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A Comparison of a Slurry and a Perfusion System to Quantify Potential and Heterotrophic Nitrification Rates in Soils

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ABSTRACT. Potential and heterotrophic nitrification rates were investigated in three soils:two Brazilian soils of pH 4.7, and one British soil of pH 6.5. Two methods were compared; a slurry-based system, and a perfusion system.

There was a retardation of nitrate-N production in the high pH British soil when measured by the slurry method, probably due to the accumulation of protons and nitrite. The perfusion was found to be the best means of quantifying both potential and heterotrophic nitrification rates, regardless of soil/microbial properties. The perfusion system most closely measured nitrification rates as they would occur in the field. Its advantage was confirmed by the relatively short time needed for nitrification to proceed at an equilibrium rate. The slurry system did not seem suitable for quantifying nitrification rates in soils where nitrifying activity is adapted to a high pH.

Both methods were used to assess heterotrophic nitrification. Acetylene was used to partition heterotrophic and autotrophic nitrification by the selective inhibition of autotrophic ammonium oxidation. The Brazilian soils had 60 to 70% of potential nitrification attributed to heterotrophic activity, The British soil had less than 15% of potential nitrification attributed to heterotrophic organisms when measured by perfusion. The slurry system gave an inhibition of nitrate-N production to zero in both the acetylene and non-acetylene treatments. Therefore, the % heterotrophic nitrification could not be ascertained by this method.

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INTRODUCTION

Nitrification is the conversion of nitrogen in organic and inorganic forms from a reduced to a more oxidized state. Biological nitrification has been mainly attributed to chemoautotrophic organisms that derive their energy requirements from the oxidation of either ammonium or nitrite. However, heterotrophic nitrification has also been shown to occur in certain soils, though its significance has been the subject of much controversy.

The inorganic pathway is mediated by two main groups of autotrophic bacteria: ammonium oxidation by the genus *Nitrosomonas*, and nitrite oxidation by the genus *Nitrobacter*. Heterotrophic nitrifiers are mainly certain fungal species, though some bacteria do exist, producing nitrite or nitrate from inorganic or organic compounds that do not represent their sole source of energy.

To quantify heterotrophic nitrification, nitrification inhibitors acting specifically against autotrophic nitrifiers have been widely used. One such inhibitor is acetylene. This acts principally by inhibiting the activity of the ammonium oxidizers (Campbell and Aleem, 1965), the rate limiting step of autotrophic nitrification, by blocking the enzyme ammonia monooxygenase, which is not found in heterotrophs. The application of this inhibitor has been shown to retard the formation of nitrate in soils (De Boer *et al.*, 1989). Therefore, any inhibition of this enzyme is almost always interpreted as an indication of chemoautotrophic nitrification (De Boer *et al.*, 1988). If no such inhibition of nitrate formation takes place, nitrification is attributed to heterotrophic organisms.

As a preliminary investigation to further research, two methods were compared for their ability to quantify potential and heterotrophic nitrification. Firstly, a slurry-based incubation (Belser, 1977) that measured both accumulated nitrate-N and rates of potential/ heterotrophic nitrification. Secondly, a perfusion system, based on leaching a known concentration of ammonium substrate through a column of soil and subsequent analysis of the perfusate for nitrate-N. A modified technique to that used by Killham (1987) was used in order to effectively partition heterotrophic and autotrophic nitrification. Both methods were tested using a British grassland soil which was known to have high rates of microbial activity. The partitioning of heterotrophic and autotrophic nitrification was carried out by the use of acetylene mixed into the substrate solution (Killham, 1987). An inorganic substrate was chosen in preference to an organic one because it was

necessary to stimulate both forms of biological nitrification. It is known that heterotrophic nitrifiers can follow an inorganic pathway (Aleem *et al.*, 1964).

MATERIALS AND METHODS

Soils

Three soils were used:a Brazilian quartz sand, a Brazilian clay and a British grassland soil. Both Brazilian soils were Oxisols that had been under a grassland vegetation. Each had a pH of 4.7. The British soil had a pH of 6.5. Mixed and composited samples were used for investigations.

Pre-treatment of soils

All soils were sieved to $<2\mu$m and incubated at 70% water holding capacity for at least three weeks prior to the commencement of experimentation. Immediately before the start of incubation/perfusion, the two Oxisols were leached with 100 ml of calcium sulphate solution in order to remove nitrate that may be attached to positive sites on the clay. The British soil was leached with 100 ml of distilled water.

Slurry method

A slurry system was set-up similar to that proposed by Belser (1977). Ten grammes of soil was placed in a 250 ml volumetric flask. The flask was sealed and 2.5 ml of flask air removed with a syringe. The flask was injected with 2.5 ml of 1% acetylene (Killham, 1987) and incubated for 24 hrs. At the end of incubation, 50 ml of 0.5 mM ammonium sulphate solution was added (this had been saturated with acetylene for 24 hrs). The flask was re-sealed and injected with a further 2.5 ml acetylene, after removal of 2.5 ml of flask air. Timing of incubation commenced on addition of the substrate. Two treatments of triplicate samples were prepared in this way: an acetylene treatment, and a non-acetylene treatment. During the incubation, the slurry was sampled at 2, 12, 16, 20, 22, and 24 hrs. Each aliquot comprised 5 ml of fully homogenised slurry which, on sampling, was immediately placed in a cold room and filtered through Whatman No. 5 filter paper. The filtrate was analyzed for nitrate-N.

Perfusion method

Triplicate glass leaching tubes (26 cm long, 4 cm internal diam.) were set-up. These were packed with 10 g cation exchange resin (Amberlite, IR-120; Na^+ form), and 20 g of soil, being the uppermost layer. Each layer was separated by a thick band of glass wool and leaching columns connected to a Watson-Marlow peristaltic pump with small-bore 0.8 mm diam. peristaltic tubing. Perfusion was carried out at a rate of 7.5 ml/h. The perfusate was collected at intervals of 2, 4 and 6 hrs and analyzed for nitrate-N. Two treatments were used; an acetylene treatment and a non-acetylene treatment.

The perfusion solution contained a 0.5 mM ammonium sulphate substrate. This solution had previously been saturated with acetylene for 24 hrs in a gas-tight flask prior to the commencement of perfusion.

Analysis for Nitrate-N

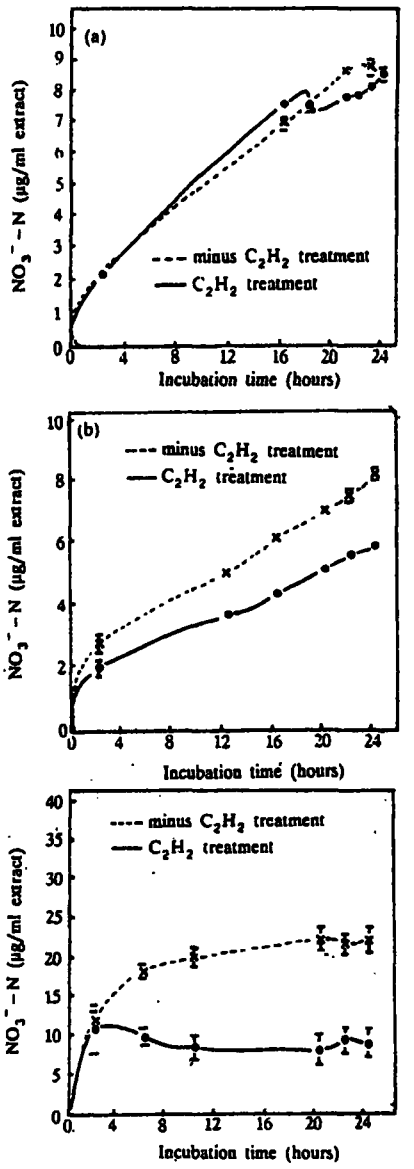
Filtrates and perfusates were analyzed colorimetrically for nitrate-N based on the technique proposed by Kamphake *et al.*, (1967). A Technicon Autoanalyzer was used for automation of the procedure, and absorption read at 525 nm. All levels were corrected for background nitrate-N in the substrate solution.

Nitrification rates were expressed as μg nitrate-N/g dry soil/h, as a mean of triplicate samples. In addition, accumulated nitrate-N (slurry incubation) was expressed as μg nitrate-N/ml extract.

RESULTS

Slurry method

The two Brazilian soils had similar levels of nitrate-N accumulation in the non-acetylene treatment throughout the 24 hr incubation (Figure 1), between 8 and 8.5 μg nitrate-N/mlct at 24 hrs. There was a linear period of nitrate-N accumulation from 0 to 2 hrs, decreasing from 2 to 24 hrs. This linear phase was most pronounced for the British soil, reaching a level of about 11 μg nitrate-N/ml extract at 2 hrs, before levelling off from 2 to 24



(a) Brazilian quartz sand
(b) Brazilian clay
(c) British soil

Figure 1. Accumulated nitrate-N as measured by a slurry system

hrs in both treatments. From 6 to 24 hrs, the level of nitrate-N accumulation remained at 18 μg nitrate-N/ml extract.

Acetylene appeared to have little effect on nitrate-N accumulation in the Brazilian quartz sand. However, the Brazilian clay did show a decrease in nitrate-N accumulation in the acetylene treatment, with 5.8 μg nitrate-N/ml extract, compared to 8.25 μg nitrate-N/ml extract in the non-acetylene treatment at 24 hrs. For the British soil, from 0 to 2 hrs, the level of nitrate-N accumulation was the same for both treatments; 11 μg nitrate-N/ml extract. At 2 hrs, treatment levels diverged. In both treatments, from 2 to 24 hrs, there was no further increase in nitrate-N accumulation.

The Brazilian clay had similar rates of nitrification for both treatments. The Brazilian quartz sand showed a greater divergence at 24 hrs. Nitrification rates for the British soil in both treatments dropped towards zero from 6 to 24 hrs, after reaching a maximum level of 32 μg nitrate-N/g dry soil/h at 2 hrs for the non-acetylene treatment.

Perfusion method

Figure 2b shows heterotrophic and potential nitrification rates. The two Brazilian soils had constant rates of both potential and heterotrophic nitrification, between 0.02 and 0.03 μg nitrate-N/g dry soil/h for potential nitrification, and between 0.016 and 0.018 μg nitrate-N/g dry soil/h for heterotrophic nitrification. The British soil had an initial flush of nitrification, followed by a constant rate at 4 and 6 hrs; the potential rates between 0.7 and 0.8 μg nitrate-N/g dry soil/h, and heterotrophic nitrification rates between 0.06 and 0.1 μg nitrate-N/g dry soil/h.

DISCUSSION

The perfusion system gave considerably lower rates of nitrification to those measured by the slurry method. However, they seem to offer a truer representation of what is happening in the field. Rates of 1 to 2 μg nitrate-N/g dry soil/h for the two Brazilian soils (Figure 2a), as measured by the slurry method, are extremely high. Rates in the order of 0.02 to 0.03 μg nitrate-N/g dry soil/h, as measured by perfusion (Figure 2b), are more consistent with rates measured for other Brazilian soils (Vitousek and

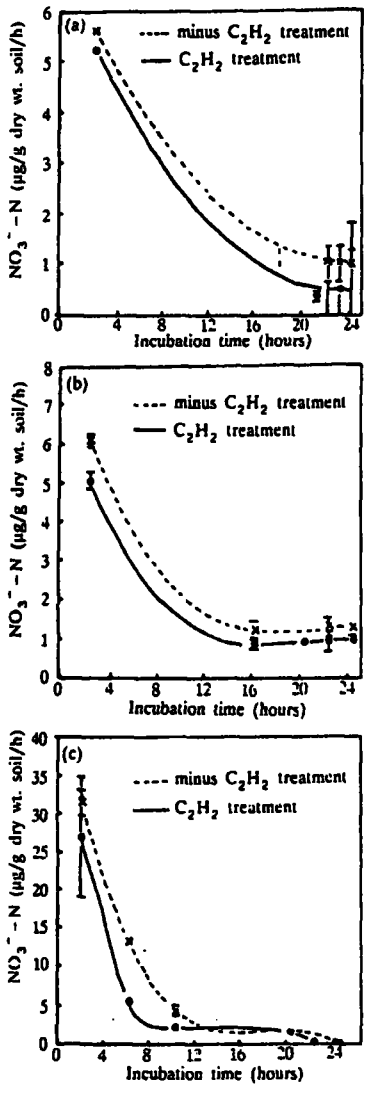


Figure 2. (a) Brazilian quartz sand
(b) Brazilian clay
(c) British soil

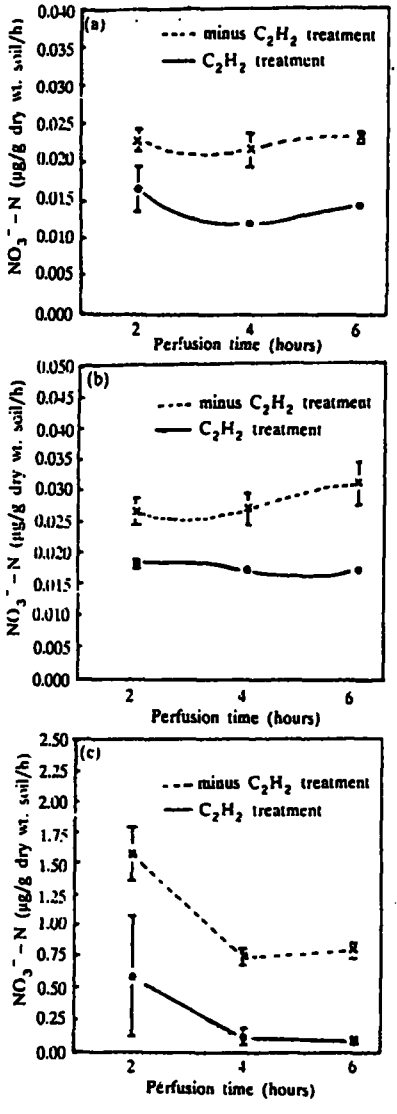


Figure 3. (a) Brazilian quartz sand
(b) Brazilian clay
(c) British soil

Figure 2. Potential and heterotrophic nitrification rates as measured by a slurry system.

Figure 3. Potential and heterotrophic nitrification rates as measured by a perfusion system.

Matson, 1988), which varied between 0.025 and 0.09 μg nitrate-N/g dry soil/h.

The slurry system produced a flush of nitrification. This was due to the sudden stimulation of the nitrifying population on addition of the ammonium substrate; its disappearance coincided with the population reaching a steady state with slurry conditions.

The decline in nitrate-N production for the British soil was drastic in the slurry incubation, where both potential and heterotrophic nitrification declined to zero. This could not be explained by either ammonium toxicity or oxygen deficiency, since the two Brazilian soils were not repressed in this way. Therefore, this fall must be attributed to a combination of conditions imposed by the slurry itself, and to the adaptation of the nitrifying population to the soil's particular chemical conditions, notably pH. Such a combination causes a tremendous flush in nitrate-N production from 0 to 2 hrs - a response to the initially favourable conditions found in this slurry system, *eg.* high pH, excess substrate, high oxygenation, and an optimum incubation temperature of 30 C (Chase *et al.*, 1968), and to the high nitrifying potential of the soil. Such conditions might raise populations of *Nitrosomonas* by several hundred million per g of soil (McLaren, 1971; Ardakari *et al.*, 1974). This flush in nitrification will lower the slurry pH; the increased proton concentration serving to retard the activity of *Nitrobacter* relative to the *Nitrosomonas*. Such a situation is pronounced in the British soil, where nitrifying activity is adapted to a pH of 6.5. Keen and Prosser (1987) showed that *Nitrobacter* became completely inactive in initially high pH soils when the pH fell to <6. As the pH is lowered, subsequent retardation of the nitrite-N oxidizers will lead to an increase in nitrite-N accumulation, with no further increase in nitrate-N production. Nitrite-N is known to be toxic to the microbial population (Bollag and Henninger, 1978) and is known to become more active as the pH decreases (Hauck and Stephenson, 1965). Such a situation will be quick to occur in the slurry system, provided the correct combination of factors is present, since there is no continuous flow of substrate and product out of the system. It seems, therefore, that it is not an increase in the H^+ concentration that causes an inactivation of Nitrobacter, but rather the accumulation of nitrite-N because of it.

The two Brazilian soils did not show such a decline in nitrate-N production. Here, the *Nitrobacter* will be adapted to a low pH, and function at a similar rate to the *Nitrosomonas*.

Acetylene was used to specifically inhibit autotrophic nitrifiers. Though the slurry system proved adequate in retarding autotrophic nitrification early in the incubation, as can be seen in the acetylene treatment of the British soil (Figure 1), the % heterotrophic nitrification of the potential could not be ascertained until after about 20 hrs incubation, due to the time taken for steady rates to be achieved (Figure 2a). A steady rate of both potential and heterotrophic nitrification was achieved after only 2 hrs in the perfusion system. Only when such a constant rate is achieved can the true contribution of heterotrophic nitrification to potential nitrification be assessed.

CONCLUSION

The perfusion system offers the best means of quantifying both potential and heterotrophic nitrification rates. It is suitable for all soils, regardless of microbial adaptation to soil chemical properties, namely pH, by avoiding the build-up of metabolic acidity. In addition, the perfusion system measures nitrification rates as they would occur in the field; measurements aided by the swift realization of equilibrium rates.

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