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QUANTITATIVE ASSESSMENT OF SOIL NITRATE DISAPPEARANCE AND N_2O EVOLUTION DURING DENITRIFICATION

NITRATE DISAPPEARANCE DURING DENITRIFICATION

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Summary—Nitrate dynamics in the soil environment often confound investigations into the denitrification process and contribute to the extreme variability witnessed. NO_3^- dynamics and evolution of N_2O were investigated in a soil chemically-treated to control the amount and form of carbon, nitrogen and the microbial population. This was achieved by removing soil organic matter with hydrogen peroxide, leaving the soil inorganic skeleton. Treatments were: 1. chemically-treated soil (low in C and N) amended with 500 mg NO_3^--N kg^{-1} and 600 mg glucose-C kg^{-1} (**Control**); 2. chemically treated soil amended with 500 mg NO_3^--N kg^{-1} and 600 mg glucose-C kg^{-1} and a culture of *Pseudomonas denitrificans*, equivalent to 10^6 denitrifiers g^{-1} of soil (**Dentreat**) and 3. field soil amended with 500 mg NO_3^--N kg^{-1} and 600 mg glucose-C kg^{-1} and a culture of *P. denitrificans* (**Soil**). Flasks were purged of O_2, shaken and anaerobically incubated with 10% C_2H_2 (v/v) for 96 h. The N_2O and CO_2 production and NO_3^- and NO_2^- concentrations were monitored. Approximately 10, 150 and 146 mg NO_3^--N kg^{-1} disappeared from the soil solution in 2 h from the **Control, Dentreat** and **Soil** samples respectively. At the same time there was no concurrent increase in soil NO_2^- concentration or N_2O evolution, whilst CO_2 concentrations bore no stoichiometric relationship with N disappearance until the end of the incubation (96 h). The production of N_2O did not reflect the microorganisms' capacity to remove NO_3^- from the soil environment. An approximate 80% balance of NO_3^- disappearance and N_2O evolution was seen for the **Dentreat** and **Soil** samples at 96 h respectively. The ability to remove large quantities of NO_3^- quickly under non-limiting conditions and then to utilise this at a later date, may benefit some soil microorganisms. Such an excessive disappearance has implications for the use of *in-situ* NO_3^- concentrations as a predictor of soil denitrification potential in the field. A proportion of soil N would be temporarily immobilised within the microbial-biomass and could later be denitrified, leading to an underestimate of potential gaseous N loss. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Soil environmental and physical variables control denitrification activity by the indigenous microbial populations. Much work has been done to examine the effect of such variables as carbon, nitrate concentration, moisture content and temperature on denitrification (Bowman and Focht, 1974; Burford and Bremner, 1975; Myrold and Tiedje, 1985). Quantification of their role remains complicated by a high degree of spatial and temporal variability, and the number of interactions and feedbacks of the process itself, and between the process and the soil system. Nitrate has been identified as one of the proximal controls in the denitrification process (Bowman and Focht, 1974; Benckiser, 1994). Discernment of the role of NO_3^-, coupled with the availability of C, in supporting the maintenance and growth of denitrifiers in soil would provide another component to our understanding of the denitrification process.

Previous attempts to measure the dynamics of NO_3^- utilisation were impeded by C limitations in field soils, high NO_3^- concentrations in relation to available C and constraints on the rate of NO_3^- diffusion (Murray *et al.*, 1989). The dependence of denitrification on NO_3^- concentration is a contentious issue, with some workers considering the process to be independent, showing poor correlations between NO_3^- and nitrous oxide (N_2O) (Burford and Bremner, 1975; Burton and Beauchamp, 1985; Myrold and Tiedje, 1985). Others have found good correlations between N_2O and NO_3^- concentration (Bowman and Focht, 1974; Murray *et al.*, 1989, Weier *et al.*, 1991). Lalisse-Grundmann *et al.* (1988) stressed the importance of simultaneously following the production of N_2O and consumption of NO_3^- due to the ambiguous behaviour of NO_3^- in the soil environment.

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Data from permanent pasture (Dendooven and Anderson, 1995; Dendooven *et al.*, 1996a), and a to lesser extent from a forest soil (Dendooven *et al.*, 1996b), indicated that NO_3^- disappearance from soil was rapid following the onset of anaerobic conditions, and was unaffected by the amendment of C_2H_2 or chloramphenicol. Approximately 25 mg NO_3^--N kg^{-1} disappeared from the soil solution within 2 h from the pasture soil. This initial NO_3^- disappearance, in excess of the microorganisms' ability to utilise the substrate (as indicated by the respiration rate), could introduce a greater degree of variability to the already highly variable denitrification process. It could complicate the use of soil factors, such as NO_3^-, as predictors of denitrification potential and lead to an underestimate of potential field losses of N_2O, with a proportion of NO_3^- temporally immobilised within the soil microbial-biomass. Losses of N due to denitrification based on a point measurement of soil NO_3^-, and a model calculation, could be underestimated (I.P.C.C., 1990).

We report a series of experiments, using an approach similar to that reported by Elliott *et al.* (1983) to quantitatively assess the uptake of NO_3^- from a sterilised soil re-inoculated with a single denitrifying microorganism and supplied with an amount of C as glucose, sufficient to support denitrifying activity for 96 h. The soil environment was chemically treated to eliminate much of the indigenous C, N and microorganisms whilst maintaining soil textural components (sand, silt and clay), which are believed to be important factors regulating denitrifying activity (Beauchamp *et al.*, 1980; Weier *et al.*, 1991). Nitrate is highly reactive in the soil environment and this may account for part of the variation inherent in denitrification studies. By reducing *in-situ* NO_3^- concentrations in the chemically-treated soil, it was assumed that any additions made would be less variable and thus easier to quantify. The chemically-treated soil system allowed the effect of adding substrate in supporting the growth and activity of the added microorganisms to be isolated. Our objectives, therefore, were to study the dynamics of NO_3^-, NO_2^- and N_2O under anaerobic conditions, and assess the relationship between NO_3^- disappearance and the evolution of N_2O and CO_2.

MATERIALS AND METHODS

Soil preparation

The soil was a sandy loam from Woburn Experimental Farm, Bedfordshire, U.K. It contained 76% sand, 13% silt and 11% clay and soil organic-C and inorganic-N contents at the time of sampling were 16.08 g C kg^{-1} and 6.12 g N kg^{-1}, respectively.

Soil samples were collected on 25 November 1994 from the plough layer (top 23 cm) and sieved (5 mm) to remove roots and coarse stones. Samples were bulked, with half stored moist at 4°C until required, the other half being air-dried.

To quantitatively assess the uptake of NO_3^- under the same C addition, it was necessary to chemically treat the soil environment to decrease the amount of available substrate, whilst maintaining soil textural components (the proportion of sand, silt and clay). We removed indigenous microorganisms from the soil by a double sterilisation procedure. Water-soluble, organic-C and NO_3^- were removed by wet oxidation and leaching, and were added back in quantified amounts with a culture of *Pseudomonas denitrificans*, as follows:

One kg of sieved air-dried soil was placed in a bag (Sterilin, U.K.) laid flat in an autoclave tray and smoothed to a depth of approximately 1 cm. The soil was autoclaved at 123°C for 30 min and left in the sealed bag at room temperature for 48 h before being re-autoclaved. The double sterilisation ensured that all soil microorganisms were effectively killed as were any spore-forming bacteria regenerated over the 48 h period.

One kg of sterile soil was oxidised using 625 ml of 6% H_2O_2 (Day, 1965). Wet oxidation was allowed to proceed at room temperature for 1 h before a further 625 ml H_2O_2 was added. The solution was heated and constantly agitated until a marked decrease in effervescence denoted the complete oxidation of available organic matter. The treatment was designed to remove organic matter, with concentrations of C reduced to 60 mg C kg^{-1} (standard error (SE) 10). The soil was transferred to a sterile leaching column and 10 l of 10 mM $CaCl_2$ solution leached through, removing NO_3^--N and ammonium (NH_4^+-N). Soil NO_3^- and NH_4^+ concentrations were reduced to 0.33 (SE 0.05) and 2.74 (SE 0.30) mg N kg^{-1} respectively.

Further experimentation to assess the potential utilisation of remaining soil C and N under anaerobic conditions revealed that 4.46 mg CO_2-C kg^{-1} and 230 μg N_2O-N kg^{-1} was produced in the mineral soil skeleton after 96 h anaerobic incubation.

Harvest of P. denitrificans

A freeze-dried culture of *P. denitrificans* (NCIMB 1656) was purchased from the National Collection of Industrial and Marine Bacteria (Aberdeen, U.K.) in April 1993. The microorganisms were kept in continuous culture on nutrient agar plates and slopes for use in denitrification studies. Microorganisms were grown for 48 h in Tryptic Soy Broth (Oxoid, U.K.) at 28°C and harvested at the exponential growth phase (where all the individual processes involved in biomass production increase at the same rate (Slater, 1988)). The culture was transferred to sterile 250 ml bottles and centrifuged at 5000 ***g*** for 10 min. The pellet was washed and re-suspended in approximately 200 ml of sterile 25% Ringers solution (Oxoid, U.K.), providing an osmotically-balanced media for the microorganisms. The process was

repeated three times and the resultant pellet suspended in 200 ml of 25% Ringers solution for inoculation into the experimental soil.

Anaerobic incubation to assess nitrate disappearance and gaseous products

Ten g sub-samples of the chemically treated soil were added to 54 250 ml Quickfit flasks (Fisons, U.K.). Twenty-seven of the flasks were amended with 10 ml of a solution containing 36 mM KNO_3 and 8 mM glucose, equivalent to approximately 500 mg NO_3^--N kg^{-1} and 600 mg C kg^{-1}, and are termed the **Control** treatments. A further 27 flasks were amended with 10 ml of a solution containing 36 mM KNO_3 and 8 mM glucose and 1 ml of *P. denitrificans*, equivalent to 10^6 denitrifiers g^{-1} of soil, these being termed the **Dentreat** samples. Ten g sub-samples of air-dried, field soil were added to 21 flasks and treated as the **Dentreat** samples, these being termed the **Soil** treatments. The flask atmospheres were isolated using greased Quickfit adaptors fitted with rubber bungs and sampling tubes to allow the collection of headspace gases. All flasks were evacuated for 5 min and the headspace replaced with N_2 to ensure anaerobic conditions. Thirty ml of the headspace gas was withdrawn and replaced by 30 ml of C_2H_2 (B.O.C., U.K.) giving an approximate headspace concentration of 10% (v/v), sufficient to prevent the reduction of N_2O to N_2 (Yoshinari and Knowles, 1976). The soils were slurried and incubated on an orbital shaker at 21°C. At the onset of the experiment and after 0.17, 0.5, 2, 6, 24, 48, 72 and 96 h, three flasks were chosen at random from the **Control** and **Dentreat** samples; and at times 0, 0.17, 0.5, 2, 6, 24 and 48 h from the **Soil** samples, and the headspace gases were analysed by gas chromatography (Hall and Dowdell, 1981). The GC (Ai 93, Ai Cambridge, U.K.) was fitted with an ECD for N_2O determination and a TCD for determination of CO_2 and C_2H_2. N_2O and CO_2 concentrations were corrected for absorption by water (Weast, 1968; Moraghan and Buresh, 1977, respectively). After gas sampling, the soil was extracted with 40 ml of distilled water, shaken for 30 min, filtered through Whatman No. 41 paper and stored at − 18°C pending analysis for NO_2^- and NO_3^-. The NO_3^- and NO_2^- concentrations of the extracts were determined on a Tecator 5010 flow injection analyzer (U.K.) (NO_3^- reduced to NO_2^- by a copperised Cd column, after which the total NO_2^- was determined by reaction with N-1-naphthylethylenediaminedihydrochloride).

Statistical analysis

Carbon dioxide and N_2O evolution was subjected to a two way analysis of variance to test for significant effects of the treatments at each sampling time. To assess the significance of NO_3^- disappearance between sampling times, mean NO_3^- concentrations were fitted to a linear model at each time point and for each treatment separately. All analyses were performed using GENSTAT™ 5 Release 3.1.

RESULTS AND DISCUSSION

Nitrate

Both the **Dentreat** and **Soil** samples showed significant ($P < 0.01$), rapid decreases in NO_3^- concentration within 2 h of anaerobic incubation (Fig. 1). Between 0 and 2 h, the measured decline in NO_3^- concentration was 150 mg N kg^{-1} (SE 27) and 146 mg N kg^{-1} (SE 31) for the **Dentreat** and **Soil** samples, respectively. The NO_3^- decrease in the first 2 h was greater than any other change during the incubation and was followed by a non-significant increase in both treatments, which could represent a release of immobilised NO_3^- by the microorganisms under anaerobiosis, or alternatively, a release of physically or chemically bound-N to the soil. Soil NO_3^- concentrations remained constant in the **Control** samples throughout the incubation (Fig. 1) indicating that any observed disappearance of NO_3^- was microbially mediated. This pattern of NO_3^- disappearance and subsequent release was also observed in permanent pasture and forest soil, where the addition of C_2H_2 was found to have no effect on the process (Dendooven *et al.*, 1996a; Dendooven *et al.*, 1996b), but the mechanism or biological benefit for this are currently unknown. However, the ability to remove large quantities of NO_3^- quickly under non-limiting conditions, and then utilise this at a later time may benefit some soil microorganisms. There are a number of mechanisms of NO_3^- entry into bacterial

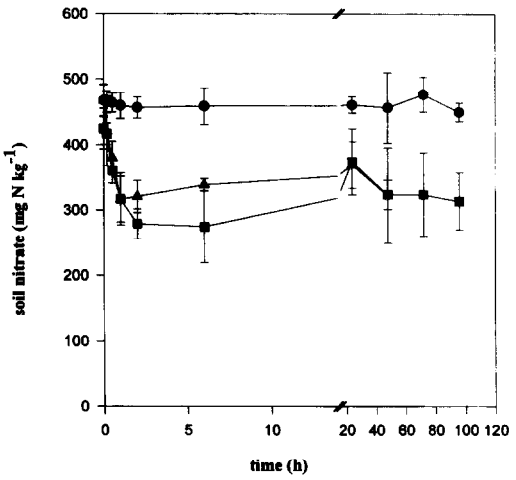

Fig. 1. The soil NO_3^- concentration (mg N kg^{-1}) from samples anaerobically incubated for 96 h at 21°C, (–●–) **Control** soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1}, (–■–) **Dentreat** modified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganisms g^{-1} soil and (–▲–) **Soil** unmodified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganism g^{-1} soil. Bars indicate ± 1 standard deviation.

cells and it is thought to be a multiphase process involving several different transport systems (Stouthamer *et al.*, 1982). Using data from pure culture studies of cyanobacterium there appear to be two transport mechanisms leading to NO_3^- entry. 1. An adenosine tri-phosphate driven transporter, whose activity may be a function of C availability. 2. A co-transport mechanism that exhibits a strong and selective requirement for Na^+ in establishing the necessary electrochemical gradient for NO_3^- transport (Rodriguez *et al.*, 1992). Further evidence was provided by an apparent sensitivity of NO_3^- transport to monensin, an ionophore which relaxes the Na^+ electrochemical gradient (Skulachev, 1985). However, the capabilities of soil microorganisms to take up NO_3^- and the controls over it have not been well studied.

Soil nitrite

Nitrite concentrations in all three treatments were highly variable. The concentration in the **Control** samples remained less than 50 μg N kg^{-1} during the incubation (Fig. 2). The NO_2^- concentration in the **Dentreat** samples increased to 2.74 mg N kg^{-1} at 24 h and decreased after this time, with concentrations remaining below 1 mg N kg^{-1} throughout the experiment. The **Soil** samples exhibited much higher soil NO_2^- concentrations, 600 μg N kg^{-1} after 2 h increasing to 10 mg N kg^{-1} at 48 h. This could be attributed to the activity of different microbial populations in the **Soil** samples as compared to the **Dentreat** samples; with mixed populations possessing differing enzyme affinities and synthesis times resulting in a differing pattern of NO_2^- production and release to the soil (Betlach and Tiedje, 1981).

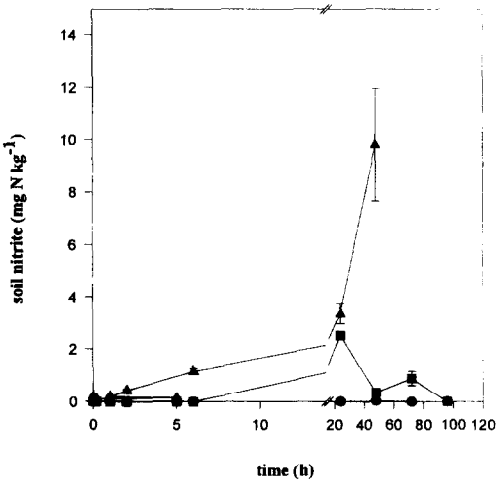

Fig. 2. The soil NO_2^- concentration (mg N kg^{-1}) from samples anaerobically incubated for 96 h at 21°C, (-●-) **Control** soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1}, (-■-) **Dentreat** modified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganisms g^{-1} soil and (-▲-) **Soil** unmodified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganism g^{-1} soil. Bars indicate ± 1 standard deviation.

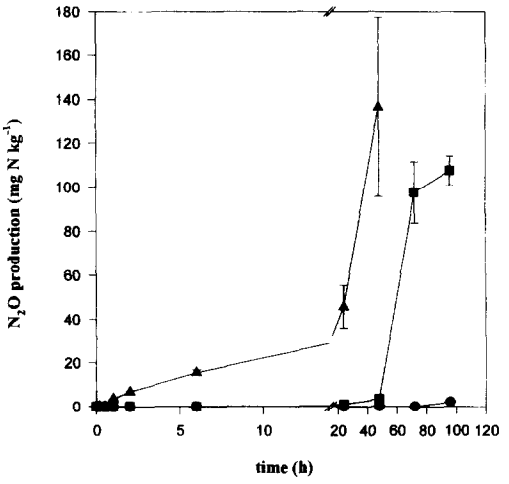

Fig. 3. The N_2O evolution (mg N kg^{-1}) from soil samples anaerobically incubated for 96 h at 21°C, (-●-) **Control** soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1}, (-■-) **Dentreat** modified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganisms g^{-1} soil and (-▲-) **Soil** unmodified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganism g^{-1} soil. Bars indicate ± 1 standard deviation.

N_2O evolution

N_2O began to evolve after 2 h in all three treatments with 0.04, 0.12 and 6.87 mg N kg^{-1} for the **Control**, **Dentreat** and **Soil** samples respectively (Fig. 3). The **Control** samples evolved less than 2 mg N kg^{-1} during the incubation, confirming that there was negligible denitrification activity in the sterilised samples. The **Dentreat** samples exhibited a lag phase of approximately 48 h, which was followed by an exponential increase to 98 mg N kg^{-1} at 72 h, with a slower increase to 108 mg N kg^{-1} at 96 h. Gaseous evolution was characteristic of microbial growth, exhibiting a lag, exponential and constant phase of production. From 2 h and onwards, the **Soil** samples evolved N_2O curvilinearly to 137 mg N kg^{-1} at 48 h.

The quantity of NO_3^--N disappearing within 2 h of anaerobic incubation was equivalent to 72% and 94% of the N_2O-N recovered from the **Dentreat** and **Soil** samples at 96 and 48 h respectively, although there were periods of apparent NO_3^- release during the incubation, notably between 6 and 24 h. We believe that this early phase of NO_3^- removal from the soil solution was the most important in determining the denitrification loss of N as N_2O. The accumulation of NO_2^- or production of N_2O could not be directly related to the disappearance of NO_3^- in the first 24 h. There was no sequential reduction of NO_3^- to NO_2^- and to N_2O as predicted by denitrification enzyme kinetics (Firestone, 1982). Soil NO_3^- was presumably either held intercellularly, for example in vacuoles, or chemically- or physically-complexed within the microbial-biomass.

CO_2 production

After 6 h of anaerobic incubation CO_2 evolution was 2.00 mg C kg^{-1} for the **Control** and **Dentreat** samples and 40 mg C kg^{-1} for the **Soil** samples (Fig. 4). The **Control** samples evolved less than 5 mg C kg^{-1} during the whole incubation, confirming there to be negligible biological activity in the sterilised soil. The **Dentreat** samples exhibited a sigmoidal pattern of evolution, characteristic of microbial growth under initially non-limiting substrate (Slater, 1988). In this treatment there was a lag phase to 48 h where CO_2 production increased to 4 mg C kg^{-1} which was followed by a 23-fold increase to 94 mg C kg^{-1} between 48 and 72 h. After 72 h, CO_2 evolved more slowly with concentrations increasing 1.4-fold to 130 mg C kg^{-1}. The **Soil** samples evolved CO_2 at a significantly greater rate than the **Dentreat** ($P < 0.05$). CO_2 concentrations increased 45-fold between 2 and 48 h from 16 to 737 mg C kg^{-1}. Soil respiration rates over 24 h from the sterilised and inoculated soil were comparable to other measurements made under similar conditions. At 24 h, average rates of 4 and 10 μg CO_2-C $g\ h^{-1}$ were found for the **Dentreat** and **Soil** samples, respectively. Elliott *et al.* (1983) reported rates of 8–10 μg CO_2-C $g\ h^{-1}$ at 15.5 h for sterilised soil re-inoculated with a $10^6\ g^{-1}$ culture of *Pseudomonas paucimobilis*.

Approximately 130 and 737 mg CO_2-C kg^{-1} evolved from the **Dentreat** and **Soil** samples after 96 h. Previous experiments showed that 4.5 mg C kg^{-1} was evolved as CO_2 from the unamended mineral soil and 600 mg glucose-C kg^{-1} was added to the samples, thus the equivalent of 0.22 and 1.22 of the available C, respectively, was utilised by the microbial biomass. There must have been an additional C source available to the **Soil** samples: indigenous C or non-viable microorganisms that could contribute to the soil C pool upon death. By converting bacterial numbers to a weight, the potential C contribution from dead microorganisms was assessed (Behera and Wagner, 1974). Assuming that all the initial biomass killed over the incubation was replaced with viable progeny, 24 mg C kg^{-1} would have been liberated to the soil system. The combination of glucose-C, microbial-biomass-C and soil-C accounts for 21% and 85% of the total CO_2 evolved from the **Dentreat** and **Soil** samples respectively. For the **Dentreat** samples, where most of the substrate was supplied by exogenous additions, it was assumed that glucose was the only significant C source available and NO_3^- was the only electron acceptor. Burford and Bremner (1975) suggested that under such conditions the ratio between CO_2-C and N_2O-N evolved should be 1-to-1.17. The ratio for the **Dentreat** samples was 1-to-0.93 indicating that slightly more CO_2 was evolved than predicted by stoichiometry. The ratio between N_2O-N evolved and NO_3^--N lost was 1-to-1.39 indicating that not all of the NO_3^- removed was reduced to N_2O. Presumably some N was either assimilated or stored within the microbial biomass. In the **Soil** samples, the ratio between CO_2 and N_2O produced was 1-to-0.19, indicating that glucose was not the only C source available to the microorganisms, and that between N_2O evolved and NO_3^- lost was 1-to-1.07.

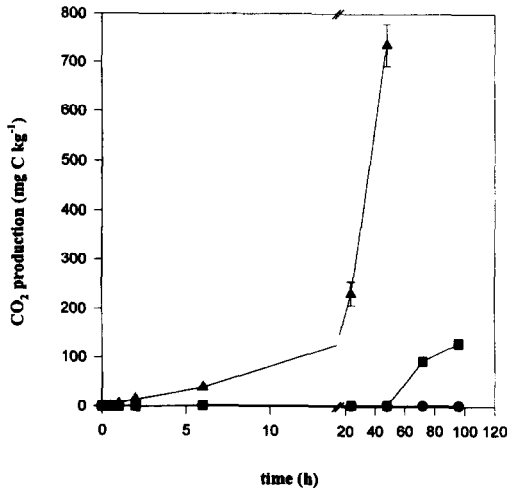

Fig. 4. The CO_2 evolution (mg C kg^{-1}) from soil samples anaerobically incubated for 96 h at 21°C, (–●–) **Control** soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1}, (–■–) **Dentreat** modified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganisms g^{-1} soil and (–▲–) **Soil** unmodified soil amended with 600 mg C kg^{-1}, 500 mg N kg^{-1} and *P. denitrificans* to a concentration of 10^6 microorganism g^{-1} soil. Bars indicate ± 1 standard deviation.

The rapid disappearance of NO_3^- under anaerobic conditions may not have been restricted to the activity of denitrifying microorganisms. In previous experiments to assess NO_3^- disappearance during denitrification, and confirm it was a repeatable phenomenon, the chemically treated soil was found to be non-sterile and 36 mg NO_3^--N kg^{-1} disappeared from the soil solution within 2 h (Table 1). Nitrite concentrations remained less than 1 mg N kg^{-1} during the incubation (Table 1), and did not accumulate under anaerobic conditions. Nitrous oxide was not produced in the system until after 6 h, although there was considerable production of CO_2 (Table 1). However, a similar pattern of NO_3^- uptake and release was seen as in the **Dentreat** and **Soil** samples. The contamination indicated that the excessive NO_3^- disappearance was not restricted to denitrifying microorganisms, but could be found in microorganisms capable of anaerobic respiration. Partial denitrifiers, i.e. not reducing NO_3^- to N_2O (Ingraham, 1981) could be excluded as there was no evidence of NO_2^- accumulation in the soil (Table 1). We did not measure NO in the head-space and it is unlikely that this highly reactive chemical species would accumulate under anaerobic conditions (Williams *et al.*, 1992). The microorganisms could have been capable of autotrophic denitrification, i.e. they were microorganisms that can obtain energy by using NO_3^- to oxidise inorganic compounds (e.g. S_2^-,

Table 1. NO_3^- and NO_2^- concentrations and the amounts of N_2O and CO_2 produced during the anaerobic incubation of the microbially-contaminated chemically-treated soil, which had been amended with 10 ml of a solution containing 36 mM KNO_3 and 8 mM glucose (approximately equivalent to 600 mg C kg^{-1} and 500 mg N kg^{-1})

Time (h)	NO_3^-	NO_2^- (mg N kg^{-1})	N_2O	CO_2 (mg C kg^{-1})
0	486.4 (40.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
0.17	490.1 (36.4)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
0.50	455.2 (40.5)	0.3 (0.1)	0.0 (0.0)	0.0 (0.0)
1	449.3 (11.1)	0.2 (0.0)	0.0 (0.0)	1.9 (0.6)
2	450.0 (60.9)	0.2 (0.0)	0.0 (0.0)	3.6 (0.6)
6	411.7 (42.6)	0.2 (0.0)	0.0 (0.0)	4.2 (2.5)
24	432.3 (31.4)	0.2 (0.0)	0.8 (0.1)	95.5 (4.9)
48	423.3 (47.9)	0.6 (0.3)	1.3 (0.1)	152.1 (4.9)
72	417.0 (22.7)	0.2 (0.0)	3.6 (0.2)	500.1 (12.0)
96	415.1 (40.8)	0.2 (0.1)	1.6 (0.1)	677.8 (36.5)

Figures in parentheses are the standard deviation of the mean of 3 values.

Fe_2^+, NH_4^+, organic compounds (Golterman, 1991; Van de Graff *et al.*, 1995). It must be stressed that greater than 70 mg N kg^{-1} was actively taken up by the contaminant population during the incubation, with less than 5% recovered as N_2O-N at 96 h.

CONCLUSIONS

Our study illustrated a phenomenon that we are not aware of in literature concerned with denitrification in soils experiencing anaerobiosis — the rapid uptake of large quantities of soil NO_3^- by microorganisms in excess of their ability to utilise the substrate under anaerobic conditions. Approximately 150 mg N kg^{-1} was actively taken up from the soil solution by denitrifying microorganisms within 2 h following the onset of anaerobiosis. Soil NO_2^- concentrations did not increase until 48 h, suggesting no significant reduction of NO_3^- until this time, and preceded the period of significant N_2O evolution after 48 h. The amounts of NO_2^- measured could not account for the reduction of disappeared NO_3^-, and N_2O production after 48 h was more closely related to the NO_3^- taken up within 2 h than the following NO_2^- accumulation. There was a lag phase between NO_3^- disappearance and N_2O evolution of 48 h. Approximately 72 to 94% of the NO_3^- taken up was evolved as N_2O at 96 h for the **Dentreat** and **Soil** samples respectively. CO_2 production could not be related to NO_3^- disappearance and was thus not a direct reflection of the ability of the microbial biomass to reduce the compound. It is possible that there is another metabolic pathway available to microorganisms that enables them to store or react with NO_3^- and couple the product or energy formed to oxidative phosphorylation.

We propose that under anaerobic conditions and with sufficient labile C, soil microorganisms are able to actively or passively take up quantities of NO_3^- in excess of their immediate energetic requirements and either store it internally or physically- or chemically-complex the ion for later use. It is possible that the release of N to the soil environment after the initial disappearance suggests that the later explanation to be the most likely, as such high intracellular concentrations of NO_3^- would be toxic unless stored in vacuoles or another such physiological reservoir. Such a phenomenon is important for our understanding of the denitrification process. Where the direct relationship between soil NO_3^- and N_2O evolution is complicated by the short-term storage of quantities of N, care should be exercised when using routinely-measured soil substances, such as NO_3^-, as predictors of potential denitrification activity. Such a phenomenon could account for some of the extreme variability encountered in denitrification studies, with mixed microbial populations possessing different abilities to remove and denitrify substrate from the soil environment, and the correlation of soil NO_3^- and N_2O production could therefore be flawed. Further work to assess the capability of microorganisms to remove NO_3^- from the soil environment under different moisture and temperature regimes could provide a more reliable estimate of the potential for N loss through denitrification. The influence of C quantity and quality on this uptake requires additional investigation. The modified soil system used in these experiments allowed the effect of substrate quantity and quality on a denitrifying microorganism, under highly-controlled conditions of temperature and moisture content to be studied. It provides a useful tool for the manipulation of the soil environment to study a highly mechanistic soil process.

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