The soil can be classified as Alfisols according to US soil taxonomy. The soil bulk density was 1.10 g cm^{-3} in field condition by measuring dry weight of soil in known volume. The fresh soil was air-dried at room temperature (22 °C), sieved (2 mm) and stored at 5 °C. Water content of the air-dried soil was 10.4% before the experiment (all data are reported in dry weight of soil hereafter). Total C and N content of the soil was 16.4, 0.01, 3.8 g C g^{-1} soil, respectively. Initial nitrate, nitrite and ammonium content of the soil was 16.4, 0.01, 3.8 g N g^{-1} soil, respectively. The soil pH was 5.9 in water (1:1) and 5.3 in 0.1N KCl solution (1:1).

2.2. Soil slurries and treatments

Four treatments were established in soil slurries, including control (CK), glucose (GLU) addition, chloramphenicol (CHL, an antibiotic) addition, and combined glucose and chloramphenicol (GLU + CHL) addition. Soil slurries with different treatments were prepared as followings: 10 g thoroughly mixed air-dry soil was weighed into a 120 ml bottle. Different volume of distilled water was added according to different treatments: 20 ml for the CK, 19 ml for the CHL treatment and GLU treatment, and 18 ml for the GLU + CHL treatment. Glucose, as an electron donor for denitrification, was provided by adding 1 ml solution making final concentration of 120 mg C ml^{-1} soil. Chloramphenicol was provided by adding 1 ml solution making final concentration of 120 μg g^{-1} soil to inhibit new enzyme synthesis during the incubation (Pell et al., 1996). It is assumed that there will be no N uptake by microorganisms in the two CHL added treatments. Chloramphenicol was intentionally provided in small quantity to minimize possible inhibition of existing enzymes (Brooks et al., 1992; Dendooven et al., 1994; Pell et al., 1996). Each bottle was sealed with a rubber stopper, evacuated for 2 min, and then refilled with pure N_2 at one atmosphere pressure to ensure an anaerobic incubation environment. Pure C_2H_2 was injected to replace 10 ml of the headspace volume of all bottles to inhibit N_2O reduction activity (Tiedje et al., 1989).

The incubation was conducted at 25 °C in a rotary shaker at 170rpm. Each treatment had 24 replicate bottles, preparing for 12 measurements with two replicate bottles each time in 3 days. At different intervals (more frequent at beginning of the incubation), two replicate bottles were used for quantifying concentrations of N_2O, NO_3^-, NO_2^- and NH_4^+. Gas samples were withdrawn by 4.5 ml using a syringe from the headspace of the bottles, and immediately transferred into a 3.0 ml vacuum vial (Venojects, Belgium) for later analysis of N_2O concentration by gas chromatograph. Then the bottles were uncovered and sacrificed by extracting with KCl (final concentration 0.1N) for 4 h. The extracted water samples were filtered (0.45 μm) and stored at −20 °C for later analysis of NO_3^-, NO_2^- and NH_4^+ concentrations.

2.3. Sample analysis and data calculation

Gas samples were analyzed in a Hewlett-Packard gas chromatograph (GC) 5890 with an electron capture detector (ECD) to determine N_2O concentration. The GC was equipped with a 1.5 m long separation column packed with Haysep (80–100 mesh). The oven, injector, and detector temperatures were 40, 120, and 325 °C, respectively. The amount of N_2O dissolved in
the water phase of the soil slurries was considered by taking Bunsen coefficient as 0.556 at 25 °C (Moraghan and Buresh, 1977). A flow injection nutrient analysis system (Aquatec, Sweden) was used for the analysis of NH₄⁺, NO₂⁻, and NO₃⁻ in the filtered water samples. Total soil carbon and nitrogen was analyzed by a CN analyzer. Soil pH in slurries was measured by a pH meter 28 (Radiometer Copenhagen).

Denitrification rate was calculated by liner regression of nitrate concentration decrease or by liner regression of N₂O concentration increase over a certain incubation period (i.e. first 10 h). Statistical analysis was conducted using SAS software, version 9.1 (SAS Institute Inc. Cary, NC, USA). Difference of the measurement between different treatments was tested using GLM procedure to determine its significance (P value <0.05). The significance level was chosen at α = 0.05.

3. Results and discussion

3.1. Kinetics of denitrification during the incubation

Denitrification activity and synthesis of new denitrifying enzymes can be completely inhibited by O₂. Trace amount of O₂ may still exist following the above soil slurry preparation protocol. Oxygen has higher priority than nitrate to obtain electrons from organic matter according to the thermodynamics of reduction–oxidation (redox) processes in soils (Ponnamperuma, 1972). At beginning of the incubation in this study, a lag phase of initiating denitrification lasted for a few hours, more obviously in the treatments without glucose addition (Fig. 1). Addition of glucose likely stimulated consumption of the remaining O₂ in the soil slurries, resulting less lag time for denitrification activity to fully function. New denitrifying enzyme synthesis may be induced during the lag phase if it is not inhibited. After the lag phase, where N₂O production rate was relatively low, accumulation of N₂O in headspace of the soil slurries showed zero-order kinetics in early phase (first 20 h) of the incubation, and later first-order kinetics when nitrate was disappearing. Without glucose addition, the results show less N₂O accumulation in the CHL treatment than control, indicating some effect of antibiotic addition on enzymes responsible for N₂O production. However, during the entire incubation period, there was no significant difference in N₂O accumulation pattern between treatments with and without GLU, regardless of glucose addition (P = 0.48 for the two treatments without glucose; P = 0.97 for the two treatments with glucose).

Dynamics of NO₃⁻, NO₂⁻ and NH₄⁺ concentration during the incubation is shown in Fig. 2. Nitrate concentration in the soil slurries showed a continuous decreasing pattern, and addition of glucose enhanced nitrate disappearance rate. The nitrate disappearance rate showed a zero-order kinetics until nitrate concentration reached approximately 6 µg N g⁻¹ soil in the soil slurries, thereafter a first-order kinetics took place. The nitrate disappearance in the soil slurries was largely responsible for the observed N₂O accumulation kinetics (Fig. 1). Negligible difference in nitrate decreasing pattern was found between the two treatments without GLU (P = 0.87), and between the two treatments with GLU addition (P = 1.00).

![Fig. 1 – Nitrous oxide accumulation with acetylene blockage during the incubation. Treatment: control (CK), glucose (GLU), chloramphenicol (CHL), and combined glucose and chloramphenicol (GLU + CHL). Points represent means of the two replicates. Vertical bars represent standard errors of the means and may not be seen when they are smaller than the symbols.](image)

The sample soil was almost nitrite free before the experiment. During the incubation, nitrite concentration in the soil slurries showed some temporal spikes mainly in the first half of the incubation (Fig. 2). Nitrite reduction rate was likely greater than nitrate reduction rate in the soil, because nitrite concentration remained in low level throughout the study. Addition of CHL and/or GLU elevated the average nitrite level in the soil slurries. Average nitrite concentration during the incubation was 0.11, 0.22, 0.27, and 0.56 µgN g⁻¹ soil for the treatment of CK, CHL, GLU, and GLU + CHL, respectively. It appeared that the antibiotic probably inhibited the synthesis of nitrite reduction enzyme during the course of incubation, since nitrate reduction was not altered by CHL addition (Fig. 2). However, statistically there was no significant difference in nitrite concentration between the two treatments without GLU (P = 0.31), and between the two treatments with GLU addition (P = 0.15).

The results show some effects of CHL addition on ammonium kinetics in the soil slurries, even though ammonium analysis covered only part of the incubation period. Without CHL, ammonium concentration in the soil slurries tended to decrease during the incubation, and such a decreasing trend was more obvious when glucose was added (Fig. 2). In CHL added treatments, however, ammonium concentration showed an increasing trend in the soil slurries. There...
Fig. 2 – Changes of nitrate, nitrite, and ammonium concentrations during the incubation. Treatment: control (CK), glucose (GLU), chloramphenicol (CHL), and combined glucose and chloramphenicol (GLU + CHL). Points represent means of the replicates (n = 2). Vertical bars represent standard errors of the means and may not be seen when they are smaller than the symbols.

was a significant difference (P < 0.01) in ammonium concentration between the treatment of CK (3.80 μg g⁻¹ soil) and CHL (5.33 μg g⁻¹ soil), and between the treatment of GLU (2.80 μg g⁻¹ soil) and GLU + CHL (4.63 μg g⁻¹ soil).

3.2. Nitrate disappearance rate is higher than N₂O production rate with C₂H₂ blockage

By comparing Figs. 1 and 2, we found that nitrate disappearance rate was substantially higher than N₂O production rate with C₂H₂ inhibition in all treatments, and the temporal and minor accumulation of nitrite could not account for this difference. Further analysis of nitrate disappearance rate and N₂O production rate with C₂H₂ blockage in the same incubation period (i.e. first 10 h, first 35 h, etc.) was summarized in Fig. 3.

In PDA measurement using C₂H₂ inhibition technique, choosing incubation period and frequency of gas sampling is a practical question. Ideally, PDA measurement should yield the maximum denitrification rate by linear regression of several N₂O measurements. Linear increase of N₂O accumulation pattern during the incubation indicates that N₂O production is not limited by nitrate as a substrate for denitrification, and is normally considered for the validity of this method. In this study, the optimum time for determining soil denitrification potential was probably from 10 h to 35 h, and the calculated linear accumulation pattern (Fig. 1) was used for regression analysis. Too short a period of incubation, such as less than 10 h, was not suggested because of (1) slow N₂O production in the lag phase, and (2) some practical difficulties to manage several-round samplings, especially for a large number of samples. Prolonged period of incubation and measurement may underestimate denitrification potential, because N₂O production would be limited by nitrate concentration in the system. For the same reasons, the optimum time of incubation and gas measurement for PDA can be shorter if additional organic matter is provided (like glucose addition in this study), and can be longer if additional nitrate is provided.

During the initial lag phase with slow N₂O production, nitrate disappearance rate was actually the highest and most variable, especially in the two treatments with GLU addition (Figs. 2 and 3). The results suggest that nitrate was consumed immediately when the anaerobic incubation started, and a large portion of this nitrate consumption did not convert to N₂O during this period. Determining denitrification rate by nitrate disappearance rate in the early phase of the incubation (i.e. <10 h in this study) should be avoided. As suggested for determining denitrification potential by N₂O production with C₂H₂ blockage, using the first 20-h measurement was probably also adequate for determining denitrification potential by nitrate disappearance rate. At end of the first 20-h incubation, nitrate disappearance rate became relatively constant (in treatments without GLU), or less variable (in treatments with GLU). If all measurements during the three-day incubation were used for regression analysis, nitrate disappearance rates and N₂O production rates with C₂H₂ blockage were in
a narrow range of 0.18–0.26 μg N g⁻¹ soil h⁻¹ regardless of different treatments (Fig. 3). However, denitrification potential determined in a prolonged incubation would be substantially underestimated due to substrate (nitrate) limitation for denitrification activity.

Regardless of different treatments, nitrate disappearance rate was consistently higher than the corresponding N₂O production rate with C₂H₂ blockage for most period of the incubation. The difference between the nitrate disappearance rate and N₂O production rate with C₂H₂ blockage was greater in the first day and smaller during the rest period of the incubation.

3.3. Mass imbalance in denitrification potential assay using C₂H₂ blockage technique

The soil slurry incubation in this study was a closed system without mass transport, except for gas samplings during the incubation. However, the sum of nitrate, nitrite and N₂O with C₂H₂ blockage during the incubation could not recover the original amount of nitrate in the sample soil. Such N imbalance was most significant at approximately 20 h after the incubation started (Fig. 4). The “missing” N may account for up to 40% of the original nitrate-N in the soil (16.4 μg N g⁻¹ soil), and could be due to some, if not all, of the following reasons:

(1) The most likely cause for the N imbalance during the incubation was NO production in denitrification (analysis of NO was not available for this study). Nitric oxide has been known as an intermediate product of denitrification (Ryden, 1981; Ye et al., 1994). A significant portion of the soil nitrate probably converted to NO at early phase of the incubation, which might be largely responsible for the higher nitrate disappearance rate than the N₂O production rate with C₂H₂ blockage. Nitric oxide production and later reduction to N₂O could interpret well the temporal nature of the N imbalance during the incubation, as shown for the treatments without glucose addition (Fig. 4).

(2) Incomplete inhibition of N₂O reduction to N₂ is always a concern in denitrification study using C₂H₂ blockage technique. A recently completed study using ¹⁵N-nitrate tracer showed a significant enrichment of ¹⁵N-N₂ in an anaerobic soil/sediment (3 rice soils and 1 lake sediment) incubation with 10% C₂H₂ (Yu et al., submitted for publication). Possible reaction of C₂H₂ and NO in gas phase (Bollmann and Conrad, 1997; McKenney et al., 1997) could cause substantial decrease of C₂H₂ partial pressure during the course of incubation. Nevertheless, N₂ formation under certain circumstances challenges validity of any C₂H₂ based techniques in denitrification study, because the denitrification rate will be underestimated if N₂O reduction to N₂ is not completely inhibited. Nitrogen gas formation could partially interpret the results in the treatments with glucose addition, where the N imbalance lasted to the end of the incubation (Fig. 4).

(3) Dissimilatory nitrate reduction to ammonium has been reported as a mechanism of nitrate consumption under anaerobic condition (Kaspar et al., 1981). In this study, only small and temporal increase in ammonium concen-
Fig. 4 – Sum of nitrate, nitrite, and nitrous oxide with acetylene blockage during the incubation. Ammonium concentration is not included, due to limited number of measurements. Treatment: control (CK), glucose (GLU), chloramphenicol (CHL), and combined glucose and chloramphenicol (GLU + CHL).

(4) Anaerobic ammonium oxidation (anammox) by nitrite to form N2 (without N2O as an intermediate product) has recently been found a unique N pathway, which may play an important role in global N cycle. Occurrence of anammox reaction has been reported in both marine and freshwater environment (Jetten et al., 2003; Penton et al., 2006). Consumption of ammonium was clearly observed in the glucose treatment without antibiotic (Fig. 2), but it remained unknown if this ammonium consumption was directly related with anammox reaction. Regardless of the possible mechanisms, N2 formation likely occurred during the incubation especially in the two treatments with glucose addition, since the N imbalance sustained to the end of incubation (Fig. 4).

(5) In the treatment without CHL, N uptake by microorganisms probably contributed to the observed N imbalance. However, microbial N uptake seems play a minor role by comparing the results of treatments with and without CHL.

4. Conclusions

This study clearly show that nitrate disappearance rate was higher than N2O production rate with C2H2 blockage in a typical PDA measurement, and the results were consistent for the treatments with and without antibiotic to inhibit new enzyme synthesis and microbial N uptake during the incubation. The information is important for interpreting nitrate removal capacity and denitrification potential in different systems that are commonly conducted by ecologists and bioengineers. Commonly applied PDA measurement to quantifying N2O production with C2H2 blockage likely underestimates the nitrate removal capacity of a system. Slight changes in nitrite and ammonium concentration during the incubation could not account for this difference. Non-determined NO and N2 production could be the major cause for the observed faster nitrate disappearance rate (than N2O production rate with C2H2 blockage), and the N imbalance during the incubation. Despite of increasing application of stable isotope in studying N cycle in soil (Ruppel et al., 2006), using C2H2 to inhibit the reduction of N2O to N2 is the simplest and most commonly used technique to quantify denitrification. Incubation time and organic matter/nitrate ratio are the most critical factors to consider in avoiding substantial underestimating denitrification potential for designing, monitoring, or constructing ecosystems. By comparing the treatments with and without antibiotic, it seemed that microbial N uptake played a minor role in N balance, and that nitrate reduction enzyme likely remained the same, but other denitrifying enzymes (reduction of nitrite, and probably NO and N2O) could be synthesized during the incubation.

Acknowledgments

The authors thank Karin Vestberg, Department of General Microbiology, University of Copenhagen, for her skillful technical assistance. This study was supported by a Danish DANIDA project in collaboration with China.

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