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

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ABSTRACT

A mixed beech and spruce forest soil was incubated under potential denitrification assay (PDA) condition with 10% acetylene (C_2H_2) in the headspace of soil slurry bottles. Nitrous oxide (N_2O) 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 N_2O production rate with C_2H_2 blockage during the incubation. Sum of nitrate, nitrite, and N_2O with C_2H_2 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 N_2 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 C_2H_2 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 (NO_3^-) and nitrite (NO_2^-) to N gases – nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2). 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.

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denitrification) are another concern. Nitrous oxide is one of the most important trace gases associated with global warming (Dickinson and Cicerone, 1986) and destruction of stratospheric ozone (Crutzen, 1981; Weiss, 1981). Other N gases (such as NO_2 and NO) are also closely involved in the atmospheric chemistry of ozone (Last et al., 1994).

Denitrification rate can be determined by nitrate reduction (disappearance) rate (since nitrite is normally in trace amount), or by N gas production rate. Quantifying nitrate disappearance rate is a straight forward 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 1.69% and 0.17%, respectively. Initial nitrate, nitrite and ammonium content of the soil was 16.4, 0.01, 3.8 μg 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 280 μg C g^{-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 170 rpm. 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 Hayesape (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 NH_4^+, NO_2^-, and NO_3^- 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 N_2O 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 $\alpha = 0.05$.

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 O_2. Trace amount of O_2 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 O_2 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 N_2O production rate was relatively low, accumulation of N_2O 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 N_2O accumulation in the CHL treatment than control, indicating some effect of antibiotic addition on enzymes responsible for N_2O production. However, during the entire incubation period, there was no significant difference in N_2O accumulation pattern between treatments with and without CHL, regardless of glucose addition ($P = 0.48$ for the two treatments without glucose; $P = 0.97$ for the two treatments with glucose).

Dynamics of NO_3^-, NO_2^- and NH_4^+ 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 $\mu g\,N\,g^{-1}$ 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 N_2O 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 ($P = 1.00$).

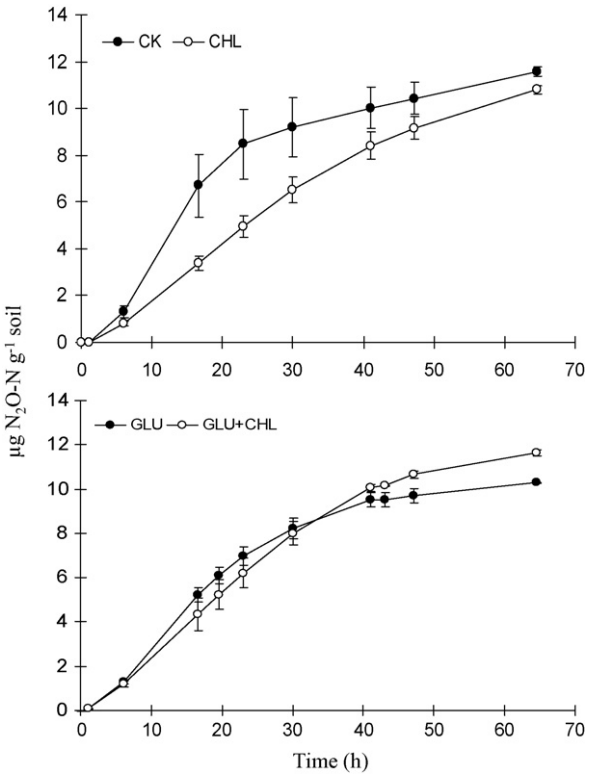

Fig. 1 – Nitrous oxide accumulation with acetylene blockage during the incubation. Treatment: control (CK), glucose (GLU), chloramphenicol (CHL), and combined glucose and chloramphenicol (GLU + CHL). Points represent means of the two replicates. Vertical bars represent standard errors of the means and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

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 $\mu g\,N\,g^{-1}$ 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

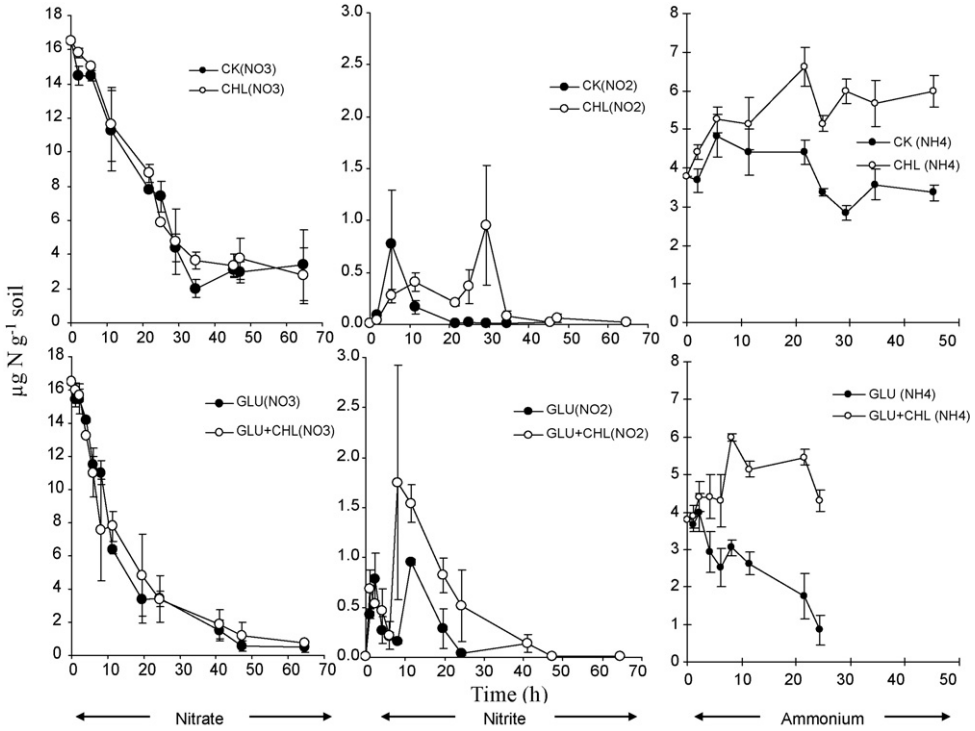

Fig. 2 – Changes of nitrate, nitrite, and ammonium concentrations during the incubation. Treatment: control (CK), glucose (GLU), chloramphenicol (CHL), and combined glucose and chloramphenicol (GLU + CHL). Points represent means of the replicates ($n = 2$). Vertical bars represent standard errors of 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 $\mu g\,N\,g^{-1}$ soil) and CHL (5.33 $\mu g\,N\,g^{-1}$ soil), and between the treatment of GLU (2.80 $\mu g\,N\,g^{-1}$ soil) and GLU + CHL (4.63 $\mu g\,N\,g^{-1}$ soil).

3.2. *Nitrate disappearance rate is higher than N_2O production rate with C_2H_2 blockage*

By comparing Figs. 1 and 2, we found that nitrate disappearance rate was substantially higher than N_2O production rate with C_2H_2 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 N_2O production rate with C_2H_2 blockage in the same incubation period (i.e. first 10 h, first 35 h, etc.) was summarized in Fig. 3.

In PDA measurement using C_2H_2 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 N_2O measurements. Linear increase of N_2O accumulation pattern during the incubation indicates that N_2O 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 first 20 h when N_2O concentration showed a linear accumulation pattern (Fig. 1), and the calculated N_2O production rate reached the maximum (Fig. 3). Within the 20 h, at least three measurements should be conducted for linear regression analysis. Too short period of incubation, such as less than 10 h, was not suggested because of (1) slow N_2O 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 N_2O 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 N_2O 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 N_2O 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 N_2O production with C_2H_2 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 N_2O production rates with C_2H_2 blockage were in

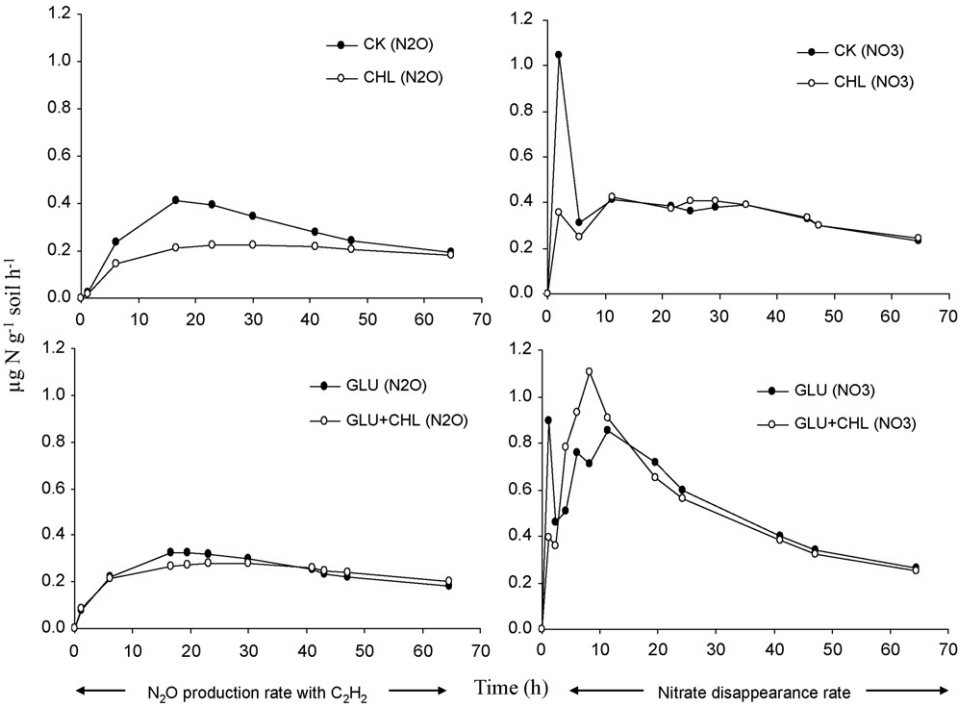

Fig. 3 – Comparison of denitrification rate determined by nitrous oxide production with acetylene blockage and determined by nitrate disappearance rate at different time. Denitrification rate is determined by linear regression of all measurements conducted from beginning of the incubation to a certain time (i.e. first 10 h, first 35 h, etc.). Treatment: control (CK), glucose (GLU), chloramphenicol (CHL), and combined glucose and chloramphenicol (GLU + CHL).

a narrow range of 0.18–0.26 $\mu g\,N\,g^{-1}$ soil h^{-1} regardless of different treatments (Fig. 3). However, denitrification potential determined in a prolonged incubation would be substantially underestimated duo to substrate (nitrate) limitation for denitrification activity.

Regardless of different treatments, nitrate disappearance rate was consistently higher than the corresponding N_2O production rate with C_2H_2 blockage for most period of the incubation. The difference between the nitrate disappearance rate and N_2O production rate with C_2H_2 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 C_2H_2 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 N_2O with C_2H_2 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 $\mu g\,N\,g^{-1}$ 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 N_2O production rate with C_2H_2 blockage. Nitric oxide production and later reduction to N_2O 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 N_2O reduction to N_2 is always a concern in denitrification study using C_2H_2 blockage technique. A recently completed study using ^{15}N-nitrate tracer showed a significant enrichment of ^{15}N-N_2 in an anaerobic soil/sediment (3 rice soils and 1 lake sediment) incubation with 10% C_2H_2 (Yu et al., submitted for publication). Possible reaction of C_2H_2 and NO in gas phase (Bollmann and Conrad, 1997; McKenney et al., 1997) could cause substantial decrease of C_2H_2 partial pressure during the course of incubation. Nevertheless, N_2 formation under certain circumstances challenges validity of any C_2H_2 based techniques in denitrification study, because the denitrification rate will be underestimated if N_2O reduction to N_2 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-

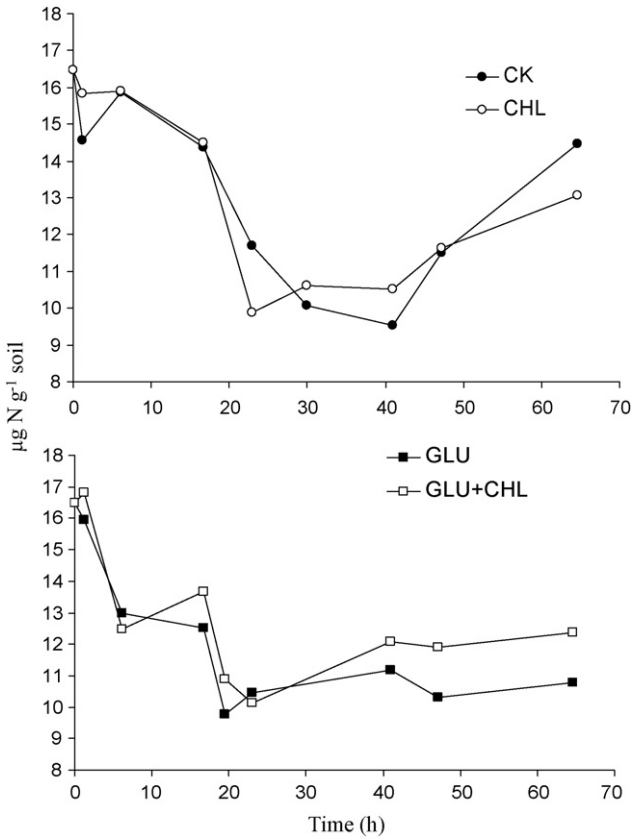

Fig. 4 – Sum of nitrate, nitrite, and nitrous oxide with acetylene blockage during the incubation. Ammonium concentration is not included, due to limited number of measurements. Treatment: control (CK), glucose (GLU), chloramphenicol (CHL), and combined glucose and chloramphenicol (GLU + CHL).

tration was observed in the treatments with antibiotic. In the treatments without antibiotic, ammonium concentration even showed a decreasing tendency, especially in glucose addition treatment where the N imbalance was the greatest (Figs. 2 and 4). Dissimilatory nitrate reduction to ammonium was unlikely a significant mechanism in this study responsible for the N imbalance and the higher nitrate disappearance rate than the N_2O production rate with C_2H_2 blockage.

(4) Anaerobic ammonium oxidation (anammox) by nitrite to form N_2 (without N_2O 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, N_2 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 N_2O production rate with C_2H_2 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 N_2O production with C_2H_2 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 N_2 production could be the major cause for the observed faster nitrate disappearance rate (than N_2O production rate with C_2H_2 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 C_2H_2 to inhibit the reduction of N_2O to N_2 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 N_2O) could be synthesized during the incubation.

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