DENITRIFICATION IN LAKE SEDIMENT AND SEWAGE SLUDGE

by

Ian Keith Nicholson

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ABSTRACT

Mass spectrometric analysis of atmospheres over lake sediment and flooded sewage sludge incubated with N-15 labeled NO_3^- was performed. The sequence of denitrification was NO_2^-, N_2O, and N_2 in lake sediment at 10C. However, added NO_3^- was reduced to NO_2^- and N_2 during incubation of sludge. Re^*, the electron acceptance rate by nitrogenous oxides during their reduction, was faster in the sludge. Addition of glucose to sediment greatly increased Re^* and reduced quantities of N_2O accumulated. The maximum of NO_2^- accumulation was greatly increased by the addition of lime to sediment. This retarded the rate of N_2O accumulation, but the effect on Re^* and the rates of NO_3^- loss and N_2 production was slight. Acidification of sludge decreased Re^* and the rate of NO_3^- loss. A lag period before N_2 production was evident, but no N_2O was detected.

In non-agitated sediment with relatively low concentrations [2.5 to 10 ppm] of added NO_3^--N, Re^* and the rate of NO_3^- loss were nearly first-order. However, the rates were almost zero-order when agitation ensured the homogeneity of the incubated sediment. Although the rates were first-order with 10 ppm added NO_3^--N in sludge, they were zero-order with higher concentrations [20 to 50 ppm]. These results indi-

cated that Re* and the rate of NO_3^- loss were rate-limited by diffusion in non-agitated sediment. Re* for sediment agitated with 10 ppm added NO_3^--N was approximately twice that of the non-agitated sediment. Trends in NO_3^- reduction, N_2O accumulation, and N_2 evolution with time in agitated sediment were similar to those generated by competitive Michaelis-Menten kinetics.

Lowering of the incubation temperature of sludge to 4C induced the accumulation of N_2O, which was not detected at a higher temperature [10C]. Also, Re* and the rate of NO_3^- loss were less.

Although addition of sludge [20.7% $CaCO_3$ equivalent] to sediment at 10C increased NO_2^- accumulation, the rate of N_2O accumulation was not retarded and the rate of N_2 production was accelerated.

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INTRODUCTION

Nitrogen is the most important essential plant nutrient that affects the yield of an agricultural crop. In order to meet the food requirements of an ever-increasing world population, the use of nitrogenous fertilizers in crop production has also steadily increased. Large projected increases in fertilizer use have raised some concern about the possible hazardous effects of nitrogenous compounds on the environment. High nitrate levels in drinking water, the role of nitrate in lake eutrophication, and more recently, the detrimental effect of nitrogen oxides on the atmosphere are the major issues. Nitrogen applied to the soil in chemical fertilizers, animal manures, or digested municipal sewage sludge, as well as nitrogen from beneath feedlots, may be leached to groundwater or denitrified. In Canada, excluding Quebec, 80 percent of sewage sludge is dumped into lakes and rivers, while in Quebec most of the 150,000 tons annual production of untreated sewage sludge is discharged directly into the St. Lawrence River and its tributaries (Gagnon 1973).

Denitrification—the reduction of nitrate to gaseous products—is often mentioned in the discussion of the above issues. Losses of fertilizer-N applied to agricultural fields, sometimes in excess of 50 percent, are blamed on this process. On the other hand, denitrification in sewage treatment and waterways is promoted as a means of reducing nitrate-N concentration. Infant and ruminant methemoglobinemia, an impairment of oxygen transport by blood due to reaction of nitrite with hemoglobin, occurs when nitrate is ingested and reduced. The nitrate maximum in drinking water is set at 10 ppm nitrogen. Nitric and nitrous oxides, gaseous products of denitrification, may be involved in atmospheric ozone destruction (Crutzen 1976; Delwiche and Bryan 1976; Keeney 1978). Carcinogenic ultraviolet light is screened by the protective ozone layer in the upper atmosphere.

The ideal agricultural solution to denitrification is to minimize nitrate removal, while the ideal environmental solution is to maximize nitrate removal and minimize production of gaseous nitrogen oxides. Lake sediment as a site of denitrification is fairly well understood. However, the effect of nitrate concentration on the forms and quantities of the nitrogen oxides and molecular nitrogen has received limited attention. Work with denitrification in agricultural soils has shown a dependence of the ratio of the produced nitrogen oxides to produced nitrogen gas on the added nitrate concentration (Cho and Mills 1979; Cho and Sakdinan 1978). The purpose of this investigation is to study the denitrification process in flooded lake sediment and sewage sludge samples, with respect to the removal of nitrate and the production of nitrogenous gases.

Chapter II

LITERATURE REVIEW

2.1 BIOLOGICAL REDUCTION OF NITROGEN OXIDES

Nitrate is reduced by microorganisms in the assimilation of nitrate-N and in anaerobic respiration. Reductase enzymes involved in the biological reduction of nitrogen oxides are flavoproteins and may contain molybdenum, iron, or other heavy metals which are alternately oxidized and reduced during electron transfer (Conn and Stumpf 1967; Lehninger 1970).

Nitrate assimilation occurs in both aerobic and anaerobic conditions and is the incorporation of inorganic-N into organic-N. However, less immobilization occurs in anaerobic conditions since microbial metabolism is less efficient and growth is slower (Bartholomew 1965). Most microorganisms preferentially assimilate ammonium over nitrate (Jansson *et al*. 1955). The presence of readily available organic matter promotes nitrogen assimilation (Bartholomew 1965). Assimilation can be written as a series of two-electron transfers:

$$\underset{(5)}{NO_3^-} \Rightarrow \underset{(3)}{NO_2^-} \Rightarrow \underset{(1)}{[HNO]} \Rightarrow \underset{(-1)}{NH_2OH} \Rightarrow \underset{(-3)}{NH_4^+} \Rightarrow \text{amino acids.} \quad \{1\}$$

The numbers in parentheses denote the oxidation states. The intermediates, nitrite, hydroxylamine, and ammonium, have been detected. The hypothetical intermediate [HNO] has not been detected, but is postulated to be hyponitrite (Campbell and Lees 1967).

In aerobic conditions, hydrogens donated by organic matter [represented by glucose] reduce oxygen to water according to:

$$C_6H_{12}O_6 + 6O_2 \Rightarrow 6CO_2 + 6H_2O. \qquad \{2\}$$

In the absence of oxygen, other compounds serve as terminal acceptors and are reduced.

The bacterial group containing the most genera able to reduce nitrate is comprised of the nitrate-respirers (Payne 1973). They reduce nitrate to nitrite by dissimilation in anoxic conditions.

Denitrification is the production of nitrogen or gaseous nitrogen oxides through the reduction of nitrate (Wijler and Delwiche 1954). Biological denitrification is carried out by facultative anaerobic bacteria in anoxic conditions. The complete reduction of nitrate to nitrogen, coupled with the complete oxidation of organic matter [represented by glucose], is written as:

$$C_6H_{12}O_6 + 4NO_3^- \Rightarrow 6CO_2 + 6H_2O + 2N_2. \qquad \{3\}$$

Payne (1976) lists the bacterial genera containing denitrifying bacteria. The number is small, but denitrifiers are widely distributed in soil, sediments, and fresh and salt water. Many carry out other transformations involved in the decomposition of organic matter such as proteolysis and ammonification (Keeney 1973). The most numerous denitrifiers are *Pseudomonas sp.*, *Achromobacter sp.* and *Bacillus sp.* (Alexander 1977). Different denitrifying species have different rates of gas production under similar environmental conditions (Valera and Alexander 1961). Organisms isolated from a waterlogged soil varied in their ability to reduce nitrate (Jordan *et al.* 1967).

The sequence of detectable products with most denitrifiers is nitrite, nitrous oxide, and nitrogen:

$$NO_3^- \Rightarrow NO_2^- \Rightarrow N_2O \Rightarrow N_2. \qquad \{4\}$$

$$(5) \qquad (3) \qquad (1) \qquad (0)$$

However, one species of Alcaligenes will reduce nitrite, but not nitrate, and some species or strains of Corynebacterium and Pseudomonas produce nitrous oxide as the terminal product (Payne 1976). Nitrous oxide is not normally detected during reduction of nitrite to nitrogen by *Pseudomonas aeruginosa*, but St. John and Hollocher (1977), using N-15 nitrite and an atmosphere of nitrous oxide, trapped tagged nitrous oxide and proved that it was a free, obligatory precursor of nitrogen in denitrification with this species.

Early studies investigating the sequence of denitrification and the gases produced in incubated soil amended with nitrate indicated that two pathways were operating. Nitric oxide was the initial gaseous product from acid soil (Wijler and Delwiche 1954; Cady and Bartholomew 1960). It did not accumulate and was further reduced to nitrous oxide and nitrogen. Cooper and Smith (1963), working with seven near-neutral soils, found nitrite to be the first product with nitrous oxide and then nitrogen as subsequent gaseous products. No nitric oxide was detected. Broadbent and Clark (1965) concluded that since nitric oxide was only produced from acid soil, it was a product of the chemical decomposition of nitrite. However, nitrite production and the subsequent reduction of nitric oxide and nitrous oxide to nitrogen were biological. This was supported by the work of Bollag _et al_. (1973). Addition of nitrite resulted in nitric oxide production from both sterile and non-sterile acid soil [pH 5.0] and, to a much lesser extent, from sterile neutral soil. However, nitrous oxide and then nitrogen were produced from non-sterile neutral soil.

Most denitrifiers are heterotrophs, but some use energy sources other than carbon. The autotroph _Thiobacillus denitrificans_ oxidizes sulphur compounds and _Paracoccus denitrificans_ oxidizes hydrogen or organic compounds (Alexander 1977). Both of these bacteria utilize carbon dioxide as a source of carbon (Delwiche and Bryan 1976). Additions of sulfur to soil columns significantly increased denitrification and numbers of _T. denitrificans_ (Mann _et al_. 1972).

Although the production of nitrogenous gases was first reported in 1886, it was not until 1946, when nitrous oxide was found in the atmosphere near the earth's surface, that large losses of nitrogen from agricultural soils were attributed to denitrification (Broadbent and Clark 1965). For this reason, much of the pioneering work on denitrification was done with soil samples. Early work with samples from aquatic systems indicated the occurrence of denitrification, but with nitrogen as the sole gaseous product (Goering and Dugdale 1966). Similar results were reported for lake 227 in the Experimental Lakes Area [ELA] in northwestern Ontario, although nitrous oxide was occasionally detected as a denitrification product (Chan 1977; Chan and Campbell 1975). The sequence of denitrification, equation {4} above, occurred in incubated sediment samples (Chen et al. 1972b; van Kessel 1978b). Nitrogen was produced at a much faster rate in lake water incubated over sediment (Goering and Dugdale 1966).

In eutrophic [nutrient-rich] lake water, numbers of denitrifying bacteria varied between 100 and 10,000 per ml, depending on the season and depth of sampling (Kuznetsov 1970). Numbers were reduced tenfold in oligotrophic [nutrient-poor] waters. During summer stagnation, denitrifiers were higher in the anoxic hypolimnion [bottom waters] than in the more aerobic epilimnion [surface waters]. Denitrifier density was high in eutrophic lake sediment with counts of three million per gram, but was reduced to 10,000 per gram in oligotrophic sediment (Kuznetsov 1970). Chan (1977) concluded that the littoral sediment zone was a major site of denitrification in a eutrophic lake. Preliminary observations indicated that assimilatory and dissimilatory nitrate reductase levels were high in shallow reaches of a small lake receiving sewage effluent (Hall et al. 1978).

The main goal of sewage treatment is to lower the biological oxygen demand [BOD] of wastes by the microbial stabilization of readily available organic-C (Taber 1976). Modern activated sludge units combine nitrification and denitrification to reduce the high nitrogen content of domestic waste (Francis and Callahan 1975; Johnson 1968; McCarty and Haug 1971). Ammonia released by decomposition of urea and other organic-N is nitrified to nitrate in a stirred, heated reactor where optimum aeration and temperature prevail. In a consecutive anaerobic reactor, the produced nitrate is denitrified. In the treatment of wastewater containing 27 ppm total-N, the aerobic digestor removed 20% of the nitrogen, while the anaerobic unit removed 54 to 64% of the remainder (Francis and Callahan 1975). In total, 60 to 80% of the original nitrogen was removed.

Non-stirred, facultative sewage lagoons may be used for secondary or tertiary anaerobic waste stabilization. McCarty and Haug (1971) reported that an uncovered pond was unsatisfactory for efficient nitrate removal by denitrification since algal growth produced oxygen, but a similar pond covered with large styrofoam blocks removed up to 90% of the initial 20 ppm nitrate-N in 10 days at 20C. They did not report whether or not harvesting of the algae increased the efficiency of nitrogen removal from the effluent.

Although studies on the gaseous products of denitrification in sewage treatment are scarce, it was found that nitrogen, but no nitrous oxide, was produced from laboratory activated sludge reactors (Johnson and Schroepfer 1964).

Work with pure cultures and cell-free extracts indicated that more than one enzyme was involved in denitrification. A resting-cell suspension of a denitrifier reduced nitrite to nitrous oxide, then nitrogen (Matsubara and Mori 1969). However, cell-free extracts reduced nitrite to both nitric and nitrous oxides, and nitric oxide to nitrous oxide alone (Matsubara and Iwasaki 1971). Separate fractions, each responsible for the reduction of nitrite, nitric oxide, and nitrous oxide, were isolated from crude extracts of the denitrifier <u>Pseudomonas perfectomarinus</u> (Cox and Payne 1973; Payne <u>et al</u>. 1971). Payne (1973) proposed that the denitrification sequence was:

$$\underset{(5)}{NO_3^-} \Rightarrow \underset{(3)}{NO_2^-} \Rightarrow \underset{(2)}{NO} \Rightarrow \underset{(1)}{N_2O} \Rightarrow \underset{(0)}{N_2}. \qquad \{5\}$$

However, although intact cells and cell-free extracts of a denitrifier reduced added nitric oxide to nitrous oxide, other results indicated that nitric oxide reductase had a high affinity for nitric oxide, which was not released to the gas phase, but immediately further reduced on the enzyme surface (Miyata *et al*. 1969). However, St. John and Hollocher (1977) failed to trap tagged nitric oxide in an atmosphere of unlabeled nitric oxide over a denitrifying culture of *Pseudomonas aeruginosa*, which was supplied with N-15 labeled nitrite. They concluded that nitrite and nitric oxide were reduced by separate pathways during denitrification in this species.

Nitrate reductase isolated from *Pseudomonas aeruginosa* contained molybdenum (Fewson and Nicholas 1961). Iron and molybdenum were involved in *Paracoccus denitrificans* dissimilatory nitrate reductase (Forget and Dervartanian 1972). Purified nitrite reductase probably contained copper (Walker and Nicholas 1961). A copper-containing enzyme catalyzed nitrous oxide formation from nitrite by a denitrifier (Suzuki and Iwasaki 1962).

2.2 FACTORS AFFECTING BIOLOGICAL DENITRIFICATION

The microbial process of denitrification, like other biological processes, is a function of environmental factors. These factors affect the growth and metabolism of both the denitrifiers and the general microbial population. Each exerts its own influence, but response to one factor may vary depending on the amount of stress applied by the others.

2.2.1 Oxygen concentration

Since denitrification is a respiratory function, the concentration of oxygen in the system is very important. Any condition which decreases the oxygen concentration or restricts oxygen supply promotes denitrification.

Denitrification has been reported in soil samples incubated under an air atmosphere (Broadbent and Stojanovic 1952). However, Bremner and Shaw (1958b) found no nitrate loss under similar aerobic conditions. Denitrification occurred when the oxygen level was less than 7%, by volume, in the atmosphere over soil amended with high levels of alfalfa (Cady and Bartholomew 1961). High biological activity created a demand for oxygen. An inverse relationship between denitrification and partial pressure of oxygen has been reported (Allison *et al*. 1960; Broadbent and Stojanovic 1952; Cady and Bartholomew 1961; Wijler and Delwiche 1954). With an aqueous medium, the dissolved oxygen concentration is more important than the atmospheric content (Focht and Chang 1975). Skerman and MacRae (1957) determined the threshold dissolved oxygen concentration, above which denitrification ceased in pure culture of *Pseudomonas aeruginosa*, to be 0.2 ppm.

Denitrification in soil increases with moisture content to above field capacity (Bailey and Beauchamp 1973b; Bremner and Shaw 1958a, 1958b; Mahendrappa and Smith 1967; Wijler and Delwiche 1954). Pilot and Patrick (1972) determined critical moisture tensions above which increased air-filled porosity and aeration rapidly decreased denitrification. As soil texture became finer, the critical moisture tension increased since small water-filled pores and increased tortuosity restricted oxygen diffusion. The oxygen diffusion rate [ODR] is directly dependent on the temperature, the cross-sectional area of the pore space, and the concentration gradient and is slower in water than in air (Taylor and Ashcroft 1972).

Maximum denitrification occurred in fully saturated or flooded conditions (Bailey and Beauchamp 1973b; Bremner and Shaw 1958b; Cho and Sakdinan 1978). This could occur in soil after a heavy rain, irrigation, or during spring thaw [temperature permitting]. In a field study with a clay loam, the highest nitrous oxide concentrations occurred when the soil was waterlogged and oxygen levels were low (Dowdell and Smith 1974). Oxygen consumption during incubation of soil was greatly reduced by a 2 cm layer of stagnant water (Khdyer 1978). In a similar study, 5 mm of quiescent water above the soil surface restricted oxygen consumption to 40 percent of the original partial pressure while all of the added nitrate was reduced (Cho and Sakdinan 1978). Slight reduction in denitrification rate in flooded ditch sediment occurred when the dissolved oxygen concentration of the overlying water was raised from 0 to 2 ppm, but further increases, up to 9 ppm, had no effect (van Kessel 1977a).

Similar findings were reported for lake sediment (Terry and Nelson 1975) and flooded swamp and marsh soils (Engler et al. 1976).

2.2.2 pH

Acid conditions reduce the metabolic activity and growth of most microorganisms (Bollag et al. 1970; Paul and Victoria 1978). Variation in pH range and optimum was found with different denitrifying species isolated from soil and data suggested a greater selective effect of pH upon their abundance than that of the general microbial population (Valera and Alexander 1961).

Most studies with mixed soil populations have found an increase in denitrification with an increase in pH from acid conditions, and the optimum in neutral to slightly alkaline reaction (Bollag et al. 1970; Bollag et al. 1973; Bremner and Shaw 1958b; Cady and Bartholomew 1960; Cooper and Smith 1963; Khdyer 1978). Wijler and Delwiche (1954) found denitrification rates increased with pH up to pH 6.0, after which they became constant, in a soil not limited by organic matter. The rate of nitrate loss in calcareous lake sediment was much faster than in noncalcareous sediment (Chen et al. 1972a). On the other hand, in a study of virgin and cultivated Alberta soils amended with glucose and nitrate, no correlation between pH and denitrification rate was observed (Khan and Moore 1968).