METHANE, NITROGEN MONOXIDE, AND NITROUS OXIDE FLUXES IN AN ORGANIC SOIL

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Trace gas fluxes in an organic soil

ABSTRACT

Field and laboratory studies were performed to estimate fluxes of the trace gases nitrogen monoxide (NO), nitrous oxide (N_2O), and methane (CH_4) in an organic soil, to determine the microbial processes involved, and to assess how soil water and nitrogen controlled flux rates. Metabolic inhibitors showed microbial nitrification to be the major NO source, regardless of the soil moisture content. Nitrification also produced N_2O, but denitrification losses of this gas from flooded, anaerobic soil were much higher. Up to 26% of nitrified N was converted to NO, but most of this (95%) was consumed microbially before it could escape across the soil surface. The NO-consuming process appeared to be co-oxidation by soil heterotrophs, not coupled to energy production. Organic matter content and CO_2 production were therefore good predictors of NO oxidation rates across soil types, and NO oxidation was stimulated by manure addition.

Soil water and nitrogen had nonlinear effects on trace gas fluxes, acting on both production and consumption. Kinetic analysis showed that nitrate was a weak noncompetitive inhibitor, but ammonium a strong competitive inhibitor of soil CH_4 oxidation at field fertilization rates. However, spatial and temporal factors complicated fertilization effects on CH_4 oxidation *in situ*. Ammonium was immobilized in surface soil and rapidly nitrified, limiting its inhibitory effect on CH_4 oxidation. Fertilizer N stimulated nitrification and denitrification and therefore gaseous N-oxide production, but other, unexpected fertilizer effects were also observed. Ammonium fertilizer decreased NO oxidation rates. Nitrate and other salts stimulated NO and N_2O losses during nitrification, an effect apparently related to soil nitrite accumulation.

The controls exerted on trace gas fluxes by soil water were mediated primarily through diffusion rates. Oxygen diffusion controlled the balance of anaerobic (methanogenesis and denitrification) versus aerobic (CH_4 oxidation and nitrification) processes. Soil moisture content also controlled the diffusion rate of atmospheric CH_4 to soil methanotrophs, and the escape of gaseous N-oxides from production sites across the soil surface.

RESUME

Des études en laboratoire et sur le terrain furent réalisés pour estimer le flux des gas atmosphériques en trace tel le monoxide d'azote (NO), l'oxide nitreux (N_2O), et le méthane (CH_4) dans un sol organique et pour déterminer les processus microbiens impliqués. L'impact régulateur des facteurs environnementaux tel le niveau d'eau et la concentration d'azote sur les flux de ces gas fut aussi étudié. Des inhibiteurs métaboliques ont démontré que la nitrification est la source principale de NO, et ce indépendemment du taux d'humidité dans le sol. Dans notre étude, la nitrification produit aussi du N_2O, mais la production de ce gas via le processus de dénitrification dans les sols innondés et anaérobiques est beaucoup plus importante. Jusqu'à 26% de l'azote nitrifié est converti en monoxide d'azote. Par contre, la majorité (95%) est consommée microbiologiquement avant de quitter le sol. Le processus de consommation du NO semble être une cooxydation par les hétérotrophes du sol, mais elle n'est pas relié à la production d'énergie. L'organicité du sol et la production de CO_2 se sont donc avérés de bons facteurs de prédiction du taux d'oxydation du NO dans les différents sols. De plus, l'addition de fumier stimule l'oxydation du NO.

Le niveau d'eau et la concentration d'azote dans le sol a un effet non-linéaire sur le flux des gas en trace. Une analyse de la cinétique du système démontra que le nitrate est un faible inhibiteur non-compétitif, mais que l'ammonium est un inhibiteur compétitif très actif au taux d'application sur le terrain. Cependant, des facteurs spatio-temporels compliquent les effets de la fertilization sur l'oxydation du CH_4 *in situ.* L'ammonium est rapidement immobilisé et nitrifié à la surface du sol, ayant pour effet de limiter son effet inhibiteur sur l'oxydation de méthane. L'azote des fertilisants stimule la nitrification et la dénitrification, et augmente ainsi la production d'oxides gazeux d'azotes. Par contre, d'autre effets surprenants des fertilisants furent aussi observés. L'ammonium diminue le taux d'oxydation du monoxide d'azote. Les nitrates et d'autre sels stimulent la production net de monoxide d'azote et d'oxide nitreux durant la nitrification, cet effet est apparemment relié à l'accumulation de nitrite dans le sol.

Le contrôle exercé par l'eau du sol sur le flux des gas atmosphériques en trace

est principalement effectué par le taux de diffusion. La diffusion de l'oxygène contrôle le ratio entre les processus anaérobiques (méthanisation et dénitrification) et les processus aérobiques (l'oxydation du méthane et la nitrification). Le taux d'humidité du sol contrôle également la diffusion du méthane atmosphérique jusqu'aux méthanotrophes et le relachement des oxides gazeux d'azote par le sol.

CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Section 4 presents the first exhaustive study over a wide range of soil moisture content relating CH_4 fluxes to diffusion rates, and comparing this relationship to the known exponential dependence of diffusion rates on gas-filled porosity (ϕ_g). A decreasing exponential relationship between CH_4 uptake and ϕ_g was documented. Diffusion limitation was only appreciable below a threshold of ϕ_g=0.2. This provides one plausible explanation for conflicting reports on the negative effect, or lack of an effect, of soil moisture on CH_4 uptake.

2. An organic soil acted periodically as both a source and a sink for CH_4 and N_2O (Section 4).

3. Although NH_4^+ was a strong inhibitor of CH_4 oxidation, applied urea failed to affect field CH_4 oxidation because it was immobilized in surface soil and rapidly nitrified. Because CH_4 oxidation in subsurface soil was not greatly limited by the diffusion rate of atmospheric CH_4, CH_4 oxidation occurred in deeper soil. The inhibition was reversible and did not persist after nitrification depleted NH_4^+ (Section 4).

4. Section 5 presents kinetic evidence for a competitive inhibition mechanism of NH_3 on soil CH_4 uptake, thereby extending observations on pure methanotroph cultures to a natural system.

5. In parallel to a previous report that soil methanotrophs possess a higher affinity for CH_4 than do known, cultured methanotrophs (Bender and Conrad 1992), soil methanotrophs were shown to have a higher affinity for NH_3 than known methanotrophs (Section 5).

6. $NaNO_3$ is a noncompetitive inhibitor of soil CH_4 oxidation (Section 5).

7. During the journal-review process for Section 6, NO oxidation by soil heterotrophs was reported by Baumgärtmer et al. (1996). Our own results provide independent confirmation of this result for a separate soil type, and also: (i) more conclusive evidence for a microbial source through the use of inhibitors and through

characterization of the temperature response, and (ii) characterization of the relative contributions of microbial and nonmicrobial processes to NO uptake at various temperatures.

8. In studies of soil NO flux, 5 Pa C_2H_2 can be used to inhibit nitrification. At higher levels (>1 kPa) C_2H_2 reacts with O_2 and NO (Section 6).

9. Soil organic matter content and soil heterotrophic activity are good predictors of NO uptake capacity across soil types (Section 7).

10. Manure additions to soils increase their aerobic NO uptake rate constants, and can decrease NO efflux rates (Section 7).

11. Microbial NO oxidation is a major regulator of the amount of soil-produced NO which effluxes to the atmosphere. Section 8 presents the first attempt to estimate, rather than just surface fluxes, gross NO production and NO oxidation rates in intact, aerobic soil columns. An estimated 95% of the NO produced from NH_4^+ fertilizer was consumed within the soil column.

12. Nitrate and other salts can increase NO and N_2O losses during nitrification (Section 8).

13. Up to 26% of nitrified NH_4^+ in a humisol can cycle through NO, a value much higher than any previous report on soil or nitrifier cultures (Section 8).

14. Fertilizer NH_4^+ decreases soil NO consumption rates as well as increasing NO production rates (Section 8).

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SECTION 1. ORGANIZATION OF THE THESIS

The body of this thesis consists of five published or submitted papers. These appear in their published or submitted form with the following exceptions. Soil classification has been standardized to the Canadian system (Canada Soil Survey Committee 1978). Stylistic formatting has been standardized. The referencing style is that used by the journal *Biology and Fertility of Soils* and all references are collected into a single section at the end of the thesis. Abbreviations and symbols have been standardized as described in "1.3 Abbreviations and symbols used in the text". The term "nitric oxide" used in Dunfield and Knowles (1997) has been replaced with "nitrogen monoxide", and nomenclature of nitrogen compounds follows the recommendations of Koppenol and Traynham (1996).

1.1 Provisions for manuscript-based theses

Faculty regulations require reproduction of the following five paragraphs for the information of examiners.

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.**

Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

1.2 Contributions of co-authors

All manuscripts published or submitted for publication are authored by Dunfield PF and Knowles R except Section 4. Dr. Knowles provided supervisory assistance regarding experimental design, and proofread the manuscripts.

In Section 4, Dr. Edward Topp (committee member) designed the field experiment. His laboratory, along with Christian Archambault (a summer student in our laboratory) was responsible for field flux measurements using chambers, gas chromatographic analyses of these samples, and extraction and measurement of soil nitrogen by colorimetric autoanalysis. All aspects of intact core experiments, experiments comparing flux measurement methods, and all data analyses were performed by myself.

The field manuring experiment examined in Section 7 was designed and maintained by Dr. EG Gregorich (Central Experimental Farm, Agriculture and Agri-Food Canada, Ottawa), and examined with kind permission.

1.3 Abbreviations and symbols used in the text

Abbreviations:

AMO	ammonia monooxygenase
ANOVA	analysis of variance
CD	calendar day
CV	coefficient of variation
d	day
d.w.	dry weight
FID	flame ionization detector
GC	gas chromatography
h	hour
ID	internal diameter
LOI	loss on ignition
MANOVA	multivariate analysis of variance
min	minute
MMO	methane monooxygenase
MPN	most probable number
pMMO	particulate methane monooxygenase
ppbv	parts per billion of volume
ppmv	parts per million of volume
rpm	revolutions per minute
SEM	standard error of the mean
sMMO	soluble methane monooxygenase
TSB	tryptic soy broth (Difco)
WHC	gravimetric soil water-holding capacity
WFPS	water-filled pore space (%)
w.w.	wet weight
y	year

Symbols:

D_{ab}	diffusion coefficient of gas a in medium b
J	soil surface flux, emission when +, uptake when -
$K_{i(app)}$	apparent Michaelis-Menton inhibition constant (Segel 1975)
$K_{m(app)}$	apparent Michaelis-Menton half-saturation constant (Segel 1975)
k_{NO}	first-order NO uptake rate constant
K_s^*	Hill half-saturation constant (Segel 1975)
n_{app}	Hill coefficient (Segel 1975)
P	statistical probability
Q_{10}	10°C temperature coefficient, relative rate increase per 10°C temperature increase
V_{max}	maximum enzyme velocity (Segel 1975)
z	soil depth
ϕ_g	fractional gas-filled soil porosity

SECTION 2: GENERAL INTRODUCTION AND OBJECTIVES

The trace gases nitrogen monoxide (NO), nitrous oxide (N_2O), and methane (CH_4) are produced and consumed through a suite of microbial processes in soil. Methane and N_2O are greenhouse gases, while gaseous N-oxides are involved in fertilizer loss, acid precipitation, and both tropospheric and stratospheric ozone chemistry.

NO and N_2O are produced primarily through nitrification and denitrification in soils, although other microbial nitrogen transformations contribute in some cases (Conrad 1996a). Denitrification usually dominates N_2O production from saturated soils, while nitrification is the predominant source in dry, aerobic soils. The same pattern does not seem to hold for NO. Nitrification may be the principle source of NO emissions to the atmosphere regardless of soil moisture content, but this is not well studied (section 3.3.3). Nitrogen fertilization of agricultural systems increases the absolute gaseous N-oxide production rates, but may also control end-product ratios (Firestone and Davidson 1989). Although gaseous N-oxide fluxes across a soil surface are the sum of both productive and consumptive processes occurring within the soil column, only the productive processes are well studied. N_2O is probably consumed only through denitrification, but several potential reductive and oxidative NO consumption mechanisms exist (Conrad 1996a).

CH_4 is produced in flooded anaerobic systems such as swamps and rice paddies, but also in periodically wet forest and agricultural soils. Drier, aerobic soils exhibit net consumption of atmospheric CH_4 (Conrad 1995). Studies suggest that CH_4 oxidation is constrained mostly by soil water content (through diffusion limitation and water stress) and by non-optimal environmental conditions (inhibitory substances and antagonistic ecological interactions). Methanotrophic activity is often confined to subsurface soil, disadvantageously distant from the CH_4 source, suggesting that conditions in surface soil are inhibitory. Nitrogen fertilizers have been shown to decrease CH_4 oxidation, and the conversion of soils to agriculture has been linked to declining CH_4 uptake capacity (King 1992; Hanson and Hanson 1996).

Nitrogen and carbon mineralization in organic soils fuels rapid microbial

metabolism (Guthrie and Duxbury 1978; Tate 1982). The high water-holding capacities of organic soils ensure that microbial activity is seldom water-limited, and that both aerobic and anaerobic processes can occur. They therefore support high potential nitrification and denitrification rates (Guthrie and Duxbury 1978). Organic soils are known to be important point sources of N_2O, with average efflux rates one to two orders of magnitude greater than mineral soils (Terry et al. 1981; Duxbury et al. 1982; Goodroad and Keeney 1984b). It is unknown whether organic soils display similarly large NO emissions, but this is probable given that N_2O and NO are produced by the same microbial processes. Because of their high water-holding capacities and carbon availabilities, organic soils may also support high localized rates of CH_4 production and consumption.

Assessing the magnitude and the environmental controls of trace gas fluxes from organic soils is therefore of much interest. This thesis describes some such studies undertaken on a fallow humisol located on the Central Experimental Farm of Agriculture and Agri-Food Canada in Ottawa. In an unpublished soil survey by the Soils Department of the Central Agricultural Farm, the site was classified as an organic muck. According to the Canada Soil Survey Committee (1978) this classification requires a surface organic layer to 40-cm depth. Although the organic topsoil at our site was in places underlain by clay or sand at only 35-cm, we preferred to accept the "organic" classification on functional grounds that all trace gas fluxes across the soil surface resulted from processes occurring in this surface layer.

2.1 OBJECTIVES

Field and laboratory studies were designed to:

1. Estimate trace gas fluxes from a humisol under various moisture and fertilization regimes.

2. Assess how water content and gas diffusion affect CH_4 fluxes. O_2 diffusion potentially determines whether the soil is a net CH_4 source or sink, and the magnitude

of atmospheric CH_4 uptake could also be diffusion-limited. As this soil had no vertical horizon structure, it presented an ideal system in which to study gas diffusion with a simple one-dimensional model.

3. Assess the effects of various forms of nitrogen fertilizer on trace gas cycling. This objective was fulfilled with laboratory examinations into the underlying mechanisms of nitrogen effects, and with flux measurements in the field and in laboratory microcosms to assess how the determined mechanisms were modified in natural, intact systems.

4. Determine the microbial processes involved in gaseous N-oxide cycling. Attempts were made to partition nitrification and denitrification as gaseous N-oxide sources, and to determine whether consumption processes attenuated net surface emissions of the gaseous N-oxides produced in soil. NO consumption processes were characterized as microbial or chemical, oxidative or reductive, and the degree to which the processes were particular to organic soils examined.

SECTION 3. LITERATURE REVIEW: PROCESSES AND ENVIRONMENTAL CONTROLS OF CH_4, NO, AND N_2O CYCLING IN NON-WETLAND SOILS

3.1 Introduction

Methane (CH_4), nitrous oxide (N_2O), and nitrogen monoxide (NO) are produced and consumed by a suite of microbial processes in soil, primarily nitrification, denitrification, methanogenesis, and methane oxidation. These processes are often studied together because their roles in trace gas metabolism overlap, and because they respond to the same key environmental controls. Nitrifiers, for example, produce gaseous N-oxides and are also able to oxidize CH_4 (Bédard and Knowles 1989). Methanotrophs can consume NO as well as CH_4 (Krämer et al. 1990). Agricultural fertilizer use influences all of these processes, providing substrate for nitrification and denitrification, while inhibiting methanogenesis and methanotrophy. This review will examine the processes involved in CH_4, NO, and N_2O cycling in soils and the principal environmental controls of these processes. Where possible, reference will be made primarily to temperate agricultural systems.

Soil fluxes contribute appreciably to global trace gas budgets. For nitrous oxide, soils appear to be the principal source, with only limited input from fuel combustion (Davidson 1991). Present estimates place the contribution of fertilized agricultural soils at only about 30% of the total N_2O produced by natural systems, due to high production rates in natural tropical ecosystems. However, these estimates are a matter of great uncertainty (Davidson 1991; Aulakh et al. 1992). There is a large (50%) discrepancy between the total estimated N_2O production and the total estimated soil emissions, suggesting either that unidentified sources exist or that emission rates from various ecosystems are poorly quantified (Conrad 1995).

Anthropogenic sources, primarily livestock farming and rice agriculture, produce about 70% of global CH_4 emissions, with the remainder coming from natural methanogenic ecosystems (Duxbury and Mosier 1993). Methanotrophs in dry, aerobic soils act as a sink for CH_4. The magnitude of this sink is up to 15% of the main CH_4 sink, reaction with atmospheric hydroxyl radicals (OH) (Duxbury and

Mosier 1993; Ojima et al. 1993). Evidence suggests that human land-use changes have diminished soil CH_4 oxidation by as much as 30% in the temperate zone (Ojima et al. 1993).

Anthropogenic combustion is the largest single source of tropospheric nitrogen monoxide + nitrogen dioxide (NO_2) ($NO + NO_2 = NO_x$). Soil NO_x emissions contribute an estimated 15-40% of the total global production (Williams, Hutchinson et al. 1992). However, NO sources must be characterized on a regional basis to understand tropospheric oxidant chemistry and gaseous nitrogen movement over non-urban areas. Models must account for the fact that an NO molecule in rural air with low NO_x mixing ratio is a more efficient producer of ozone (O_3) than an NO molecule in an industrial plume (Sillman et al. 1990). Because of the short tropospheric lifetime of NO, the soil source strength can exceed that of industrial sources in natural and agricultural areas, especially in summer when temperatures favour biological activity (Williams, Hutchinson et al. 1992; Williams, Guenther et al. 1992; Stohl et al. 1996).

It has been suggested that the springtime O_3 maximum observed in rural areas results from NO production in fertilized fields (Stohl et al. 1996). Fertilized agricultural systems account presently for about 40% of all NO produced by soils, a figure which should rise to over 50% by 2025 based on predicted land-use changes (Yienger and Levy 1995). The percentage is already much greater in intense agricultural zones such as the US and Europe, where fertilized soils account for up to 70-80% of soil NO emissions (Williams, Guenther et al. 1992; Stohl et al. 1996). However, all soil NO emission estimates are based on limited experimental data and on predictive models constructed with few controlling variables, generally temperature, moisture, and land use. Better understanding of the biotic factors affecting NO flux should lead to better mechanistic models (Williams, Hutchinson et al. 1992; Conrad 1996a).

The following review examines the controls of soil trace gas fluxes. Since they pertain to the major experimental work of the thesis, the sections on CH_4 oxidation and NO cycling cite the primary research papers. Because of the sheer

magnitude of the literature in peripheral areas such as the biochemistry of microbial metabolism, these areas are outlined primarily with reference to reviews. Several exhaustive recent reviews deal with soil N_2O flux (Sahrawat and Keeney 1986; Eichner 1990; Aulakh et al. 1992; Williams, Hutchinson et al. 1992; Bouwman et al. 1993; Conrad 1995; Conrad 1996b), nitrification (Focht and Verstraete 1977; Prosser 1989), denitrification (Focht and Verstraete 1977; Knowles 1982; Tiedje 1988; Kuenen and Robertson 1994; Ye et al. 1994), NO chemistry and biochemistry (Williams, Hutchinson et al. 1992; Fontecave and Pierre 1994; Conrad 1996a; Conrad 1996b; Wink et al. 1996), methanogenesis (Jones et al. 1987; Oremland 1988; Conrad 1989; Garcia 1990; Boone 1991; Jones 1991; Knowles 1993; Conrad 1995), and methanotrophy (Topp and Hanson 1991; King 1992; Knowles 1993; Reeburgh et al. 1993; Conrad 1995; Mancinelli 1995; Conrad 1996b; Hanson and Hanson 1996).

3.2 Methane

3.2.1 Atmospheric chemistry

The CH_4 mixing ratio in the atmosphere is increasing at 0.5-1% per year (Duxbury and Mosier 1993; Hanson and Hanson 1996), a trend attributable either to increasing production rates or decreasing oxidation rates. The rate of CH_4 accumulation has slowed in recent years (Khalil and Rasmussen 1994). The present mixing ratio of about 1.72 parts per million of volume (ppmv) is approximately 200 times lower than that of carbon dioxide (CO_2). However, because it is more effective at absorbing IR radiation and has a longer lifetime, CH_4 is estimated to contribute 15-25% to the atmospheric greenhouse effect (Lelieveld et al. 1993).

Hydroxyl radicals are the main oxidants of CH_4. Microbial oxidation in soils presently accounts for $<15\%$ of the total CH_4 sink (Born et al. 1990; Duxbury and Mosier 1993), but this ratio may increase. Although the atmospheric OH trend is a matter of question (Khalil and Rasmussen 1994), depletion of atmospheric OH may occur as CH_4 mixing ratios continue rising. Such a depletion would lead to an increased tropospheric lifetime for CH_4 and provide positive feedback on global warming (Lelieveld et al. 1993). However, because soil CH_4 oxidation is

concentration-dependent, the magnitude of this sink will rise and provide negative feedback.

3.2.2 Methanogenesis

Methanogens are obligate anaerobes which couple CH_4 production from CO_2 and organic substrates to ATP generation (Jones et al. 1987). The group consists of over 65 putative species, comprising three orders of the urkingdom Archaebacteria. They are present in such anaerobic environments as hydrothermal vents, animal guts, soils, wetland sediments, rumen, and tree heartwood (Garcia 1990). The taxonomy (Garcia 1990), biochemistry (Jones et al. 1987; Rouvière and Wolfe 1988; Jones 1991), bioenergetics (Daniels et al. 1984), and ecology (Oremland 1988; Conrad 1989; Boone 1991) of methanogens have all been the subject of recent reviews.

Methanogenic energy substrates include, in decreasing order of free energy yield per mol of CH_4: carbon monoxide (CO), dihydrogen (H_2) + CO_2, formate (HCOOH), methanol (CH_3OH), methylamines, dimethylsulfate, and acetate. Propanol, butanol, and other alcohols are also thought to be utilized by some species (Garcia 1990). Carbon dioxide reduction to CH_4 by H_2 is the most widely distributed pathway among known species. Binding of CO_2 to methanofuran is the initial step of the methanogens' novel metabolic pathway, in which 1-carbon intermediates derived from CO_2 are passed through a series of carriers while being progressively reduced to CH_4. Alternate substrates enter into the cycle at these intermediate steps. Acetate enters at the final intermediate: Coenzyme M. The acetate is split, with the methyl group binding to Coenzyme-M while the carboxyl group provides electrons for its subsequent reduction. This final step, in which methyl-Coenzyme M is cleaved, is the CH_4-producing step (Jones et al. 1987).

The energy yield from methanogenesis is low. The Gibbs standard free energy yield is -135.6 kJ mol^{-1} CH_4 for the H_2/CO_2 reaction, and only -31 kJ mol^{-1} CH_4 for acetate reduction, about enough to form one ATP molecule. A proton-motive force is involved in ion transport in methanogens, and is probably also coupled to energy generation through a membrane-bound sodium/proton antiporter (Jones et al. 1987;

Rouvière and Wolfe 1988).

Acetate, H_2, and perhaps formate are the major environmental methanogenic substrates. Although the fermentation (acetate-using) pathway is the least energetically favourable of all methanogenic pathways, acetate production during soil organic matter decomposition makes it ecologically important. Radiolabel studies show acetate to be the origin of about 70% of the methane produced in freshwater environments (Whiticar et al. 1986; Conrad 1989), including lake sediments (Conrad, Goodwin et al. 1987) and rice paddies (Thebrath et al. 1992). The remainder comes primarily from H_2/CO_2.

Schoell (1988) notes three substrate-use patterns. Fermentation is the dominant CH_4 production pathway in freshwater, warm sediments with regular organic carbon input. CO_2 reduction dominates in cold, saltwater sediments dominated by "old" organic carbon. These three factors generally covary, but not always. Hence buried, old peats produce CH_4 from H_2/CO_2 even though the environment is freshwater (Coleman et al. 1988). Acetate fermentation seems to require constant fresh organic matter input. Paddy soil incubated anaerobically for long time periods gradually loses its fermentation methanogenesis, but CO_2 reduction remains constant (Conrad et al. 1989). Very little is known about the degradation of "old" organic carbon (Conrad 1989).

3.2.3 Environmental controls of methanogenesis

Methanogens are obligate anaerobes, and are killed by exposure to O_2. Many of their enzymes are irreversibly denatured by O_2. However, the absolute sensitivity, and time to death, varies among species (Daniels et al. 1984). In their natural habitats, methanogens can survive prolonged periods of soil drying and aeration (Mayer and Conrad 1990). Methanogenesis can be induced in agricultural and forest soils that are rarely or never flooded (Mayer and Conrad 1990), suggesting that the organisms survive in anaerobic microsites or through protective associations.

Methanogens must compete with other organisms for H_2, acetate, and formate. In ecosystems where oxidizing agents other than CO_2 are present, methanogens are

nearly always outcompeted (Oremland 1988). In natural systems, oxidizing agents tend to be used up preferentially in order of decreasing redox potential. Aerobic respiration dominates until O_2 is exhausted, then NO_3^- reduction, SO_4^- reduction, and finally fermentation and CO_2 reduction to CH_4 (Oremland 1988). Bacteria using higher redox potential reactions gain more energy from the same substrate. They can therefore maintain H_2 (and other substrates) at concentrations below the minimum utilization thresholds for bacteria using less favourable redox pathways (Cord-Ruwisch et al. 1988). Such a mechanism allows complete competitive exclusion. The concentration of dissolved H_2 in sediments is in fact an indicator of the predominant redox pathway (Lovley and Goodwin 1988).

Because of the inhibition of methanogenesis by O_2 and other oxidants, CH_4 production has only rarely been measured in non-wetland soils. Net production is occasionally observed in forest soils, especially in the organic layers where O_2 consumption and carbon mineralization rates are high enough to create methanogenic conditions (Yavitt et al. 1990; Adamsen and King 1993; Castro et al. 1993; Amaral and Knowles 1997a). Periodically flooded forest and agricultural soils also produce CH_4 (Bronson and Mosier 1993; Dunfield et al. 1995; Yavitt et al. 1995; Macdonald et al. 1996). The scarcity of these reports may reflect the fact that few field studies include early-springtime measurements in temperate climates when soils can be flooded for extended periods. Yavitt et al. (1995) observed methanogenic potential throughout a hardwood forest spodosol, and soil CH_4 concentrations 200 times air saturation during spring and fall. Macdonald et al. (1996) even noted net CH_4 emission from a fertilized agricultural soil after a severe summer rainfall event. This is surprising in view of the inhibitory effect of NO_3^- on methanogenesis, and microsites depleted in NO_3^- may have been involved. Although detailed investigations of CH_4 production in these systems are lacking, it is likely that methanogenesis in these cases is regulated by the same factors described for wetlands.

Most methanogens are mesophilic, with temperature optima of around 35°C. Some thermophilic but no psychrophilic species have been isolated (Oremland 1988). Methanogenic systems such as rice paddies exhibit seasonal and diel fluctuations in

CH_4 emissions (Schütz et al. 1990). The effects of temperature on ecosystems are complex, however. Activation energies for methane production vary in time and space within ecosystems, because temperature effects are not confined to methanogens. In a paddy soil, for example, decreased temperature resulted in decreased H_2 production, and therefore also less H_2-dependent methanogenesis (Conrad, Schütz et al. 1987).

Most methanogens are pH neutrophilic (Conrad 1989; Garcia 1990). One slightly acidophilic species has been isolated (Patel et al. 1990), and two species have pH optima of 5 when grown on methanol + H_2 (Boone 1991). Enrichment cultures from acid peat showed methanogenic activity at pH 3.1 and growth at pH 5.3, but no isolates were obtained (Williams and Crawford 1985). CH_4 production has been reported for acid peats (Williams and Crawford 1984; Williams and Crawford 1985; Moore and Knowles 1990) and tropical wetlands (Tathy et al. 1992) with pH values down to 3.7, suggesting that as yet unknown acid-tolerant species exist. However, methanogens in acid environments generally operate under non-optimal conditions (Goodwin et al. 1988; Dunfield et al. 1993).

Methanogens are substrate-limited in many natural environments. Organic matter additions to rice paddies, for example, usually increase CH_4 emissions (Bouwman 1991). Many soils and wetlands receive only periodic inputs of organic matter, and the hydrolysis of large carbon-polymers is often the rate-limiting step in decomposition (Oremland 1988).

3.2.4 Methanotrophy

Methanotrophs catalyse the oxidation of CH_4 to CO_2. These organisms are classified based on their carbon assimilation pathway and morphology into three groups: type I, type II, and type X (Hanson and Hanson 1996). Methane is the most reduced form of carbon, and its activation energy is so high that the first step in its oxidation is an endothermic hydroxylation. This reaction is catalysed by a monooxygenase requiring O_2 and a reductant for the excess O atom:

$$CH_4 + O_2 + 2H^+ + 2e^- \rightarrow CH_3OH + H_2O \qquad (3.1)$$

Two forms of methane monooxygenase (MMO) are known to exist, a particulate form (pMMO) for which reduced cytochrome-*c* is the reductant, and a soluble form (sMMO), which uses NADH + H^+. The pMMO is common to all methanotrophs, but its synthesis requires high (1 μmol g $cells^{-1}$) concentrations of copper. The sMMO occurs in several type II and type X, and a single type I strain (Hanson and Hanson 1996). Of the two forms, sMMO has a broader substrate specificity and a slightly lower affinity for CH_4.

Beyond the initial reaction catalysed by MMO, methanotrophic metabolism is identical to methylotrophy. The product of MMO, methanol (CH_3OH), feeds into the electron transport chain at the level of methanol dehydrogenase (Anthony 1982). Methanol oxidation to formaldehyde (HCOH) contributes to the proton-motive force by releasing $2H^+$ in the periplasm, while O_2 reduction consumes $2H^+$ in the cytoplasm (Anthony 1990). Formaldehyde is either assimilated into cell material by the ribulose monophosphate or serine pathways, or is further oxidized to HCOOH and then to CO_2. Formate and formaldehyde oxidation are directly coupled to NAD^+ reduction, which is primarily cycled back into supporting MMO function (Anthony 1982).

There is also an anaerobic CH_4 oxidation pathway utilizing SO_4^- as the oxidant, but because of its reduced redox potential this reaction is much less important than the aerobic pathway in terrestrial systems (Mancinelli 1995).

3.2.5 Methane consumption by ammonia oxidizers

Ammonia (NH_3) oxidation is catalysed by the enzyme ammonia monooxygenase (AMO), in a process directly analogous to methane oxidation (Prosser 1989):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \qquad (3.2)$$

Ammonia (NH_3) rather than ammonium (NH_4^+) is the substrate for AMO, but at pH values typically found in soil, almost all of the NH_4^+/NH_3 acid-base pair is in the NH_4^+ form, so the process is often referred to as ammonium oxidation. Like pMMO, AMO is membrane-bound and associated with intracytoplasmic membrane stacks. Because of the similarity of both the enzymes and the substrates, CH_4 is

oxidized by AMO (Jones and Morita 1983), and NH_3 by MMO (next section).

Environmental CH_4 oxidation is therefore potentially mediated by ammonia oxidizers rather than true methanotrophs. Although reported $K_{i(app)}$ (CH_4) values in nitrifiers are generally much higher than the $K_{m(app)}$ (CH_4) values in methanotrophs, some are comparable (Bédard and Knowles 1989). The lowest CH_4 affinity reported for nitrifiers is 6.6 μM in *Nitrosococcus oceanus* (Ward 1987), but this estimate is probably invalid since the inhibition pattern was not simple competition (Ward 1987; Ward 1990). Interpretation is therefore difficult. Studies with the marine nitrifier *Nitrosococcus oceanus* show that some of the methanol formed from CH_4 oxidation is incorporated into cell biomass (Ward 1987), although growth on CH_4 does not seem possible (Bédard and Knowles 1989).

Several lines of evidence suggest that nitrifiers in aerobic soils are not involved in CH_4 oxidation. Increasing the ambient CH_4 mixing ratio by 2-5 orders of magnitude generally does not affect soil nitrification rates (Megraw and Knowles 1987a; McCarty and Bremner 1991; Dunfield and Knowles 1995), although such increases do stimulate CH_4 oxidation rates. The maximum specific CH_4 oxidation rate documented in nitrifier cultures is 5 times lower than the minimum rate documented for methanotrophs (Bédard and Knowles 1989). Bender and Conrad (1994a) preincubated four upland soils and a rice paddy soil with NH_4^+ to stimulate the nitrifying populations, but observed unchanged CH_4 consumption rates after the NH_4^+ was depleted. Although NH_4^+ inhibited CH_4 oxidation and CH_4 inhibited nitrification, the nitrifying population did not detectably contribute to CH_4 oxidation in these soils. Maximum potential nitrification activity occasionally occurs in the same subsurface soil horizons as maximal methane oxidation (Amaral and Knowles 1997a), but this may simply represent similar environmental preferences and inhibitor sensitivities (see section 3.2.9.5), and in other soils the zones of maximum nitrification and maximum methane oxidation are spatially separate. Soil nitrification rates and NH_4^+ and NO_3^- pools are often highest in surface organic soil, while the maximum CH_4 oxidation activity occurs lower (Castro et al. 1994; Schnell and King 1994; Kruse and Iversen 1995). Judging from all this evidence, a functional overlap of methanotrophs and

nitrifiers in CH_4 metabolism is not noteworthy in aerobic soils.

3.2.6 NH_3 as a substrate for methane oxidizers

Methane monooxygenase can oxidize a variety of substrates besides CH_4, and these should therefore compete with CH_4 for the active site of the enzyme (Hanson and Hanson 1996). One such cosubstrate is NH_3, which is oxidized to nitrite (NO_2^-) via hydroxylamine (NH_2OH) (Dalton 1977). Pure culture studies with *Methylomonas methanica* (Ferenci et al. 1975), *Methylosinus trichosporium* (O'Neill and Wilkinson 1977), and both *Methylococcus capsulatus* cells (Carlsen et al. 1991) and cell-free extracts (Dalton 1977) have shown that NH_3 acts as a competitive inhibitor of CH_4 oxidation. Two of these studies (O'Neill and Wilkinson 1977; Carlsen et al. 1991) noted a significant pH effect on the $K_{i(app)}$ measured as NH_4^+ concentration. The $K_{i(app)}$ was more constant for NH_3, suggesting that this is the inhibitory species for MMO, just as it is the substrate for AMO. Both sMMO and pMMO oxidize NH_3 (Hanson and Hanson 1996). The sensitivity to NH_3 varies among different methanotroph species (Whittenbury et al. 1970), but the affinities for CH_4 and NH_3 are generally on the same order of magnitude (Ferenci et al. 1975; O'Neill and Wilkinson 1977; Carlsen et al. 1991).

Inhibition patterns more complex than simple enzymatic competition between CH_4 and NH_3 are occasionally evident in methanotrophs. For example, *Methylococcus capsulatus* cells grown under copper excess (10 μM) conditions showed a competitive pattern, but cells grown without Cu did not, suggesting a more complex inhibition mechanism for sMMO (Carlsen et al. 1991). The inhibition in low-Cu cells was dependent on the O_2 concentration. Dalton (1977), using cell-free extracts from *Methylococcus capsulatus* (grown with low Cu), observed agreement with a competitive model at high substrate concentrations, but slight negative cooperativity at low concentrations. Although inhibition patterns are not always strictly competitive, MMO is almost certainly the catalyst for NH_3 oxidation, as indicated by O_2 uptake stoichiometry and by similar responses of CH_4 and NH_3

metabolism to various inhibitors (Topp and Hanson 1991).

The requirement of MMO for the cosubstrates O_2 and a reductant can complicate the competitive pattern of NH_3 inhibition (Bédard and Knowles 1989). Several reports have noted that while large CH_4 additions inhibit NH_3 oxidation by methanotrophs, smaller CH_4 additions actually stimulate it (O'Neill and Wilkinson 1977; Knowles and Topp 1988; Megraw and Knowles 1989; King and Schnell 1994b; Schnell and King 1994). The former effect is presumably competitive, the latter stemming from energetic limitation at low CH_4 concentrations, and insufficient reducing power for optimal MMO function. Hydroxylamine (Hubley et al. 1975) and NO_2^- (O'Neill and Wilkinson 1977; Jollie and Lipscomb 1991; King and Schnell 1994b) produced during NH_3 oxidation are themselves inhibitors of methanotrophic activity, and further complicate the CH_4 and NH_3 responses.

The common deviation from simple competitive interactions at low substrate ($< K_{m(app)}$) concentrations in both methanotrophs and nitrifiers is not trivial, since environmental concentrations of these molecules are typically low. Effects of inhibitors in the field are therefore difficult to predict.

Whether CH_4 oxidizers act as nitrifiers in natural environments is uncertain. The maximum specific NO_2^- production rate determined in methanotroph cultures is still 20 times lower than the minimum rate by nitrifiers, and methanotrophs possess a lower affinity for NH_3 (Bédard and Knowles 1989). Therefore, methanotroph nitrification should only be ecologically important where conditions favour large methanotroph populations. Production of NO_2^- in the layer of maximum methanotrophic activity of Lake Mendota was interpreted as a potential indication of methanotroph nitrification (Harrits and Hanson 1980). Methane-dependent nitrification was observed in a polluted sediment (Roy and Knowles 1994) and in a humisol after enrichment of the resident methanotrophs (Megraw and Knowles 1989). Preincubation with CH_4 did not increase nitrification in several other soils, although the methanotrophic population was significantly stimulated (Bender and Conrad 1994a).

3.2.7 Soil methanotrophic activity

Methane oxidizers exist in most methanogenic habitats with an aerobic interface, and attenuate net CH_4 emissions from these environments (King 1990, Conrad and Rothfuss 1991; Topp and Hanson 1991; King 1992; Reeburgh et al. 1993). Atmospheric CH_4 oxidation (i.e. net uptake at 1.7 ppmv CH_4) has also been reported for tropical forests (Keller et al. 1983; Castro et al. 1994), savannah (Seiler et al. 1984), temperate forests (Keller et al. 1983; Steudler et al. 1989; Born et al. 1990; Yavitt et al. 1990; Crill 1991; Whalen et al. 1992; Adamsen and King 1993; Schnell and King 1994; Yavitt et al. 1995; Kruse et al. 1996; Amaral and Knowles 1997a), grasslands and meadows (Mosier et al. 1991; Neff et al. 1994), landfill cover soils (Whalen et al. 1990), desert (Striegl et al. 1992), tundra (Whalen and Reeburgh 1990), heathland (Kruse and Iversen 1995), agricultural soils (Mosier et al. 1991; Bronson and Mosier 1993; Crill et al. 1993; Hansen et al. 1993; Hütsch et al. 1993; Topp 1993; Dunfield et al. 1995), and from wetlands during periods when the water table is low (Harriss et al. 1982). Experiments with autoclaved soil and $^{14}CH_4$ show the activity to be biologically mediated (Whalen et al. 1990; Yavitt et al. 1990; Bender and Conrad 1992; Whalen et al. 1992; Schnell and King 1995; Roslev et al. 1997).

Whether soil methanotrophs require occasional exposure to super-atmospheric CH_4 concentrations and proximity to methanogenic sites for growth is unknown. Soil CH_4 uptake rates can be stimulated by incubation under >1000 ppmv CH_4 (Bender and Conrad 1992; Nesbit and Breitenbeck 1992; Bender and Conrad 1995; Schnell and King 1995), but no study has yet demonstrated increasing activity through incubation with <200 ppmv CH_4 (King and Schnell 1994a; Bender and Conrad 1995; Schnell and King 1995). In forest soils $^{14}CH_4$ is incorporated into cell material even at atmospheric mixing ratios (Schnell and King 1995; Roslev et al. 1997), but this may not support population growth. Methanotrophic activity in a forest soil could be maintained, but not stimulated, for several months on 1.7 ppmv CH_4, but the potential activity declined when completely deprived of CH_4 (Schnell and King 1995).

In environments such as landfills (Whalen et al. 1990), swamps (Harriss et al.

1982), and peats (Macdonald et al. 1996), methanotrophs are certainly stimulated by proximity to a CH_4 source, and continue to consume atmospheric CH_4 under transiently dry conditions when the source retreats into deeper soil. The highest reported potential CH_4 oxidation rates are associated with methanogenic environments (Whalen et al. 1990). Infrequent and transient CH_4 production in normally aerobic soils (outlined above) may similarly support the growth of methanotrophs. Nesbit and Breitenbeck (1992) studied a variety of Louisiana soils and found that those from periodically flooded sites had the highest CH_4 oxidation potentials. However, the kinetic properties of CH_4 oxidation differ between elevated-CH_4 and atmospheric-CH_4 environments (see next section), suggesting that different organisms predominate in the two ecosystem types.

Alternatively, soil methanotrophic bacteria may survive through mixotrophic growth (Patel et al. 1978), although most pure culture evidence is to the contrary (Hanson and Hanson 1996). Methanotrophic bacteria isolated from a landfill soil grew best on complex media (Jones and Nedwell 1993), and the enhanced survival ability of methanotrophic bacteria under anoxic compared to oxic conditions could result from an anaerobic metabolism (Roslev and King 1994). Mixotrophic CH_4-consuming yeasts exist (Wolf and Hanson 1979), and may be ecologically important. Addition of eukaryotic inhibitors to soils occasionally (Schnell and King 1995) but not always (Bender and Conrad 1994b) decreases atmospheric CH_4 oxidation. However, no effect of adding potential mixotrophic substrates was observed on CH_4 oxidation in a forest soil (Schnell and King 1995).

Typically, aerobic soils can consume CH_4 down to thresholds <0.5 ppmv. (Born et al. 1990; Whalen and Reeburgh 1990; Whalen et al. 1990; Yavitt et al. 1990; Bender and Conrad 1992; Adamsen and King 1993; Koschorreck and Conrad 1993; Dunfield et al. 1995; Kruse et al. 1996). Sediments usually display threshold values >2 ppmv (reviewed in Mancinelli 1995), and in one spruce forest soil methanotrophs were unable to consume CH_4 below 10 ppmv (Yavitt et al. 1990). The threshold is not a true enzyme kinetic parameter (since there is always a finite chance of enzyme and substrate meeting) but the result of physiological or ecological factors.

In methanotrophs the non-zero threshold could result from reductant limitation. Thus, cultured methanotrophs can consume atmospheric methane, but only temporarily since this level of CH_4 this does not supply sufficient maintenance energy (Roslev and King 1994; Schnell and King 1995). In nature, the threshold could also represent a balance between CH_4 production and consumption.

3.2.8 Substrate affinity

Methane half-saturation constants in methanotroph cultures range from 1-70 μM (Ferenci et al. 1975; O'Neill and Wilkinson 1977; Carlsen et al. 1991, and references; Conrad 1984). Samples from methanogenic environments such as sediments and landfill cover soils display $K_{m(app)}$s in the same range (reviewed in Whalen et al. 1990; Topp and Hanson 1991), as do nonmethanogenic soils preincubated under a 20% CH_4 atmosphere (Bender and Conrad 1992).

The $K_{m(app)}$ for CH_4 oxidation in forest and agricultural soils is several orders of magnitude lower, at 30-90 nM (Bender and Conrad 1992; Dunfield and Knowles 1995). In ocean water with CH_4 concentrations < 1 μM, $K_{m(app)}$ values of 70-500 nM were measured (Ward and Kilpatrick 1990). Thus, methanotrophs in aerobic soils and other low-CH_4 habitats seem to be adapted to reduced CH_4 levels, although a "normal" methanotrophic affinity can be induced by CH_4 enrichment (Bender and Conrad 1992). Even in the super-micromolar range, the CH_4 affinity of soil methanotrophs adjusts itself to the concentration of available CH_4 (Kightley et al. 1995).

The substrate affinity of pMMO is somewhat higher than that of sMMO, but both are still > 1 μM, so neither approaches the $K_{m(app)}$ measured in aerobic soils (Bédard and Knowles 1989; Bender and Conrad 1992). CH_4 oxidizers in aerobic soils may therefore employ a CH_4-oxidizing system unlike that of known methanotrophs. Either uncharacterized species are involved, or an as yet unknown enzyme is induced by the known "classical" methanotrophs living in aerobic soils. Methanotroph counts based on MPNs using > 1000 ppmv CH_4 do not correspond well to oxidation rates of

<2 ppmv CH_4 in soils, indicating that the counted organisms are not those responsible for field CH_4 oxidation (Bender and Conrad 1995). While soil CH_4 oxidation activity can be maintained for months at 1.7 ppmv CH_4 (Schnell and King 1995), calculations based on measured kinetic constants indicate that "classical" methanotrophs are incapable of such extended survival (Conrad 1984). Direct study confirms this (Roslev and King 1994; Schnell and King 1995).

This substrate affinity paradox is directly analogous to nitrifiers, where the $K_{m(app)}$ (NH_3) values measured in pure cultures are up to 3 orders of magnitude greater than those measured in crude environmental samples (reviewed in Prosser 1989). Immunofluorescence study suggests that the organisms in natural marine samples are closely related to culturable species (Ward 1986), and that high-affinity enzymes are therefore induced in natural systems.

3.2.9 Environmental controls of soil CH_4 consumption

3.2.9.1 Cultivation and nitrogen fertilization. Conversion of natural ecosystems to agricultural use has been linked to declining CH_4-oxidizing activity. Comparison of adjacent cultivated and pristine ecosystems reveals CH_4 oxidation rates around an order of magnitude less in the cultivated sites (Keller et al. 1990; Mosier et al. 1991; Bronson and Mosier 1993; Hütsch et al. 1994; Lessard et al. 1994). The activity loss is greatest in the surface soil, suggesting that the effect is either mediated through surface-applied agrochemicals, or is related to a less stable surface microclimate in agricultural than in forest systems (Keller et al. 1990). Although declining CH_4 oxidation rates have been noted in unfertilized cultivated soils (Mosier et al. 1991), and although a variety of agrochemicals inhibit soil CH_4 oxidation (Topp 1993; Syamsul Arif et al. 1996), nitrogen fertilization is usually postulated as the cause of methanotrophic decline in agricultural soils.

NH_4^+ additions inhibit CH_4 oxidation in laboratory studies of soils and sediments (Conrad and Rothfuss 1991; Nesbit and Breitenbeck 1992; Adamsen and King 1993; Bosse et al. 1993; Bronson and Mosier 1994; King and Schnell 1994a; Schnell and King 1994; Dunfield and Knowles 1995). Nitrate (NO_3^-) is also

inhibitory of CH_4 oxidation, occasionally at similar concentrations as NH_4^+ (Adamsen and King 1993; Kightley et al. 1995), and occasionally only at much higher concentrations (Nesbit and Breitenbeck 1992; Adamsen and King 1993; Bronson and Mosier 1994; Dunfield and Knowles 1995). Field studies have also shown that nitrogen fertilizers, especially in the form of NH_4^+ or urea, reduce the influx rates of atmospheric CH_4 into soils (Steudler et al. 1989; Mosier et al. 1991; Bronson and Mosier 1993; Hansen et al. 1993; Hütsch et al. 1993; Castro et al. 1994; Crill et al. 1994; Hütsch et al. 1994; King and Schnell 1994a; Neff et al. 1994; Schnell and King 1994; Sitaula et al. 1995; Macdonald et al. 1996), and decrease net CH_4 oxidation in sediments (King 1990; Conrad and Rothfuss 1991; Bosse et al. 1993). Fertilization with KNO_3 also inhibited CH_4 oxidation in a drained peat soil (Crill et al. 1994), although the causative factor may still have been NH_4^+, displaced from exchange sites by K^+, or allowed to accumulate through mineralization after KNO_3 alleviated biotic nitrogen limitation.

In some cases the fertilizer effect is evident only after long-term, repeated applications (Hütsch et al. 1993; Hütsch et al. 1994). Study of the Broadbalk wheat experiment showed decreased soil CH_4 oxidation potential after 140 y of NH_4NO_3 applications, but no significant effect of a single fertilization event (Hütsch et al. 1993). In grassland, 130 years of $(NH_4)_2SO_4$ applications decreased CH_4 uptake compared to native grassland (Hütsch et al. 1994). Similar long-term $NaNO_3$ fertilization had no effect, so the inhibition was related to NH_4^+ rather than absolute nitrogen status. The inhibition remained even three years after the cessation of $(NH_4)_2SO_4$ fertilization (Hütsch et al. 1994). In these extended experiments organic N additions were less inhibitory than inorganic N additions, or even stimulated CH_4 oxidation (Hütsch et al. 1993; Willison et al. 1996). This final effect potentially resulted from increased soil methanogenesis, and the resultant stimulation of resident methanotrophs.

The conclusion from these studies is that NH_4^+ inhibition is mediated not by an immediate effect on methanotrophic bacteria or the MMO enzyme, but rather through long-term changes in soil chemistry, microbial populations, or ecological

interactions. Many soil properties change with long-term fertilization. Nitrogen turnover rates rather than the absolute N levels may control CH_4 oxidation (Mosier et al. 1991; Hütsch et al. 1993). An explanation advanced by the Broadbalk group (Hütsch et al. 1993; Hütsch et al. 1994; Willison et al. 1996) was an increased dominance of nitrifiers over methanotrophs in fertilized soil. This hypothesis assumes that methanotrophs and nitrifiers compete for growth factors, and that stimulation of nitrifiers through continued fertilization gradually displaces soil methanotrophs.

Antagonistic interactions between methanotrophs and nitrifiers have not clearly been demonstrated in aerobic soils. Castro et al. (1994) suggested that extended fertilization of a pine plantation caused a switch in the dominant CH_4-consuming group from methanotrophs to nitrifiers. This analysis was indirect, based on an assay relating soil CO oxidation rates to CO oxidation rates by pure cultures of methanotrophs and nitrifiers. This is of questionable applicability to soil, where methanotrophs behave differently than in pure culture. It is uncertain how this population shift would affect net CH_4 uptake in any case. Sitaula and Bakken (1993) noted a negative correlation between nitrification rates and CH_4 oxidation rates in a spruce forest, although high nitrification rates may simply have indicated high NH_3 availability and therefore NH_3-mediated inhibition of CH_4 oxidation. Evidence for a lack of antagonistic interactions between nitrifiers and methanotrophs was given by Bender and Conrad (1994a), who showed that soil nitrification rates were unchanged after the methanotroph population was increased by three orders of magnitude, and by Yavitt et al. (1993), who noted that drastic changes in nitrification were caused by liming a forest soil, but that these changes were not correlated to altered CH_4 oxidation rates.

Methanotrophs and nitrifiers could compete for O_2. Megraw and Knowles (1987a) showed that the nitrification rate of a humisol decreased under CH_4-enriched atmospheres, although CH_4 had no direct effect on nitrifiers at the concentrations used. They proposed that enrichment of methanotrophs had imposed competition for O_2 and for assimilable NH_4^+. Roy and Knowles (1994) documented similar O_2 competition in a polluted sediment. However, the applicability of these results to

aerobic soil where the growth of methanotrophs is likely CH_4-limited (and nitrifiers NH_3-limited) is questionable, except under very wet conditions. Competition between methanotrophs and nitrifiers for assimilable NH_4^+ is also unlikely to explain the long-term fertilization effect, since NH_4^+ limitation is obviously alleviated by fertilization.

Several of the field studies cited at the beginning of this section documenting decreased soil CH_4 oxidation rates in agricultural systems were also based on experiments examined after several (>4) years of fertilization (Hansen et al. 1993; Castro et al. 1994), or many years after a fertilization event (Mosier et al. 1991). Other studies show a more immediate effect (Steudler et al. 1989; Crill et al. 1994; Neff et al. 1994; Sitaula and Bakken 1995), and some show no effect at all (Bronson and Mosier 1993; Castro et al. 1993; Torn and Chapin 1993; Dunfield et al. 1995). Fertilization with 37 or 120 kg NH_4NO_3-N ha^{-1} over one year inhibited CH_4 oxidation in forest soils (Steudler et al. 1989). Strong inhibition resulted from 100 kg N ha^{-1} as NH_4Cl or urea applied once to a drained peat (Crill et al. 1994), 90 kg NH_4NO_3-N ha^{-1} applied once to a pine stand (Sitaula and Bakken 1995), and 250 kg urea-N ha^{-1} y^{-1} applied in two consecutive years to a meadow (Neff et al. 1994). However, Bronson and Mosier (1993) observed no effect of up to 150 kg urea-N ha^{-1} addition on CH_4 oxidation in a clay soil under wheat, or of 218 kg urea-N ha^{-1} on a clay loam under corn. No effect of three years application of 32 kg NH_4Cl-N ha^{-1} y^{-1} was evident in a spruce-fir stand (Castro et al. 1993), and no effect of 10 years fertilization with 50 kg NH_4NO_3-N ha^{-1} y^{-1} was observed on net CH_4 flux in a tundra soil (Torn and Chapin 1993). The net conclusion from these studies must be that both long-term and short-term effects of fertilization are possible, but that there are considerable study-specific and site-specific factors determining susceptibility.

The immediate effects of fertilization may be due to several factors, the first being enzyme competition between NH_3 and CH_4. Dunfield and Knowles (1995) concluded from a kinetic analysis that NH_3 did act as a competitive inhibitor of CH_4 oxidation in an organic soil, thereby extending observations on pure methanotroph cultures. However, other effects of fertilizer and side-effects of the enzymatic competition are also possible. Inhibition by dissolved salts has been noted. The

sensitivity to KNO_3 is similar to the sensitivity to KCl or NaCl (Nesbit and Breitenbeck 1992; Adamsen and King 1993; Dunfield and Knowles 1995; Kightley et al. 1995). Large ranges of NH_4^+ and NO_3^- are not generally tested, so the relative inhibition of these compounds cannot be well compared. The patterns are occasionally similar (Adamsen and King 1993; Kightley et al. 1995), but in agricultural soils NO_3^- and other salts are generally less inhibitory than NH_4^+ (Nesbit and Breitenbeck 1992; Bronson and Mosier 1994; Dunfield and Knowles 1995), indicating a specific NH_4^+ effect. Furthermore, the mechanism of NO_3^- inhibition is noncompetitive, unlike the NH_3 mechanism (Dunfield and Knowles 1995).

Competition for the MMO active site is obviously relieved as soon as the offending NH_3 is nitrified or otherwise removed from soil, but inhibition of CH_4 oxidation often persists (Mosier et al. 1991; Nesbit and Breitenbeck 1992; Hütsch et al. 1994; Schnell and King 1994). Simple competition therefore does not completely explain observed inhibition patterns. Absolute NH_4^+ concentrations also do not always correlate well with CH_4 oxidation rates in soil (Sitaula and Bakken 1993; Crill et al. 1994; Neff et al. 1994; Macdonald et al. 1996). Crill et al. (1994) observed higher NH_4^+ concentrations in urea-treated versus NH_4Cl-treated soil, but less inhibition of CH_4 uptake. There is generally a better correlation of CH_4 oxidation rates to nitrification rates than to absolute NH_4^+ concentrations, but this may simply mean that nitrification rates are a better index of available NH_3 (Sitaula and Bakken 1993; Neff et al. 1994).

King and Schnell (1994a; Schnell and King 1994) observed that NH_4^+ inhibition of CH_4 oxidation increased with increasing CH_4 mixing ratios between 1 and 100 ppmv, in apparent contradiction to a competitive model. Above 100 ppmv the trend reversed. The initial, low-CH_4 effect probably resulted from alleviation of reductant limitation. Increased MMO activity after CH_4 addition presumably accelerated NO_2^- production from NH_3. The authors concluded that the inhibition manifested by NH_4^+ was in fact primarily due to NO_2^-, the endproduct of methanotroph NH_3 oxidation (Bédard and Knowles 1989). NO_2^- inhibits CH_4 oxidation in soil and in methanotroph cultures (O'Neill and Wilkinson 1977; Jollie

and Lipscomb 1991; King and Schnell 1994a; King and Schnell 1994b; Schnell and King 1994; Dunfield and Knowles 1995).

With this model, enzyme competition is the root but not the direct cause of CH_4 oxidation inhibition. NO_2^- may have toxic, nonreversible effects (King and Schnell 1994a; Schnell and King 1994). As previously described, stimulation of soil CH_4 uptake can be accomplished with >1000 ppmv CH_4, but not with <200 ppmv CH_4 (Bender and Conrad 1992; Nesbit and Breitenbeck 1992; King and Schnell 1994a Bender and Conrad 1995; Schnell and King 1995). Therefore, the methanotrophic population may only slowly recover from a toxin.

Such an irreversibility could explain both the immediate and long-term fertilization effects. The key factors are the susceptibility and onset time to irreversible (or slowly reversible) inhibition. In the Broadbalk experiment examined by Hütsch et al. (1993), there was in fact a small (nonsignificant) decrease in CH_4 oxidation with a single NH_4^+ fertilization event. This inhibition could accumulate over time with repeated applications. On the other hand, inhibition is not always irreversible. Bronson and Mosier (1993) noted a depression of CH_4 oxidation immediately after urea addition to a corn field, but this disappeared after nitrification of the fertilizer. Vital factors controlling the susceptibility of soils to inhibition by NH_4^+ and the degree to which inhibition is reversible should include NH_4^+ fixation on soil minerals and pH. The former controls dissolved concentrations (Mancinelli 1995) and the latter controls the NH_3/NH_4^+ ratio. NH_3 rather than NH_4^+ is the inhibitor of MMO.

A final noteworthy possibility is that the fertilization of agricultural soils decreases their methanogenic potential through NO_3^- redox effects, and therefore deprives methanotrophs of a CH_4 source.

3.2.9.2 Soil water and diffusion limitation. Soil CH_4 uptake rates decline after rainfall events (Steudler et al. 1989) and are severely reduced in saturated soil (Nesbit and Breitenbeck 1992). A trend of decreasing CH_4 uptake with increasing soil moisture content is often observed (Whalen et al. 1990; Adamsen and King 1993;

Koschorreck and Conrad 1993; Lessard et al. 1994; Sitaula et al. 1995). There is a strong correlation among soil texture, gas transport resistance, and CH_4 uptake (Born et al. 1990; Dörr et al. 1993). Artificial soil compaction decreases (Hansen et al. 1993) and ploughing increases soil CH_4 uptake (Kruse and Iversen 1995). All these studies suggest that CH_4 oxidation is limited by the diffusion rate of atmospheric CH_4 into soils, and that restriction of CH_4 diffusion occurs as gas-filled porosity decreases. However, no consistent effect of moisture was observed in a hardwood forest (Crill 1991), or in several spruce-fir forests (Castro et al. 1993). Whalen and Reeburgh (1990) measured much higher gas relaxation rates than CH_4 oxidation rates in a porous tundra soil and concluded that delivery of atmospheric CH_4 to soil methanotrophs was not limiting.

The discrepancies among these studies may arise from variations in moisture ranges tested and in the diffusion properties of particular soils. Dunfield et al. (1995), studied a wide range of moisture content in a humisol and noted an exponential relationship between CH_4 uptake and H_2O content. There was a distinct threshold water content (ϕ_g=0.2) above which diffusion limitation set in and below which rates were constant. A similar analysis of a forest soil demonstrated diffusion limitation even at low water contents (ϕ_g=0.3-0.5) (Kruse et al. 1996).

Koschorreck and Conrad (1993) suggested that limitation of atmospheric CH_4 diffusion to methanotrophs occurred during CH_4 movement through water films rather than through the gas-filled pore space of soil. This interpretation would explain the effect of water content as well as would diffusion limitation through soil air. However, declining soil-air CH_4 mixing ratios with depth to minima <0.5 ppmv are commonly observed (Born et al. 1990; Whalen et al. 1992; Adamsen and King 1993; Dörr et al. 1993; Koschorreck and Conrad 1993; Dunfield et al. 1995; Kruse et al. 1996). This in itself indicates that transport through soil air is limiting, that methanotrophs located away from the soil surface are operating at sub-atmospheric CH_4 mixing ratios. Because it is a first-order process, CH_4 oxidation is therefore limited by CH_4 mixing ratios in subsurface soil. If transport through water films was much slower than through soil air, such gradients would not be observed.

Another possible explanation for the inhibitory effect of high moisture on soil CH_4 influx is stimulation of methanogenesis. As previously described, many soils have a methanogenic as well as a methanotrophic potential. Thus, a strong correlation between H_2O contents and CH_4 fluxes (ranging from equal extremes of net efflux to net influx) was observed in peat soils (Macdonald et al. 1996). Net CH_4 efflux from a flooded swamp switched to net influx during summer drought (Harriss et al. 1982).

At extremely low moisture contents CH_4 oxidation, like any microbial activity, becomes limited by water stress (Whalen et al. 1990; Nesbit and Breitenbeck 1992; Bender and Conrad 1995; Kruse et al. 1996; Schnell and King 1996). Torn and Harte (1996) therefore observed a hyperbolic dependence of CH_4 uptake on H_2O content in montane soils. An optimum moisture content of 50% water-holding capacity (WHC) was bordered by zones of water stress limitation and diffusion limitation.

Some studies show that water stress has long-term effects. Soil CH_4 uptake is nearly eliminated by air drying (Nesbit and Breitenbeck 1992; Syamsul Arif et al. 1996), and may not be recovered under atmospheric CH_4 mixing ratios (Nesbit and Breitenbeck 1992), or recover only after a lag phase of several days (Syamsul Arif et al. 1996). However, methanotrophs in desert sand are able to withstand extended drought, and recover within hours of rainfall (Striegl et al. 1992). Most methanotrophs produce exospores and cysts capable of surviving drought (Hanson and Hanson 1996).

3.2.9.3 pH. Adjustment of native soil pH by only 1 unit up or down inhibited CH_4 oxidation in a neutral agricultural soil (Syamsul Arif et al. 1996). CH_4 oxidation was strongly decreased by acidity in soils which were limed to pH values from 4.8-6.3 for 90 y (Hütsch et al. 1994), suggesting little adaptation to acid pH. A small adaptation of methanotrophs to pH in peats soils was noted, but these organisms were still inhibited by natural acid conditions (Dunfield et al. 1993).

CH_4 oxidation does occur in soils with $pH < 4.5$ (Heyer and Suckow 1985;

Steudler et al. 1989; Born et al. 1990; Moore and Knowles 1990; Nesbit and Breitenbeck 1992; Adamsen and King 1993; Dunfield et al. 1993; Sitaula et al. 1995), and methanotrophic enrichments were obtained from acid peats (Heyer and Suckow 1985). Methanotrophic yeasts are acid-tolerant (Wolf and Hanson 1979). Acidity is also not always inhibitory. Yavitt et al. (1993) noted no effect of liming on CH_4 oxidation in a hardwood forest, although this study ignored the mineral horizon, the zone of maximal CH_4 uptake. In a pine forest, irrigation with increasingly acidic water actually increased CH_4 oxidation rates (Sitaula and Bakken 1995). Such an effect could conceivably have resulted from lower NH_3/NH_4^+ ratios in more acid soils, although the authors found no supporting evidence for this possibility. If true, lower sensitivities of more acid soils to N fertilization would be expected, but this was not evident.

3.2.9.4 Temperature. There is only a weak temperature effect on soil CH_4 oxidation, with measured Q_{10}s of 1-2 (Born et al. 1990; Crill 1991; King and Adamsen 1992; Dörr et al. 1993; Crill et al. 1994), and little seasonal variation (Born et al. 1990; Dörr et al. 1993). The effect of temperature is often too small to be significantly noticeable (Castro et al. 1993). The limited temperature dependence may reflect the fact that diffusion of atmospheric CH_4 rather than enzyme function is the major limiting factor (King and Adamsen 1992; Dörr et al. 1993). When diffusion limitation is alleviated, the temperature response increases. In studies employing non-intact systems and saturating CH_4 concentrations, the temperature response is more typical of an enzymatic process (Whalen et al. 1990; Dunfield et al. 1993), but still low compared to other microbial processes, notably methanogenesis (Dunfield et al. 1993). The temperature response in an organic soil was increased by fertilization (Crill et al. 1994), perhaps because the absolute CH_4 oxidation rate was retarded and therefore less diffusion-limited. During early months of the year in temperate climates, when CH_4-oxidizing populations may not be established, the temperature response is greater than during the summer (Crill 1991).

Psychrophilic methanotroph communities containing *Methylococcus* spp.

(optimum 3.5-10°C) have been enriched from tundra soils (Omel'chenko et al. 1992). Significant CH_4 oxidation rates have been measured in tundra soils at 7°C (Whalen and Reeburgh 1990) and even under snow cover at near 0°C (Sommerfeld et al. 1993). Low-temperature activity may contribute a significant proportion of net annual uptake (Sommerfeld et al. 1993).

3.2.9.5 Inhibitors. In forest soils there is a pronounced subsurface optimum for CH_4 oxidation, with organic horizons lacking appreciable activity (Crill 1991; Adamsen and King 1993; Koschorreck and Conrad 1993; Yavitt et al. 1993; Bender and Conrad 1994b; Schnell and King 1994). It is noteworthy that in some cases the nitrification rate is maximal in the same mineral horizons as CH_4 oxidation (Amaral and Knowles 1997a), but in some cases is maximal in the organic layers (Yavitt and Newton 1990; Schnell and King 1994). The CH_4 oxidation optimum occurs along with NH_4^+ and NO_3^- concentration minima, which may indicate inhibition by these ions (Adamsen and King 1993; Schnell and King 1994). Alternately, other inhibitors may be present. Monoterpenes and other phenolic derivatives of plant litter are potential inhibitors of CH_4 oxidizers (White 1994; Amaral and Knowles 1997b). Extreme fluctuations in surface moisture could prevent establishment of methanotrophs (Whalen et al. 1992), as air-drying can impose non-reversible inhibition (Nesbit and Breitenbeck 1992).

Subsurface depth optima were also observed in landfill cover (Whalen et al. 1990), meadow (Bender and Conrad 1994b), and fallow agricultural soils (Dunfield et al. 1995), but not in drained peat (Crill et al. 1994), tundra (Whalen and Reeburgh 1990), or plowed agricultural soils (Bender and Conrad 1994b). Paradoxically, CH_4-consuming potential can occur much deeper than the relaxation depth (mean path length of a CH_4 molecule from the atmosphere) (Whalen et al. 1992), although this is not always the case (Koschorreck and Conrad 1993). Sample handling can decrease CH_4 oxidation activity, and hotspots of CH_4 oxidation occur (Whalen et al. 1992). These observations all indicate that factors other than diffusion from the overlying atmosphere can control CH_4 oxidation.

3.2.9.6 Other factors. There is some evidence that clay minerals and other surfaces enhance methanotrophic activity (Weaver and Dugan 1972; Kurdish and Kigel 1992). Whether this is an effect on enzyme activity or an ecological effect is uncertain, but it accents a deficiency in liquid culture studies. Organisms in natural soil environments behave much differently than those in liquid laboratory culture.

Ecological interactions between methanotrophs and other soil organisms (such as nitrifiers) are not well studied, but potentially influence CH_4 oxidation rates (Mancinelli 1995). Competition among methanotrophic species affects the composition of the methanotrophic community. In a chemostat study of competition between a type I (*Methylomonas albus*) and a type II (*Methylosinus trichosporium*) methanotroph, the outcome was affected by CH_4, Cu, and NO_3^- concentrations (Graham et al. 1993). As only type II and X methanotrophs express nitrogenase (Hanson and Hanson 1996), *M. trichosporium* dominated under N-limited conditions. Under Cu-limited conditions it also outcompeted the type I species because it could express sMMO. The type I methanotroph *M. albus* dominated under low CH_4 concentrations because of a higher specific growth rate (Graham et al. 1993). By growing methanotrophs in CH_4 and O_2 counter gradients, Amaral et al. (1995) also demonstrated the competitive superiority of type I species under CH_4-limited conditions. Although soil methanotroph communities appear to be dissimilar to these organisms, similar competitive mechanisms may occur in nature.

O_2 concentrations affect the distribution of methanotrophs in the environment and their competitive interactions. In aquatic systems, methanotrophs may prefer microaerobic conditions because of their N_2-fixing activity (Rudd et al. 1976). A similar condition might regulate CH_4 oxidation and its response to H_2O content in N-poor soils. The pMMO also has a higher affinity for O_2 than the sMMO (Joergensen 1985; Green and Dalton 1986).

3.3 Gaseous nitrogen oxides

3.3.1 Atmospheric chemistry

Tropospheric N_2O absorbs infrared radiation, contributing to global warming. It is oxidized slowly in the atmosphere, and has a mean lifetime of 110-150 years (Williams, Hutchinson et al. 1992). Upon reaching the stratosphere, N_2O absorbs short wave radiation to produce singlet oxygen and N_2. Stratospheric NO can then be formed by the reaction of N_2O with singlet oxygen. Through a series of further reactions, this NO causes stratospheric O_3 depletion (Williams, Hutchinson et al. 1992).

In contrast to N_2O, NO has a short tropospheric residence time. Eventually it is oxidized to HNO_2 and HNO_3, elements of acid deposition (Logan 1983). NO also plays a role in tropospheric oxidant chemistry and thereby, indirectly, in global warming. The experimental basis for the following brief outline has been reviewed by Logan (1983) and Williams, Hutchinson et al. (1992).

NO is oxidized in the troposphere by O_3:

$$NO + O_3 \rightarrow NO_2 + O_2 \qquad (3.3)$$

but is regenerated in sunlight:

$$NO_2 + O_2 + h\nu \rightarrow NO + O_3 \qquad (3.4)$$

This loop equilibrates within minutes for any given sunlight intensity (Williams, Hutchinson et al. 1992).

Atmospheric oxidations of CH_4, nonmethane hydrocarbons, and CO give rise to organic peroxyl radicals (RO_2) and hydroperoxyl radicals (HO_2), which also oxidize NO:

$$RO_2 + NO \rightarrow NO_2 + RO \qquad (3.5)$$

$$HO_2 + NO \rightarrow NO_2 + OH \qquad (3.6)$$

The resultant detour of NO from the closed NO/NO_2 loop of reactions (3.3-3.4) leads to net O_3 production, with no net change in NO_x. In essence, NO_x drives a catalytic system whereby light energy generates O_3 free radicals. However, when NO_x mixing ratios are very low, net ozone depletion occurs:

$$RO_2 + O_3 \rightarrow OH + 2O_2 \qquad (3.7)$$

Therefore, the tropospheric NO_x mixing ratio is a key determinant of the net production or consumption of O_3. O_3, in turn, produces OH:

$$O_3 + h\nu + H_2O \rightarrow 2OH + O_2 \qquad (3.8)$$

The free radicals OH and O_3 are initiators of many other tropospheric processes. OH, for example, is the principal initiator of CH_4 oxidation.

Most soil NO_x emission is in the form of NO rather than NO_2. NO_2 efflux has occasionally been measured from grassland (Galbally and Roy 1978; Williams et al. 1987), lucerne (Johansson and Granat 1984), and from bare soil (Williams et al. 1988; Slemr and Seiler 1991), but almost always at much lower (<10%) efflux rates than NO. This preference for NO production can deplete tropospheric ozone immediately adjacent to the soil surface through reaction (3.3) (Kim et al. 1994), but may eventually lead to O_3 production as outlined above.

NO_2 has a high adsorption rate (Judeikis and Wren 1978) and adsorption capacity (Wellburn 1990) compared to NO, and under most conditions there is a net deposition onto soil and other inorganic surfaces (Slemr and Seiler 1991; Baumgärtner et al. 1992; Kim et al. 1994), and onto plants (Voldner et al. 1986; Slemr and Seiler 1991; Weber and Rennenberg 1996). NO_2 deposited onto inorganic surfaces may desorb under high temperatures (Baumgärtner et al. 1992), be reduced and reemitted as NO through reactions coupled to Fe^{3+} and SO_2 oxidation (Baumgärtner et al. 1992), or dismute into HNO_2 and HNO_3 (Ghiorse and Alexander 1976). NO_2 dismutation also occurs in plant tissues, but most plant NO_2 oxidation occurs through reactions with antioxidants (Ramge et al. 1993).

Ecosystems may therefore be a net NO_x sink once the plant cover is considered (Wesely et al. 1982; Slemr and Seiler 1991), especially in polluted areas (Weber and Rennenberg 1996). Where this occurs, soil NO production can still be critical in offsetting the net deposition rate (Wesely et al. 1982). The net production or consumption of NO_x depends on ambient NO and NO_2 mixing ratios, upon variables such as leaf area index and stomatal resistance which control the rate of NO_2 deposition (Wellburn 1990; Yienger and Levy 1995), and upon factors controlling the soil NO emission rate.

Most estimates of soil NO flux are based on the use of field chambers. While the general applicability of this technique, and the conclusion of net NO_x production from grasslands has been verified against gradient (Parrish et al. 1987) and eddy-correlation techniques (Stocker et al. 1993), chambers cannot be used over high plant cover for obvious reasons. Soil-produced NO_x would be trivial if it failed to escape the plant canopy in such systems, and chamber measurements of questionable value. However, models and studies using micrometerological gradient techniques have verified that a portion of soil-emitted NO_x reaches the atmosphere rather than being adsorbed onto leaves, and survives long enough to influence tropospheric O_3 chemistry even over a dense tropical forest (Bakwin et al. 1990; Jacob and Wofsy 1990). One recent model predicts that on average 50% of soil NO_x emissions are trapped within plant canopies (Yienger and Levy 1995).

3.3.2 Processes of gaseous nitrogen oxide production

Increasing evidence suggests that almost any process in which nitrogen is converted through the +1 to +2 oxidation states can produce NO and N_2O as byproducts. Thus, sources of NO and N_2O include dissimilatory nitrite and nitrate reduction as well as classical denitrification, autotrophic ammonia oxidation, and heterotrophic nitrification (Conrad 1996a). Because NO_2^- can undergo chemodenitrification in soil (see section 3.3.2.4), any NO_2^--producing process is a potential indirect source of N-oxide gases.

Gaseous N-oxide production is not limited to bacteria, but is evident in algae (Weathers 1984) and nitrate-reducing fungi (Bollag and Tung 1972; Shoun et al. 1992), although the metabolic significance of the processes in these organisms is not fully understood.

One study does suggest that processes other than nitrification and denitrification can contribute significantly to soil N_2O flux, especially in soils with low denitrification and nitrification potentials and low absolute flux rates (Robertson and Tiedje 1987). However, especially in agricultural systems, nitrification and denitrification appear to be the main sources (see section 3.3.3).

3.3.2.1 Denitrification. Denitrification in the strict sense is the reduction of oxidized forms of nitrogen coupled to electron transport phosphorylation. Coupling to energy generation distinguishes denitrification from other NO_3^--reducing processes described in the next section. The sequence of denitrification is:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (3.9)$$

The series consists of four enzymatic steps, catalysed sequentially by nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos). It has only recently been clearly demonstrated that NO is an obligate intermediate in this pathway, through studies of Nos- and Nir- mutants, through demonstration of NO as the endproduct of isolated Nir, and through isolation of Nor itself (reviewed in Ye et al. 1994).

Most denitrifiers contain the entire suite of denitrification enzymes. There are a few exceptions, but because of the diversity and ubiquity of denitrifiers, it is unlikely that lack of a particular enzyme plays a role in natural systems (Firestone and Davidson 1989). Different denitrifying species do exhibit different N-oxide production rates (Anderson and Levine 1986; Remde and Conrad 1991a). The particular denitrifying species present in soil may therefore affect soil nitrogen oxide fluxes.

Most denitrifiers are facultative aerobes for which aerobic conditions both inhibit and repress synthesis of denitrifying enzymes (Smith and Tiedje 1979), although apparent inhibition may simply be competition for electrons rather than true enzyme regulation (Knowles 1982). There are exceptions to the anaerobic generalization such that, especially when coupled to nitrification, denitrification can continue at near air-saturation O_2 levels. Constitutive denitrification enzyme production was observed in a *Thiosphaera* sp. which was limited by electron transfer even at air saturation (Robertson and Kuenen 1984). Aerobic denitrification occurs during the temporal transition from anaerobic to aerobic conditions because the denitrifying enzymes are not immediately inhibited or broken down (Lloyd et al. 1987; Remde and Conrad 1991b; Kuenen and Robertson 1994). However, aerobic denitrification has not been widely documented, and an important role in N-oxide

production in soils, except transiently, is doubtful.

3.3.2.2 Nitrate reduction by nondenitrifiers. Gaseous N-oxides are produced during assimilatory (Bleakley and Tiedje 1982) and dissimilatory nitrate reduction (Smith and Zimmerman 1981; Bleakley and Tiedje 1982; Anderson and Levine 1986; Ji and Hollocher 1988; Kalkowski and Conrad 1991). A variety of pathways exist, including nitrate respiration ($NO_3^- \rightarrow NO_2^-$), and the two-step dissimilatory reduction to ammonium ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$) (Conrad 1996a). Of these, nitrate respirers have the higher N_2O production per mol NO_3^- reduced (Bleakley and Tiedje 1982). Because NO_2^- is the endproduct of nitrate respirers, chemodenitrification may account for NO and N_2O production by these organisms (Anderson and Levine 1986) (section 3.3.2.4).

Although the (nondenitrifying) nitrate reducers produce comparable amounts of NO as denitrifiers per molecule of NO_3^- reduced (Kalkowski and Conrad 1991), and in some soils reduce NO_3^- at similar rates as denitrifiers (Näegele and Conrad 1990b; Blösl and Conrad 1992), detailed study of soil N-oxide production by these organisms is lacking.

3.3.2.3 Nitrification. The pathway of NH_3 oxidation to NO_3^-:

$$NH_3 \rightarrow NH_2OH \rightarrow (NOH) \rightarrow NO_2^- \rightarrow NO_3^- \qquad (3.10)$$

is carried out by two distinct groups of chemolithotrophic bacteria. Ammonia oxidizers carry out the oxidation to NO_2^- (where NOH is a postulated intermediate), while nitrite oxidizers complete the oxidation to NO_3^-. The ammonia oxidizers comprise five genera (Prosser 1989). Most studies have been performed on *Nitrosomonas* spp., although recent 16S rRNA evidence suggests that *Nitrospira* spp. are more widespread in soil (Hiorns et al. 1995). The nitrite oxidizers comprise four genera, but only *Nitrobacter* spp. are well characterized (Prosser 1989).

Although anaerobic NH_3 oxidation occurs (Mulder et al. 1995), in soils it is a strictly aerobic process. Ammonia oxidation by AMO, like methane oxidation by MMO, requires molecular oxygen and reducing power (equation 2.2), while

hydroxylamine and nitrite oxidations are energy-yielding processes through the electron transport chain.

Ammonia-oxidizing bacteria in pure culture produce both N_2O and NO during growth (Yoshida and Alexander 1970; Goreau et al. 1980; Lipschultz et al. 1981). This can theoretically occur in two ways, through decomposition of the hypothetical intermediate NOH, or through reduction of the product NO_2^-. Evidence for the decomposition pathway include NO production by purified hydroxylamine oxidoreductase (Hooper and Terry 1979), and N_2O production by *Nitrosomonas* spp. that is independent of NO_2^- concentrations under fully aerobic conditions (Ritchie and Nicholas 1972; Hynes and Knowles 1984).

However, a great deal of evidence implicates NO_2^- reduction as the major process, especially at low pO_2. Gaseous N-oxide production proceeds under fully anaerobic conditions (Ritchie and Nicholas 1972) and is positively related to NO_2^- concentration at pO_2=0.005 atm (Anderson and Levine 1986). With hydrazine added as an alternate electron donor to hydroxylamine, *Nitrosomonas europaea* reduced NO_2^- to NO and N_2O at an 84% recovery rate, suggesting that the major reaction is denitrification rather than NOH decomposition (Remde and Conrad 1990). Poth and Focht (1985) clearly showed that ^{15}N-labelled NO_2^- was incorporated into N_2O, and furthermore that all the N_2O produced during growth on $^{15}NO_2^-$ and $^{14}NH_4^+$ was accounted for by NO_2^- reduction. *Nitrosomonas* may possess the entire suite of denitrification enzymes save NO_3^- reductase (Poth and Focht 1985; Poth 1986).

The N_2O/NO_3^- production ratios in various ammonia oxidizers increase with declining pO_2 (Goreau et al. 1980; Lipschultz et al. 1981; Poth and Focht 1985; Anderson and Levine 1986; Remde and Conrad 1990), further demonstrating that the process is primarily NO_2^- denitrification, operating to relieve oxidant shortage. The O_2 effect is less clear for NO than for N_2O. Contradictory results show that O_2 may (Lipschultz et al. 1981) or may not (Anderson and Levine 1986) affect NO production by *Nitrosomonas europaea*. It certainly affects NO production less than N_2O production (Remde and Conrad 1990). The synthesis of NO reductase may be less O_2-sensitive, or NO production may be a combined effect of denitrification and

chemodenitrification. Remde and Conrad (1990), after inhibiting NO and N_2O but not NO_2^- formation with chlorite, estimated NO and N_2O production in *Nitrosomonas* cultures as only 15% due to chemodenitrification, so this explanation of the O_2 effect is unlikely. Anderson et al. (1993) suggested the varying experimental results regarding O_2 sensitivity could be reconciled by considering the NO_2^- concentrations under which experiments were carried out. The low pO_2 optimum for both NO and N_2O production by *Nitrosomonas* disappeared under conditions of excess NO_2^-, indicating that O_2 and NO_2^- competed for electrons.

The use of NO_2^- as an electron acceptor may preserve O_2 for use by AMO under low O_2 conditions, and may prevent NO_2^- toxicity. NO_2^- reduction to NO, a proton-consuming reaction, is also a potential pH-regulating reaction to aid in deprotonation of NH_4^+ (Conrad 1996a).

Although *Nitrobacter* species do not appear to be involved in aerobic NO and N_2O production during autotrophic nitrification, other than through regulation of NO_2^- concentrations, under anoxic conditions these organisms produce NO from dissimilatory NO_3^- and NO_2^- reduction (Ahlers et al. 1990).

3.3.2.4 Chemodenitrification. The maximum NO production rate during nitrification of soil fertilizer N occurs coincidentally with the maximum NO_2^- concentration (Steen and Stojanovic 1971). Addition of chlorate, an inhibitor of NO_2^- oxidation, increases soil NO and N_2O production (Tortoso and Hutchinson 1990). These data imply that NO_2^- reduction is the primary source of N-oxide gases in soil as well as in nitrifier cultures. Although in pure *Nitrosomonas* cultures most NO_2^- reduction is enzymatic (Anderson and Levine 1986; Remde and Conrad 1990), the conditions and potential reactions in soil are much different. Adding NO_2^- and NH_4^+ to sterile soil at similar concentrations as observed during active nitrification resulted in similar NO production rates (Steen and Stojanovic 1971). A great deal of the NO and some of the N_2O produced during soil nitrification may arise through chemical reactions of NO_2^-.

Nitrous acid undergoes chemical decomposition:

$$3HNO_2 \rightarrow 2NO + HNO_3 + H_2O \qquad (3.11)$$

$$2HNO_2 \rightarrow NO + NO_2 + H_2O \qquad (3.12)$$

(Nelson and Bremner 1970). Of the possible chemical reactions in soil, Nelson (1982) concluded that chemical decomposition was the principal non-microbial NO source, but Blackmer and Cerrato (1986) disagreed based on a study of various sterilized soils in which organic matter content was a significant predictor of NO production. Product ratios do not always correspond to dismutation reactions, and concurrent CO_2 production indicates participation of organic materials in gaseous N-oxide-forming reactions (Stevenson et al. 1970; Blackmer and Cerrato 1986; McKenney et al. 1990).

Several studies have concluded that soil lignins, humic and fulvic acids, and phenolics are involved in chemodenitrification to NO (Stevenson et al. 1970; Steen and Stojanvic 1971; Chalk and Smith 1983; Blackmer and Cerrato 1986; McKenney et al. 1990), but the natures of the reactions are uncertain. Soil organic constituents are also responsible for N_2O-forming reactions from NO_2^- (Nelson and Bremner 1970; Davidson 1992). The chemistry of soil constituents is complex, so such reactions may also be complex. Humic materials may catalyse reactions of NO_2^- with NH_4^+ or Fe^{2+}, or may enter directly into the reactions (Steen and Stojanovic 1971). Fe^{2+} can directly oxidize NO_2^- to NO (Chalk and Smith 1983), and microbial processes drive this reaction by reducing Fe^{3+} back to Fe^{2+} (Brons et al. 1991). NO_2^- also reacts with soil organic amino or nitroso compounds (van Slyke reactions), giving a greater-than-unity yield of N-gases (Stevenson et al. 1970; Keeney et al. 1979; McKenney et al. 1990).

Stüven et al. (1992) suggested that chemodenitrification was also driven by hydroxylamine, a nitrification intermediate. Soil studies show that excreted NH_2OH is chemodenitrified to N_2O (Bremner et al. 1980; Chalk and Smith 1983), but NH_2OH also serves as an electron donor for nitrite reductase (Hooper and Terry 1979), so the root cause of hydroxylamine effects is open to question.

3.3.2.5 Heterotrophic nitrification. Heterotrophic as well as autotrophic nitrifiers produce NO and N_2O (Papen et al. 1989; Anderson et al. 1993). These heterotrophs carry out various steps of the pathway given above for autotrophic nitrifiers (equation 2.10), and an analogous pathway involving organic-N:

$$\begin{array}{ccccccccc} NH_3 & \rightarrow & NH_2OH & \rightarrow & NOH & \rightarrow & NO_2^- & \rightarrow & NO_3^- \\ \uparrow & & \uparrow & & \uparrow & & \uparrow & & \\ R\text{-}NH_2 & \rightarrow & R\text{-}NHOH & \rightarrow & R\text{-}NO & \rightarrow & R\text{-}NO_2 & \rightarrow & NO_3^- \end{array} \quad (3.10)\ (3.13)$$

(Prosser 1989), where R represents a variety of organic compounds. The pathways are probably only partial in various bacteria, and the cycle can link with autotrophic nitrifiers at several points (Prosser 1989). Heterotrophic nitrification probably serves several cellular functions including production of iron-chelating hydroxamates and regulation of reducing status (Kuenen and Robertson 1994). Like autotrophic ammonia oxidizers, many heterotrophic nitrifiers are also denitrifiers (Castignetti and Hollocher 1984; Robertson et al. 1988) and produce more NO and N_2O under reduced pO_2 atmospheres (Robertson et al. 1988; Anderson et al. 1993). Therefore the mechanisms of NO and N_2O production may be essentially reductive as in autotrophic nitrifiers, with nitrification supplying the substrate to be denitrified.

In the field, it is conceptually difficult to separate the effects of heterotrophic and autotrophic nitrification. Heterotrophic nitrifers are often less affected by nitrification inhibitors than autotrophic nitrifiers, but this is not always the case (reviewed in Conrad 1996a). A few facts attest to the potential of these organisms to contribute to aerobic NO and N_2O flux. Some heterotrophic nitrifiers have higher pO_2 optima than autotrophic nitrifiers, and also higher ratios of gaseous N-oxides to other products under fully aerobic conditions (Anderson et al. 1993). Absolute nitrification rates per cell are generally lower in the heterotrophic version of nitrification, but only by 1-2 orders of magnitude. This could easily be compensated for by higher cell densities in soil (Robertson et al. 1988; Kuenen and Robertson 1994).

Tortoso and Hutchinson (1990) found that adding glucose to an agricultural soil did not stimulate nitrification-based NO and N_2O fluxes, and therefore the activity

was due to autotrophic rather than heterotrophic processes. However, it has since been demonstrated that soil heterotrophs consume NO (see section 3.3.5.3), a process which is also stimulated by glucose (Dunfield and Knowles 1997). These opposite effects could compensate for each other.

3.3.3 Partitioning nitrification and denitrification as NO and N_2O sources in the field

Nitrification has been shown to be the dominant NO source in a variety of soils through the use of the nitrification inhibitors acetylene (C_2H_2) (Davidson 1992; Davidson et al. 1993), nitrapyrin (Tortoso and Hutchinson 1990; Remde and Conrad 1991c; Hutchinson et al. 1993), and dicyandiamide (Skiba et al. 1993). Few studies have examined saturated soil where denitrification could be relatively more important. In two that did include near-saturated soil, or soil wetted to a degree where denitrification became a major N_2O source, nitrification remained the principal NO source (Davidson 1992; Skiba et al. 1993).

While NO is an obligate denitrification intermediate, very little escapes the soil. When soil air is rapidly stripped under anaerobic conditions, a high proportion of denitrified N is recoverable as NO (McKenney et al. 1982; Johansson and Galbally 1984). The ratio of NO production to NO consumption is higher in anaerobic versus aerobic conditions, therefore the compensation point increases under denitrifying conditions and increased NO efflux might be expected (Remde et al. 1989; Krämer and Conrad 1991). However, denitrification usually only occurs in near-saturated soils where diffusion is slow compared to microbial NO reduction, and most NO is reduced to N_2O rather than emitted (Drury et al. 1992). In a natural system, retarded NO diffusion through saturated soil can compensate for the increased NO production/NO consumption ratio brought about by anaerobic conditions. At steady-state conditions, which may occur in severely diffusion-restricted soil, the maximum NO reduction velocity is much higher than that for NO_2^-, ensuring that NO cannot accumulate to toxic concentrations (Goretski et al. 1990), and ensuring that NO is only a minor byproduct of denitrification. A flooded soil may also possess an aerobic

surface layer where NO produced by denitrification in deeper soil is oxidized.

Remde and Conrad (1991c), by using nitrification inhibitors and $^{15}NO_3^-$, presented direct evidence for a nitrate reduction or denitrification source for NO emitted from soil under an aerobic atmosphere. This study examined an acid soil with little nitrification activity, and constitutes the only clear evidence of an NO_3^--reductive source of emitted NO. However, a great deal of circumstantial evidence also exists. Sugar additions can stimulate soil NO emissions (Bakwin et al. 1990; Slemr and Seiler 1991), implicating a denitrification or heterotrophic nitrification source. Several studies have documented increased NO emissions after NO_3^- fertilization (reviewed in Williams, Hutchinson et al. 1992). This immediately suggests increased nitrate respiration or denitrification, but indirect effects are also possible. One is the controversial "priming" effect, stimulation of organic nitrogen mineralization by inorganic N addition (Hauck and Bremner 1976). Skiba et al. (1993) observed an apparent increase in NO production from nitrification rather than denitrification following NO_3^- fertilization. Although the lack of unfertilized controls in this study makes interpretation difficult, the authors noted an apparent increase of organic N mineralization after NO_3^- addition. NO_3^- is also a noncompetitive inhibitor of *Nitrobacter* (Boon and Laudelout 1962). This inhibition may be a salt effect and is especially evident at low pH (Hunik et al. 1993). Any inhibition of *Nitrobacter* will lead to higher soil NO_2^- levels and increased chemodenitrification rates.

Lack of stimulation by NH_4^+ fertilizer is also not necessarily proof of an NO_3^- reduction NO source. Nitrification in acid soils often does not respond to exogenously added NH_4^+, and may be primarily heterotrophic (e.g. Martikainen 1984). However, several studies where both effects are evident, where NO_3^- stimulates and NH_4^+ does not affect soil NO flux, are compelling evidence for NO production through an NO_3^--reductive process (Bakwin et al. 1990; Sanhueza et al. 1990; Remde et al. 1991c; Cárdenas et al. 1993; Saad and Conrad 1993a). It is probably noteworthy that all these studies involved acid soils. In one, NO_3^- reduction was clearly shown to be the major NO source (Remde and Conrad 1991c).

Therefore, considerable evidence attests to the potential of denitrification to be

the origin of soil NO emissions, especially in acid soils, but this is rarely conclusive. In soils with active autotrophic nitrification, denitrification is usually secondary to nitrification. The predominance of nitrification is partly a function of higher per cell NO production rates in autotrophic nitrifiers compared to denitrifiers or nitrate respirers (Anderson and Levine 1986). Gaseous diffusion rates in dry, aerobic, nitrifying soils are faster than in denitrifying soils. Also, in saturated soils, nitrification will occur in the aerobic surface soil, while denitrification will predominate in deeper layers. Hence nitrifiers should control surface flux.

In contrast to NO, N_2O is frequently a combined product of soil denitrification and nitrification. This conclusion arises from data showing stimulation of N_2O emissions by NH_4^+-based and/or NO_3^--based fertilizers in various sites and under various conditions (reviewed in Sahrawat and Keeney 1986; Aulakh et al. 1992). Several studies have demonstrated partial inhibition of N_2O emissions, or inhibition under certain conditions only, by nitrification inhibitors (Freney et al. 1979; Robertson and Tiedje 1987; Klemedtsson et al. 1988a; Davidson et al. 1993; Martikainen et al. 1993). Production of gaseous N-oxides directly through nitrification rather than through denitrification of the endproduct NO_3^- is easily distinguishable in these studies by examination of an NO_3^- fertilizer treatment. Often nitrification is the major N_2O source below water saturation, and denitrification the major source above saturation (Aulakh et al. 1984; Davidson et al. 1986; Parton et al. 1988; Davidson 1992; Davidson et al. 1993; Martikainen and De Boer 1993; Skiba et al. 1993; Mummey et al. 1994), although there are many exceptions. Nitrification can be the primary source in wet conditions (Klemedtsson et al. 1988a; Hutchinson et al. 1993) while studies using $^{13}NO_3^-$ show that this can be the major precursor of N_2O even in aerobic soil (Speir et al. 1995a).

3.3.4 Processes of N_2O consumption

The only known biotic N_2O sink is denitrification. Since denitrifiers both produce and consume N_2O, in theory soils can act as a net N_2O sink, but only in rare instances is this evident (Letey et al. 1981; Ryden 1983; Smith et al. 1983; Dunfield et al.

1995). In most cases the soil N_2O compensation point is much greater than the atmospheric mixing ratio, so there is a net flux out of soil (Conrad 1994). Net N_2O uptake should occur only in flooded anaerobic systems where denitrifying enzymes are present but N-limited. In these cases net production of N_2O is greatly limited while N_2O consumption potential remains, reducing the compensation point to below ambient levels.

In soils that are net N_2O sources, denitrification may still be a sink for N_2O produced from other processes. This effect was proposed by Robertson and Tiedje (1987) based on the fact that a 100% O_2 atmosphere stimulated N_2O production in soil cores, whereas if nitrifiers and denitrifiers were N_2O sources the opposite would be expected. However, in acid soils such as those used in this study, nitrifiers may be aggregated and operate under localized O_2-limited conditions (De Boer, Tietema et al. 1991), which could be alleviated by a 100% O_2 atmosphere.

Donoso et al. (1993) documented net N_2O uptake by tropical savannah soils during the dry season. Although denitrification was improbable, the mechanism was not determined. Some reports of aerobic N_2O consumption by bacteria exist (Vedenina and Zavarzin 1977, 1979), but whether this is an oxidative process, perhaps related to H_2O_2 metabolism, or an example of aerobic denitrification is unclear.

3.3.5 Processes of NO consumption

Soil NO emissions are the net results of productive and consumptive processes. It was recognized early that the amount of NO_x recovered from soil samples was inversely related to the thickness of the sample, indicating that uptake mechanisms attenuated NO release (Smith and Chalk 1979). Some soils even act occasionally as net sinks for tropospheric NO (Slemr and Seiler 1991; Skiba et al. 1994).

3.3.5.1 Sorption and chemical reactions. NO sorbs to soil, both physically and through chemical coordination with certain transition metals on clay surfaces (Mortland 1965). Sorbed NO is eventually converted to NO_3^- (Mortland 1965;

Prather at al. 1973; Stark and Firestone 1995). When coordinated with transition metals, NO^+ is susceptible to reaction with O_2 (Kanner 1996). Direct oxidations of NO by soil constituents are also possible.

The above studies were performed with extremely high (>1000 ppmv) NO mixing ratios. NO at these mixing ratios is rapidly oxidized by O_2 to NO_2, which then disproportionates in H_2O to form nitric and nitrous acids:

$$2NO + O_2 \rightarrow 2NO_2 \qquad (3.14)$$

$$2NO_2 + H_2O \rightarrow HNO_2 + HNO_3 \qquad (3.15)$$

$$NO + NO_2 + H_2O \rightarrow 2HNO_2 \qquad (3.16)$$

As all of these reactions are second-order in NO_x, they proceed slowly at tropospheric mixing ratios. The half lives are typically >1000 h (Galbally and Roy 1978, Schwartz 1984). At lower mixing ratios, soil NO uptake is drastically reduced by sterilization, and therefore principally a result of microbial activity (Johansson and Galbally 1984; Remde et al. 1989; Baumgärtner et al. 1996; Dunfield and Knowles 1997).

3.3.5.2 Denitrification. Reduction of NO to N_2O by denitrifiers assures that NO is merely a byproduct and not a primary endproduct of soil denitrification (Schuster and Conrad 1992). The NO compensation point is generally higher in anaerobic than in aerobic conditions, so it is unlikely that denitrifying bacteria act frequently as NO sinks (Johansson and Galbally 1984; Remde et al. 1989; Remde and Conrad 1991b; Rudolph and Conrad 1996; Rudolph et al. 1996). The conditions necessary for net NO uptake by anaerobic soils are probably the same as described above for N_2O: enzyme presence under severe nitrogen limitation. Both NO production and NO reduction are stimulated by excess NO_2^- and NO_3^-, but production much more so than reduction. The compensation point therefore decreases with decreasing soil nitrogen (Baumgärtner and Conrad 1992b). Denitrification becomes "leakier" under conditions of N-excess.

Remde and Conrad (1991a) proposed from a similar kinetic response of NO uptake under anaerobic and aerobic incubations that NO reduction operated in two

largely aerobic moist cambisols (at 30% H_2O w.w.), perhaps in anaerobic microsites. While they lost almost all of their NO-producing capacity, denitrifiers retained a portion of their NO-consuming activity when transferred from anaerobic to aerobic conditions (Remde and Conrad 1991b). If NO uptake was not due to oxidative reactions similar to those documented in soil heterotrophs (see section 3.3.5.3), this finding suggests that NO reductase is less O_2-sensitive than NO_2^- reductase, and that denitrifiers can be an NO sink when soil is drying out and traversing from anaerobic to aerobic conditions. This possibility is contradicted by other studies (McKenney et al. 1994).

Methanotrophs also consume NO (Krämer et al. 1990). As NO consumption occurred concurrently with N_2O production, the authors assumed a reductive NO-consuming process, although the NO/N_2O ratio was far from stoichiometric. Either further reduction of N_2O to N_2, or oxidative removal of NO as discussed below was also occurring.

3.3.5.3 Oxidative reactions. Until recently it was believed that only microbial denitrification was a soil NO sink. However, NO consumption occurs in soils with little denitrifying activity (Hutchinson et al. 1993). Recent studies show that the primary uptake mechanism in aerobic soils is oxidation, ultimately to NO_3^- (Baumgärtner et al. 1996; Dunfield and Knowles 1997).

The oxidation of NO is exothermic at standard conditions and presents a potential energy source (Conrad 1996a). *Nitrobacter* spp. produce NADH while oxidizing NO to NO_2^- (Freitag and Bock 1990). A positive correlation was noted between NO uptake rates and counts of NO_2^- oxidizers in a cambisol (Baumgärtner and Conrad 1992a), suggesting that these organisms may contribute to soil NO turnover.

Aerobic soil heterotrophs also oxidize NO to NO_3^- (Baumgärtner et al. 1996; Koschorreck et al. 1996). Their specific activity is much lower than denitrifiers, but NO oxidation is evident in the most numerous soil bacteria (Baumgärtner et al. 1996), so NO oxidation rate constants in aerobic soils (Baumgärtner et al. 1996; Dunfield

and Knowles 1997) are comparable to those in denitrifying soils (Remde and Conrad 1989; Remde and Conrad 1991c). Although NO is a potential energy source, even at very high mixing ratios (1 ppmv) the harvestable energy from measured NO oxidation rates in a *Pseudomonas* sp. was only a tiny fraction of the organism's maintenance energy (Koschorreck et al. 1996). NO oxidation is therefore probably a result of non-coupled reactions. Several such oxidative reactions occur in biological systems. These have been well studied because of the roles NO plays in signal transduction and pathogenicity, and are reviewed by Fontecave and Pierre (1994), Halliwell (1994), and Wink et al. (1996).

The primary pathways leading to NO_2^- and NO_3^- production in cells are: (i) The reaction of NO with the superoxide anion (O_2^-) to give oxoperoxonitrate ($ONOO^-$), which decomposes to NO_3^- when protonated (Wink et al. 1996), or possibly is oxidized to NO_2^- through metal-catalysed reactions (Fontecave and Pierre 1994); (ii) reactions with carbon-centred, -oxyl, or -peroxyl radicals (Padmaja and Huie 1993; Wink et al. 1996) which are transient products of enzymatic reactions; and (iii) formation of metal-nitrosyl adducts through NO binding on enzyme cofactors, especially ferrous ions. Once bound, NO may react with O_2 or with oxygen-containing compounds to form NO_2^-, NO_3^-, or $ONOO^-$ (Fontecave and Pierre 1994; Kanner 1996; Wink et al. 1996). Such an interaction is well characterized in mitochondrial cytochrome-*c* oxidase (Torres and Wilson 1996), and in myoglobin and haemoglobin, where the affinities for NO are actually much greater than those for O_2 (Fontecave and Pierre 1994).

Nitrogen monoxide may act as either an antioxidant or an oxidant in cells (Wink et al. 1996), but reactive nitrogen oxide species such as $ONOO^-$ formed through NO oxidation pathways are toxic. Among other things, they degrade proteins necessary for DNA repair (Wink et al. 1996). It has been suggested that $ONOO^-$ decomposes into NO_2 and OH (Beckman et al. 1990), but the energetics of this reaction make it doubtful (Koppenol 1996). Unlike NO, NO_2 is sufficiently reactive to initiate lipid peroxidation, a chain reaction leading to loss of membrane function (Halliwell 1994). It is in an organism's best interest to convert reactive oxygen

species such as NO_2 and $ONOO^-$ (and perhaps NO) into less harmful forms such as NO_3^-. A suite of antioxidant defenses performs these functions, including vitamin C (ascorbate), vitamin E, glutathione, uric acid, carotenoids, and flavonoids (Halliwell and Gutteridge 1989; Halliwell 1994). Oxidation of NO_x in plants by ascorbate is an example (Ramge et al. 1993). These defenses may mediate NO oxidation by cells, and the complete oxidation to NO_3^- should be rapid.

3.3.6 Environmental controls of NO and N_2O flux

Firestone and Davidson (1989) likened NO and N_2O production to flow through a leaky pipe, where the production rate is first a function of the total flow rate and second a function of the leakiness of the pipeline. The flow rate corresponds to absolute rates of nitrification and denitrification, and the leakiness to those factors which influence the ratios of various products. Thus the primary controls of nitrification are O_2 and NH_4^+, of denitrification O_2, NO_3^-, and organic carbon. Among the factors affecting end-product ratios are enzyme regulation mechanisms and the diffusion rates of intermediates through soil. How some key environmental factors affect these two levels of control are outlined in the following sections.

3.3.6.1 Carbon and nitrogen. Obviously, addition of a carbon source such as glucose (Speir et al. 1995b) or manure (Paul et al. 1993) to soils may increase N_2O production from denitrification, if the rate is not limited by nitrogen scarcity or O_2 excess. Correlations between various indices of microbially-available soil organic carbon and denitrification capacity have been documented (e.g. Burford and Bremner 1975; reviewed in Aulakh et al. 1992). Organic soils display large N_2O emissions, typically one to two orders of magnitude greater than mineral soils (Terry et al. 1981; Duxbury et al. 1982; Goodroad and Keeney 1984b). Incorporation of plant residues or manure into soil also generally stimulates denitrification, provided the C/N ratio is small enough that N immobilization into microbial biomass does not occur (reviewed in Aulakh et al. 1992).

Nitrogen monoxide fluxes are frequently stimulated by NH_4^+-based fertilizers

(Remde and Conrad 1991c; Slemr and Seiler 1991; Baumgärtner and Conrad 1992a; Hutchinson and Brams 1992; Schuster and Conrad 1992; Saad and Conrad 1993a; Skiba et al. 1993; Parsons and Keller 1995), and also occasionally by NO_3^--based fertilizers, as previously described (Bakwin et al. 1990; Sanhueza et al. 1990; Remde and Conrad 1991c; Slemr and Seiler 1991; Cárdenas et al. 1993; Saad and Conrad 1993a). Nitrogen fertilization also frequently increases N_2O efflux (reviewed in Sahrawat and Keeney 1986; Eichner 1990; Aulakh et al. 1992). The form of fertilizer affects total N_2O losses. There is a general (non-significant) trend across agricultural systems towards higher N_2O emissions from ammonium-based fertilizers (Eichner 1990). It would not be surprising were this trend significant, since NH_4^+ provides fuel for nitrifiers and, after it is oxidized, also for denitrifiers. Fertilizers of varying types also affect soil pH and other aspects of soil chemistry, thereby altering microbial and chemical N-oxide production rates. Although the initial fertilizer effect is usually stimulatory, improved plant growth brought about by early season fertilization can ensure dense root growth and lead to lower N pools available for nitrification and denitrification later in the year (Aulakh et al. 1992).

For the purpose of predictive modelling, investigators often attempt to correlate NO and N_2O fluxes with measurements of nitrogen pool sizes and turnover rates. To give a few examples: NO (Anderson and Levine 1987; Williams and Fehsenfeld 1991; Williams, Hutchinson et al. 1992; Cárdenas et al. 1993) and N_2O fluxes (Bronson et al. 1992; Williams, Hutchinson et al. 1992) have often been significantly correlated to soil NO_3^- concentrations. This does not, of course, necessarily entail a denitrification source. The presence of excess NO_3^- in soil indicates a high N/C ratio, and N availability for non-assimilatory processes like autotrophic nitrification and denitrification (Davidson 1991). NO and N_2O fluxes are also often correlated to soil NH_4^+ concentrations (Williams, Hutchinson et al. 1992; Skiba et al. 1993). Fluxes of N_2O were significantly correlated to nitrification rates in a forest soil, but not to N-mineralization rates (Sitaula and Bakken 1993). Finally, on a broad across-ecosystem scale in the Amazon, Matson et al. (1990) could explain N_2O fluxes reasonably well based on soil N concentrations and turnover rates, but

within ecosystems the flux variability was controlled by other factors.

The Achilles heel of all such studies attempting to predict gas fluxes based on nitrogen indices is that these indices are closely related to other controlling factors including soil water, O_2, organic carbon, and plant growth (e.g. Grundmann et al. 1988; reviewed in Aulakh et al. 1988). Thus, low soil NO_3^- concentrations may result from leaching in wet soils, or previous removal by denitrification. In both cases the potential for denitrification is high. Many of the controls of gaseous N-oxide flux are nonlinear, field variability is great, and the variables controlling nitrification and denitrification are disparate. Either broad landscape models integrating over large areas or complicated models of specific conditions may be necessary to predict N_2O flux (Aulakh et al. 1992). NO prediction may be somewhat simpler than N_2O because of the predominance of nitrification over denitrification.

A wide range of fertilizer loss rates have been reported in the literature. Losses of nitrified nitrogen as NO vary from 0.06-11% (reviewed in Williams, Hutchinson et al. 1992; Yienger and Levi 1995). The range for fertilizer N_2O losses is even higher, in part due to the higher contribution of denitrification, and ranges from 0-20% (reviewed in Eichner 1990; Williams, Hutchinson et al. 1992). Few studies integrate fluxes over entire annual cycles, so loss estimates are generally lowest-case scenarios.

The influence of nitrogen availability on nitrification and denitrification rates and resultant NO and N_2O fluxes is obvious. Perhaps less obvious are the effects on denitrification end-product ratios. The various N-oxide reductases are regulated by abundances of denitrification pathway intermediates in the environment. In soil, NO_3^- is the most studied and likely the most important of these, since this species occurs at higher concentrations than other intermediates. NO_3^-, (Blackmer and Bremner 1978; Gaskell et al. 1981; Knowles 1982), NO_2^- (Gaskell et al. 1981), and NO (Gaskell et al. 1981) all inhibit N_2O reductase activity in denitrifiers, but to different degrees, and not in all organisms (Betlach and Tiedje 1981). NO_3^- may also inhibit NO reductase (Payne and Riley 1969). Inhibition is not absolute, a small amount of NO_3^- is necessary to stimulate the production of N_2O reductase (Blackmer and Bremner 1979).

The N/C ratio rather than the absolute NO_3^- concentration is probably the key factor regulating the inhibition of N_2O (and other N-oxide) reductases. Thus, inhibition by NO_3^- is less severe in organic than in mineral soils, and N_2O reduction is stimulated when organic residues are incorporated into soil (reviewed in Sahrawat and Keeney 1986). Soils with high NO_3^- availability relative to C availability typically display very leaky denitrification, with high proportions of N_2O/N_2 (Firestone et al. 1980; Weier et al. 1993) and NO/N_2 (Baumgärtner and Conrad 1992b) as products.

3.3.6.2 Ambient NO mixing ratios and the compensation point. The NO flux across a soil surface is a balance of productive and consumptive processes occurring within the soil column. Nitrogen monoxide production by nitrifiers is virtually zero-order, but consumption is first-order (Galbally and Johansson 1989; Remde et al. 1989), so a compensation point exists where production and consumption processes are in balance (Conrad 1994). The direction of soil flux therefore depends on ambient NO mixing ratios, which range from <0.05 parts per billion of volume (ppbv) in clean rural air, to over 100 ppbv in polluted urban air (Logan 1983). Soil compensation points overlap this range, with measurements varying from 0.5-1500 ppbv in dry aerobic soils (Galbally and Roy 1978; Johansson and Galbally 1984; Johansson and Granat 1984; Remde et al. 1989; Slemr and Seiler 1991; Baumgärtner and Conrad 1992a; Kim et al. 1994). In anaerobic denitrifying soils the compensation points are generally higher than in aerobic soils (Johansson and Galbally 1984; Remde et al. 1989; Rudolph and Conrad 1996; Rudolph et al. 1996). Although only infrequently has net NO uptake been measured in the field (Slemr and Seiler 1991; Skiba et al. 1994), there is a negative correlation between ambient NO mixing ratios and soil NO emission rates (Slemr and Seiler 1991).

Because NO consumption is concentration-dependent, it exerts more control on net flux as the NO mixing ratio rises (Galbally and Johansson 1989; Remde et al. 1989). However, the importance of consumptive processes is not limited to polluted areas. Where production rates are rapid compared to diffusion in soil, soil NO mixing ratios are much higher than those in the (unpolluted) overlying atmosphere. A

model based on measured NO diffusion, NO production, and NO consumption rates in a loam soil predicted that compensation NO mixing ratios were reached within <3 cm of the soil surface (Galbally and Johansson 1989). A slightly higher compensation depth of 10 cm was found in a denitrifying beech forest soil (Rudolph et al. 1996). During nitrification and denitrification of fertilizer nitrogen, NO mixing ratios in soil air reach values several orders of magnitude higher than in ambient air (Rudolph and Conrad 1996; Dunfield and Knowles 1997), and most of the NO produced can be consumed before escaping to the atmosphere (Dunfield and Knowles 1997).

3.3.6.3 Soil water. The effects of H_2O are complex because they act through nitrogen mineralization, product diffusion out of soil, and O_2 diffusion. They are compounded by differences in structure and in C and N status of various soils. Water effects are not absolute but rather show pronounced hysteresis.

Bursts of NO production often follow rainfall events (Johansson and Granat 1984; Anderson and Levine 1987; Williams et al. 1987; Johansson et al. 1988; Davidson 1992; Davidson et al. 1993; Hutchinson et al. 1993; Stocker et al. 1993). The use of nitrification inhibitors in three of these studies showed this to result from nitrification (Davidson 1992; Davidson et al. 1993; Hutchinson et al. 1993). Such rainfall "pulses", when included in a recent mechanistic NO flux model, accounted for 25% of total soil NO_x emissions (Yienger and Levy 1995). N_2O production bursts also follow rainfall (Terry et al. 1981; Duxbury et al. 1982; Goodroad and Keeney 1984b; Anderson and Levine 1987; Davidson et al. 1993; Mummey et al. 1994).

Davidson (1991, 1992; Davidson et al. 1993) theorized that NO_2^- accumulates in thin water films of drying soil, and undergoes chemodenitrification upon rewetting. The concurrent production of H^+ along with NO_2^- by ammonia oxidizers acidifies these microsites and accelerates chemodenitrification rates. Drying and rewetting cycles also stimulate carbon and nitrogen mineralization from organic matter (Birch 1958; Birch 1960; Agarwal et al. 1971; Davidson et al. 1993), so the "pulsing" effect may simply reflect increased microbial activity, especially nitrification of transiently high NH_4^+ levels (Birch 1960; Davidson et al. 1993; Mummey et al. 1994), but

perhaps also heterotrophic nitrification and denitrification (Groffman and Tiedje 1988; Drury et al. 1992). The severity and length of the antecedent drying period controls the amount of nitrogen mineralized upon rewetting, as well as the amount of NO_2^- accumulated, and therefore also controls the magnitudes of NO and N_2O pulses (Birch 1960; Davidson et al. 1993; Stocker et al. 1993). Denitrifying and nitrifying bacteria survive extended drought and become active within minutes to hours after wetting soil (Smith and Parsons 1985; Rudaz et al. 1991; Davidson et al. 1993; Dendooven et al. 1994). Any preformed denitrifying enzymes are immediately uninhibited, and synthesis derepressed in 1-3 h (Smith and Tiedje 1979).

After the initial burst of activity, high moisture content may then decrease NO efflux rates relative to dry soil (Johansson and Granat 1984; Anderson and Levine 1987; Davidson et al. 1993). Negative correlations between soil moisture content and NO emissions have frequently been noted (Smith and Chalk 1979; Shepherd et al. 1991; Drury et al. 1992; Cárdenas et al. 1993; Davidson et al. 1993; Skiba et al. 1993; Yamulki et al. 1995). It has been suggested that the reduced diffusion rate in wet soil facilitates reduction of NO to N_2O before it can escape to the atmosphere (Davidson et al. 1993; Yamulki et al. 1995), but a similar argument applies to oxidative NO consumption. Thus a decreasing ratio of NO to other products with increasing water content is evident in both anaerobic (Drury et al. 1992; Schuster and Conrad 1992) and aerobic systems (Hutchinson et al. 1993). Supporting evidence for this diffusion effect is the observation that coarse-textured soils generally produce more NO than fine-textured soils (Bakwin et al. 1990).

Other studies have shown no effect (Skiba et al. 1994; Parsons and Keller 1995) or a positive effect (Slemr and Seiler 1991; Williams and Fehsenfeld 1991) of H_2O on NO flux. These contradictory results probably reflect differences in soil moisture ranges tested, differences in soil texture, and perhaps differences in the dominant microbial NO source. The most likely pattern is an inhibition when soil water is near and above saturation due to inhibited nitrification and diffusion (Johansson and Granat 1984; Anderson and Levine 1987; Stocker et al. 1993), water-stress of nitrifying and denitrifying bacteria at very low moisture, and an optimum

midrange. Precisely such a pattern was observed when a wide moisture range was examined in a tropical savannah (Cárdenas et al. 1993). Optimum flux occurred between 30-60% water-filled pore space (WFPS), with inhibition above and below this range.

With regard to N_2O, increasing soil H_2O nearly always increases production (Goodroad and Keeney 1984a; Davidson et al. 1986; Anderson and Levine 1987; Klemedtsson et al. 1988a; Shepherd et al. 1991; Davidson et al. 1993; Paul et al. 1993; Yamulki et al. 1995; Maag and Vinther 1996). Presumably this is an effect of oxygen tension on denitrification, whether it be classical heterotrophic denitrification or NO_2^- reduction by ammonia oxidizers. It has been shown using nitrification inhibitors that increasing soil moisture content leads not only to more rapid denitrification, but also to higher N_2O/NO_3^- ratios from nitrification (Bremner and Blackmer 1979; Davidson et al. 1986; Davidson et al. 1993; Maag and Vinther 1996). This trend holds as long as AMO activity does not become O_2-limited. Therefore, optimum N_2O production through nitrification occurred at 90% WHC in a loam soil. At 100% WHC, AMO became O_2-limited (Klemedtsson et al. 1988a).

Several lines of evidence suggest that microaerobic conditions, and not complete anaerobiosis, promote denitrification-based N_2O emissions as well. Because carbon and nitrogen mineralization rates are higher in the presence of O_2, denitrification may be limited by complete anaerobiosis (Abou Seada and Ottow 1985). Denitrifying enzymes also show different thresholds for regulation by O_2. N_2O reductase is the most O_2-sensitive (Hochstein et al. 1984; Lloyd et al. 1987), and therefore low pO_2 favours incomplete reduction to N_2O, while full anaerobiosis promotes further reduction to N_2. Increasing water content will limit O_2 diffusion and create the reduced pO_2 conditions necessary for denitrification. However, similar to the situation discussed for NO, extremely high water contents will slow the escape of N_2O from soil and encourage further reduction to N_2 (Mahendrappa and Smith 1967; Drury et al. 1992).

From these and similar studies a model of gaseous N-oxide flux can be made in relation to WFPS, which is the measure of soil water content most closely related

to diffusion (reviewed in Linn and Doran 1984; Davidson 1991; Aulakh et al. 1992; Williams, Hutchinson et al. 1992). The nitrification rate in soil is optimized at 50-70% WFPS. More NO_2^- reduction occurs at the wetter end of this optimum range due to low pO_2, but above 70% WFPS, AMO becomes O_2-limited. The 60% WFPS optimum seems to be relatively constant for aerobic microbial processes in soil, and represents the transition from water limitation to O_2 limitation (Linn and Doran 1984). The principal habitat of aerobic denitrifiers seems to be soil microbiology laboratories, and denitrification in the field generally only begins above a critical threshold of about 70% WFPS (reviewed in Aulakh et al. 1992). At WFPS > 80%, diffusion is severely impaired and gaseous N-oxides are effectively reduced to N_2. The WFPS optimum for soil N_2O efflux (80%) is higher than the NO optimum (60%) (Anderson and Levine 1987; Davidson 1992; Yamulki et al. 1995) because of a higher potential contribution from denitrification.

This is an effective conceptual model. Of course, the ranges given vary with other soil physical and chemical factors. Carbon excess leads to rapid microbial metabolism and produces anaerobic microsites. These can contribute to N_2O flux even when the mean soil WFPS < 70% (reviewed in Aulakh et al. 1992). Localized carbon-rich, anaerobic zones may represent the major sources of N_2O in aerobic soils (Parkin 1987). The existence of anaerobic microsites in soil aggregates has been demonstrated directly using O_2 microsensors (Sexstone et al. 1985).

There are also temporal effects of soil wet-up and wet-down. The "pulsing" effect on total nitrification and denitrification rates has already been described. There are also temporal effects on end-product ratios. The various denitrifying enzymes show differential inhibition and repression by O_2. NO_3^- reductase is the least sensitive (Knowles 1982; Drury et al. 1991), and may persist under extended aerobic conditions (Dendooven and Anderson 1994). Although the pattern probably changes with soil type and denitrifier species, the order of increasing inhibition on further denitrification pathway steps in *Pseudomonas fluorescens* is NO_2^-, NO, and N_2O reductases (McKenney et al. 1994). Therefore as a soil is saturated or dries out and the O_2 tension changes, the ratios of denitrification endproducts undergo a series of

changes. This occurs in microsites during and following rainfall events, and also during spring melt at temperate latitudes.

Derepression of NO_3^- and NO_2^- reductases occur quickly (1-3 h) after the onset of anaerobiosis, but N_2O reductase is synthesized much slower (16-30 h) (Firestone and Tiedje 1979; Smith and Tiedje 1979; Dendooven and Anderson 1994). This leads to transiently high N_2O/N_2 (and perhaps NO/N_2) ratios just after soils are wetted. During drying a similar trend occurs. The N_2O reductase is either quickly degraded or inactivated by O_2 when soil denitrifiers are switched from anaerobic to aerobic conditions (Hochstein et al. 1984; Lloyd et al. 1987; Dendooven and Anderson 1995), while NO_2^- (Hochstein et al. 1984; Lloyd et al. 1987; Dendooven and Anderson 1995) and NO reductases (Remde and Conrad 1991b) persist for longer. This leads to increased relative proportions of N_2O and NO in denitrification products.

Diffusion rates are also dependent on soil structure. In one study, the stimulatory effect of soil H_2O on net N_2O reduction was more evident in a coarse-textured than in a fine-textured soil, presumably because the water films were thicker in the coarse soil and presented a longer diffusion path between the microbes and the air (Drury et al. 1992). On the other hand, coarser textures promote faster gas diffusion rates vertically through the soil column, which may increase the N_2O/N_2 ratios of denitrification when intact systems are studied (Maag and Vinther 1996).

3.3.6.4 pH. Chemodenitrification to NO is pH-dependent. This process accelerates with increasing acidity (Chalk and Smith 1983; Blackmer and Cerrato 1986; McKenney et al. 1990), and accounts for an increasing proportion of the total (chemical + microbial) NO_2^- reduction (McKenney et al. 1990).

Denitrifiers have an optimum pH range of about 6-8 (Knowles 1982). Although low-pH adapted species exist and probably dominate in acidic soils (Parkin et al. 1985), there is a generally declining denitrification potential with increasing soil acidity. This is perhaps as a side effect of decreased carbon and micronutrient availability (reviewed by Aulakh et al. 1992).

Denitrification product ratios are also pH-dependent. In the 3.5-8 range

representative of most soils, increasing acidity corresponds to increasing N_2O/N_2 ratios (Wijler and Delwiche 1954; Blackmer and Bremner 1978; Firestone et al. 1980; Koskinen and Keeney 1982; Näegele and Conrad 1990b), perhaps through inhibition of N_2O reductase (Blackmer and Bremner 1978; Gaskell et al. 1981). The proportion of NO also increases with increasing acidity (Koskinen and Keeney 1982; Näegele and Conrad 1990b). Part of the pH effect, especially in acid soils, may result from the activity of nitrate respirers, perhaps mediated through NO_2^- chemodenitrification (Näegele and Conrad 1990b).

Autotrophic nitrifiers in pure culture have 6-8.5 pH optima and are incapable of growth under acid conditions, probably due to substrate limitation at the high NH_4^+/NH_3 ratios in acid soils (Prosser 1989). Nitrate production in acid soils is therefore usually attributed to autotrophic nitrification in alkaline microsites (Prosser 1989), or to heterotrophically nitrifying fungi (Stroo et al. 1986; Prosser 1989). Recent studies do show that the pH optima for autotrophic nitrifiers decrease when growing in a biofilm (Allison and Prosser 1993), or in aggregates (De Boer, Klein Gunnewiek et al. 1991), so these organisms may be more important than previously believed in acid soils.

pH effects on nitrification are complex. For example, opposite effects of pH adjustment were observed on aerobic NO and N_2O fluxes from an alkaline agricultural soil versus an acidic forest soil (Näegele and Conrad 1990a). In a coniferous soil, pH adjustment effects depended on the soil horizon. Lowered pH from 6 to 4 increased N_2O production through nitrification in mineral and organic horizons but decreased it in the litter layer (Martikainen and De Boer 1993; Martikainen et al. 1993). The complexity of these findings results in part because absolute rates and product ratios are both altered in experiments where soil pH is artificially altered, and because various pH-adapted flora exist. The acidophilic autotrophic nitrifiers in forest soils (De Boer, Tietema et al. 1991) actually operate at higher total NH_4^+ oxidation rates as well as higher N_2O/NO_3^- ratios under increasingly acid conditions (Martikainen and De Boer 1993). The higher N_2O/NO_3^- ratios in acid soils may result from chemodenitrification. Also, aggregation under acid conditions may create reduced

pO_2 microsites (De Boer, Tietema et al. 1991).

3.3.6.5 Temperature. There is usually a strong positive temperature effect on NO emissions (Johansson and Granat 1984; Williams et al. 1987; Williams et al. 1988; Williams and Fehsenfeld 1991; Saad and Conrad 1993a; Stocker et al. 1993; Skiba et al. 1994). Because of this dependence, fluxes show diurnal variation (Anderson and Levine 1987; Shepherd et al. 1991; Williams and Fehsenfeld 1991; Yamulki et al. 1995) and plant harvest can increase NO efflux due to loss of shading (Hutchinson and Brams 1992). In temperate mineral soils, both NO consumption and NO production exhibit mesophilic bell-shaped temperature responses with optima of 25-30°C (Saad and Conrad 1993a), although this pattern may vary (Dunfield and Knowles 1997). Microbial NO uptake processes should partially compensate for the variability of NO production with temperature, since their regulation is similar.

Most Q_{10} (15-25°C) values for the soil flux studies cited above range from 2-4, similar to those of nitrifiers and denitrifiers (reviewed in Focht and Verstraete 1977; Knowles 1982; Williams, Hutchinson et al. 1992; Bouwman et al. 1993). The ranges and optima for denitrification include much higher temperatures than for autotrophic nitrifiers. Denitrification peaks at 40-70°C and can continue to 75°C (Focht and Verstraete 1977; Knowles 1982; McKenney et al. 1982; Aulakh et al. 1992), although at high temperatures most of the activity may be chemodenitrification (Keeney et al. 1979). Direct temperature effects on microbial denitrification are complicated by indirect effects mediated through O_2 solubility and O_2 consumption (Aulakh et al. 1992). Thus, temperature also affects the time to onset of anaerobic conditions, and the response time of denitrifiers to anaerobiosis (McKenney et al. 1984).

As with pH, it is difficult to generalize temperature responses because of microbial adaptation. There can be a marked adaptation of nitrifying bacteria to low temperatures (20°C optimum) in temperate zones (Malhi and McGill 1982), while tropical NO emissions show no temperature effect from 20-45°C, suggesting high-temperature tolerance (Johansson et al. 1988; Cárdenas et al. 1993). Denitrifying bacteria have been isolated with NO and N_2O production optima from 8-40°C (Aulakh

et al. 1992; Saad and Conrad 1993b).

Temperature may also affect the gaseous N-oxide ratios of nitrification and denitrification. The NO/N_2 (Bailey 1976) and N_2O/N_2 (Nömmik 1956; Maag and Vinther 1996) ratios from soil denitrification decrease with increasing temperature. At very high temperatures (>40°C) the trend reverses (Keeney et al. 1979). Both extremely low and high temperatures encourage a leaky N cycle. This can probably be explained based on an enzyme kinetic model, whereby any decrease in total denitrification rate, such as that brought about by a non-optimal temperature, will increase the ratios of N_2O and other denitrification intermediates relative to N_2 (Betlach and Tiedje 1981). This effect may be especially important in temperate soils, where maximum N_2O fluxes occur during spring thaw when soils are cool and saturated (e.g. Goodroad et al. 1984). Low temperatures also promote high N_2O/NO_3^- ratios from nitrification (Maag and Vinther 1996), and a similar enzyme-kinetic causative argument probably holds.

3.3.6.6 Other factors. Immobilization of nitrogen by microbes (Mummey et al. 1994) and plants (Aulakh et al. 1992) decreases the amount available for nitrification and denitrification. Root exudates and root decay provide carbon substrates, so most denitrifiers in soil occur in close proximity to plant roots (Linne von Berg and Bothe 1992). The net effect of plants is therefore an increased soil C/N ratio, which may either stimulate or reduce total denitrification depending upon which factor is limiting (reviewed in Knowles 1982). Plant transpiration also controls soil water and severely limits anaerobic conditions conducive to denitrification (Aulakh et al. 1992).

3.4 Acetylene, a specific inhibitor of everything

The processes outlined in this literature review can be studied using specific inhibitors. Inhibitors of nitrification, denitrification, methane oxidation, and methanogenesis have been comprehensively reviewed (Knowles 1982; Bédard and Knowles 1989; Knowles 1993). The inhibitor sensitivities of nitrification and methane oxidation are very similar, although allylsulfide shows promise as a

differential inhibitor (Roy and Knowles 1996). One of the most popular inhibitors for soil studies is C_2H_2, although it can hardly be considered specific. Acetylene inhibits N_2O reduction, N_2 fixation, hydrogenases, NH_3 oxidation, methanotrophy, methanogenesis, and ethylene oxidation (Knowles 1982).

Klemedtsson et al. (1988b) described a process for characterizing the N_2O sources from soil which exploits the differential sensitivities of nitrification and N_2O reduction to C_2H_2. 5 Pa C_2H_2 inhibits nitrification without affecting denitrification, while 10 kPa C_2H_2 inhibits N_2O reduction by denitrifiers. Their technique functions well for N_2O, but C_2H_2 effects on NO are complicated because of chemical reactions. Acetylene was noted by Paul et al. (1993) to react with NO, an effect which Dunfield and Knowles (1997) found to be O_2-dependent and postulated as a potential complicating factor in measuring aerobic denitrification. The mechanism of this reaction has since been better elucidated (McKenney et al. 1997), whereby C_2H_2 serves as a catalyst for reaction (3.14).

3.5 Concluding remarks

The major environmental controls of trace gas fluxes have been outlined. Soil water and soil nitrogen play key roles in all major processes described, but the exact natures of these controls cannot be predicted from simple, linear models. The influence of H_2O is mediated through diffusion of products and substrates. Diffusion of O_2 is especially important, as it regulates the relative rates of anaerobic versus aerobic metabolisms. Nitrogen in its various soil forms provides substrate for trace N-oxide gas production and also affects CH_4 metabolism, both productive and consumptive. The processes regulating trace gas fluxes are in principle clear, but the relative importances of these processes in various ecosystems, and the interactions among them, are still poorly understood.

PREFACE TO SECTION 4

Section 4 presents results of a field experiment designed to assess N_2O and CH_4 emissions from a fertilized humisol. Several findings of Section 4, the rapid nitrification rate and the N_2O efflux stimulation after watering and fertilizer addition, were the basis for more detailed studies of gaseous N-oxide fluxes presented in Sections 6-8.

Field CH_4 oxidation was not inhibited by fertilization. This result is discussed in relation to Section 5, a closer examination of the kinetic mechanisms and the severity of inhibition by various forms of nitrogen. Although Section 5 showed NH_4^+ to be a potent potential inhibitor of CH_4 oxidation at field application rates, temporal and spatial factors explain the lack of any significant effect observed *in situ*.

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Dunfield PF, Topp E, Archambault C, Knowles R (1995) Effect of nitrogen fertilizers and moisture content on CH_4 and N_2O fluxes in a humisol: measurements in the field and intact soil cores. Biogeochem 29:199-222

SECTION 4. EFFECTS OF NITROGEN FERTILIZERS AND MOISTURE CONTENT ON CH_4 AND N_2O FLUXES IN A HUMISOL: MEASUREMENTS IN THE FIELD AND INTACT SOIL CORES

4.1 Abstract

Field and laboratory studies were conducted to determine effects of nitrogen fertilizers and soil water content on N_2O and CH_4 fluxes in a humisol located on the Central Experimental Farm of Agriculture Canada, Ottawa. Addition of 100 kg N ha^{-1} as either urea or $NaNO_3$ had no significant effect on soil CH_4 flux measured using chambers. Fertilization with $NaNO_3$ resulted in a significant but transitory stimulation of N_2O production. Inorganic soil N profiles and the potential nitrification rate suggested that much of the NH_4^+ from urea hydrolysis was rapidly nitrified. CH_4 fluxes measured using capped soil cores agreed well with fluxes measured using field chambers, and with fluxes calculated from soil gas mixing ratio gradients with Fick's diffusion law. This humisol presents an ideal, vertically homogeneous system in which to study gas diffusion, and the influence of gas-filled porosity on CH_4 uptake. In soil cores gradually saturated with H_2O, the relationship of CH_4 flux to gas-filled porosity was an exponential rise to a maximum. Steepening CH_4 mixing ratio gradients partially compensated for the decreasing diffusion coefficient of CH_4 in soil matrix air as water content increased, and diffusion limitation of CH_4 oxidation occurred only at water contents $>130\%$ (dry weight), or gas-filled porosities <0.2.

4.2 Introduction

Nitrous oxide (N_2O) and methane (CH_4) are trace atmospheric constituents important in global warming and ozone chemistry (Duxbury and Mosier 1993). Microbial oxidation of atmospheric CH_4 occurs widely in aerobic soils, an important phenomenon in light of increasing atmospheric CH_4, and declining CH_4 oxidation by atmospheric OH (Ojima et al. 1993). Soil microorganisms are the major global source of N_2O, primarily through the processes of nitrification and denitrification. Denitrifiers can also consume N_2O, but soils are not considered a significant net N_2O

sink (Duxbury and Mosier 1993). Increased soil N_2O production upon addition of N fertilizers is well documented (Eichner 1990).

Conversion of natural ecosystems to cultivation has been linked to declining CH_4-oxidizing activity (Keller et al. 1990; Mosier et al. 1991; Bronson and Mosier 1993; Lessard et al. 1994). Nitrogen fertilization is often cited as a cause of this trend, but field studies show inconsistent results. Fertilization with 37 or 120 kg NH_4NO_3-N ha^{-1} over one year inhibited CH_4 oxidation in forest soils (Steudler et al. 1989), and strong inhibition resulted from 100 kg N ha^{-1} of KNO_3, NH_4Cl, or urea applied once to a drained peat (Crill et al. 1994). However, Bronson and Mosier (1993) observed no effect of up to 150 kg urea-N ha^{-1} addition on CH_4 oxidation in a clay soil under wheat, or of 218 kg N ha^{-1} in a clay loam under corn.

Study of the Broadbalk wheat experiment revealed decreased soil CH_4 oxidation potential under long-term (140 year) but not short-term fertilization (Hütsch et al. 1993). Nitrogen may affect CH_4 flux through long-term changes in microbial populations and ecological interactions rather than direct inhibition of extant populations. The nitrogen turnover rate rather than the absolute N level may be a controlling factor (Mosier et al. 1991; Hütsch et al. 1993). On the other hand, NH_4^+ (or more correctly, NH_3) inhibits CH_4 oxidation in pure cultures of methanotrophs (Bédard and Knowles 1989), and in soils and sediments (Nesbit and Breitenbeck 1992; Adamsen and King 1993; Bosse et al. 1993; Bronson and Mosier 1994). NH_3 probably inhibits CH_4 oxidation through competition with CH_4 for the active site of methane monooxygenase, and may also compete with CH_4 for ammonia monooxygenase in nitrifiers (Bédard and Knowles 1989). Nitrite and hydroxylamine produced through NH_3 oxidation are also inhibitory of methanotrophs (Hubley et al. 1975; King and Schnell 1994b). Both long-term and short-term effects of fertilization therefore seem plausible.

The diffusion rate of atmospheric CH_4 into soils may limit the microbial CH_4 oxidation rate (Dörr et al. 1993). Soil texture (Dörr et al. 1993), soil compaction (Hansen et al. 1993), and soil moisture content (Steudler et al. 1989; Adamsen and King 1993; Koschorreck and Conrad 1993; Lessard et al. 1994) all affect the CH_4

oxidation rates of soils, perhaps due to restriction of CH_4 diffusion as gas-filled porosity decreases. However, controlled studies of water effects in intact soil systems are lacking.

Because of high water-holding capacities, humisols can be seasonally flooded and present alternating anaerobic and aerobic conditions for microbial growth. Organic soils represent a unique environment of carbon and nitrogen cycling (Tate 1982). The following paper examines effects of moisture content and nitrogen fertilizers on CH_4 and N_2O fluxes in a humisol.

4.3 Materials and methods

4.3.1 Field nitrogen fertilization. All studies were undertaken in 1993 on a humisol located on the Central Experimental Farm of Agriculture Canada in Ottawa, Ontario. An organic layer (loss on ignition 70.6%; pH in H_2O 7.2; density 2.43 g cm^{-3}; bulk density 0.41 g cm^{-3}) was underlain by a clay pan at 30-40 cm depth. An uncharacterized plant community dominated by perennial grasses colonized the study area. Twelve 2 m × 2 m plots in a 6 m × 8 m block were randomly assigned to three treatments: no fertilizer addition, or 100 kg N ha^{-1} as urea (soluble) or $NaNO_3$ fertilizer broadcast onto plots followed by irrigation with 10 l H_2O per plot on calendar day (CD) 195.

Each plot contained three, 0.282 m^2 acrylic chamber collars, protruding 5 cm above the soil surface. These were used as described in Lessard et al. (1994) to determine N_2O and CH_4 fluxes at one-week intervals from CD 159 to CD 285 (8 June to 12 October), between 1100 h and 1500 h on each day. Soil temperature was estimated using thermistors buried to 7.5-cm depth in each chamber, and soil moisture by oven drying (50°C) to constant weight soil from 0-15 cm depth, pooled from at least three, 2.5 cm diameter cores.

CH_4 was measured using either a Varian (4.8-m Porapak N column; oven temperature 60°C; detector temperature 390°C) or a Shimadzu (1.2-m Porapak Q column; oven temperature 50°C; detector temperature 110°C) gas chromatograph with a flame ionization detector and a 0.5-ml sample loop. N_2O and O_2 were measured

using a Varian or a Perkin-Elmer (1.2-m Porapak Q column; oven temperature 40°C; detector temperature 275°C) gas chromatograph with an electron capture detector (^{63}Ni) and a 0.5-ml sample loop. Injection ports were used in other experiments when <2-ml samples were available.

For negative CH_4 fluxes (i.e. net CH_4 consumption), rate constants from first-order regressions of ln[CH_4] versus time were used to calculate flux at 1.7 ppmv CH_4. N_2O fluxes, and positive CH_4 fluxes, were estimated by linear regression. For statistical analyses the three chambers within each plot were pooled, and repeated measures analyses of variance (ANOVAs) performed with fertilizer treatment as a grouping factor and sampling day as a repeat factor, using SYSTAT (SYSTAT Inc. Evanston, Illinois). Homogeneity of variance was checked using residual plots. All multivariate probabilities given are the Pillai trace statistic, and multiple comparisons were done by the Bonferroni method.

Three 2.5 cm diameter soil cores per plot, divided into 0-3, 3-6, 6-12, 12-18, 18-24, and 24-30 cm depth intervals, were pooled at several dates after fertilization. A portion was frozen immediately and later extracted (2.5 g fresh wt. of soil: 10 ml of 2 *M* KCl) for colorimetric determination of NH_4^+ and NO_3^- using an automated analysis system described previously (Megraw and Knowles 1987a). A small amount of activated charcoal was added to decolorize extracts after this was shown not to affect N recovery. Within two days of sampling on CD 215, CD 222, and CD 229, 5.0 g $\pm$0.1 g subsamples (fresh wt.) of each plot $\times$ depth were placed in 60-ml serum vials containing ambient (2 ppmv) CH_4, and CH_4 mixing ratios determined at 24-h intervals for 3-4 days.

4.3.2 Potential nitrification. Soil sampled from 0-20 cm depth on CD 222 (81% H_2O) and stored at 12°C was slurried at a 1:3 weight ratio of soil : distilled deionized H_2O, and 15-ml aliquots distributed into 60-ml serum vials. NH_4Cl solutions (5 ml) were added to triplicate flasks at initial concentrations of 19 to 450 μg NH_4^+-N g soil^{-1}. Slurries were incubated at 25°C under ambient (2 ppmv) CH_4 on a gyratory shaker at 250 rpm. One-ml slurry samples were removed 1.5 h and 8 h after adding NH_4Cl and frozen immediately. For analyses, samples were thawed by centrifuging 5

min (4°C, 13000 × g), resuspended by manual shaking, and centrifuged again for 15 min. NH_4^+, NO_3^- and NO_2^- in the supernatant were analyzed colorimetrically (Megraw and Knowles 1987a).

4.3.3 Flux estimation methods comparison. On CD 201 and CD 222 (July 20 and August 10), various methods of estimating CH_4 and N_2O fluxes in unfertilized soil were compared. These involved: (i) field chambers as described above, in control plots only; (ii) soil cores with an enclosed headspace; (iii) field gas mixing ratio gradients; and (iv) gas mixing ratio gradients in soil cores.

Cores were 50 cm long, 7.8 cm diameter polyvinyl chloride tubes sharpened at one end. These were driven with a sledgehammer to 40-cm depth, such that the entire organic horizon and some of the clay pan was sampled. No compaction was evident. After removing cores, 0.5 cm diameter holes previously drilled at 5-cm intervals were fitted with plug-type rubber septa (for Shimadzu gas chromatographs), so air in the soil matrix and over the soil surface (headspace) could be sampled with syringes. Core bottoms were fitted with plastic caps, and a gas-tight seal assured with Terostat sealant (Teroson GmbH, Heidelberg, Germany). Soil cores were taken from within 3 m of the experimental plots, stored at 22.5-25°C, and gas fluxes determined within 2 (CD 201) or 5 (CD 222) days. For enclosure flux determination, core tops were capped as described above and 2-ml headspace samples removed at 30- to 60-min intervals for 2-3 h. When sampling enclosed cores, 1 atm pressure was maintained by reinjecting an equal volume of air.

Gas mixing ratio gradients were determined in cores by sampling 2 ml of headspace and soil matrix air through the 5-cm spaced ports, and in the field by inserting a 1.5-mm ID stainless steel tube to 5-, 10-, 20-, and 30-cm depths and withdrawing 10 ml of soil matrix air into syringes fitted with nylon valves. On CD 201, a field gradient was sampled within 20 cm of each coring site for direct comparison. Flux was calculated using Fick's law (Whalen et al. 1992; Koschorreck and Conrad 1993). For CH_4:

$$J = D_{CH_4\,soil} \times d[CH_4]/dz \qquad (4.1)$$

where J is flux (in μmol m^{-2} d^{-1}) and $D_{CH_4\,soil}$ is the binary diffusion coefficient of CH_4

in soil matrix air (in $m^2\ d^{-1}$), estimated as described below. CH_4 mixing ratios decreased linearly to 20-cm depth (e.g. Fig. 4.1), so $d[CH_4]/dz$ (in $\mu mol\ m^{-4}$) was estimated as the slope of the mixing ratio gradient.

To determine $D_{CH_4\ soil}$ in equation (4.1), CH_4 oxidation in two soil cores was halted using methyl fluoride (CH_3F) (Oremland and Culbertson 1992). One ml ($\cong 1\%$ of gas phase) of CH_3F (Matheson Gas Products, Ville St. Laurent, Quebec) was injected into each side port, 24 h and 2 h before injecting 0.05 ml ($\cong 100$ ppmv) CH_4 into the headspace. Addition of CH_3F to cores completely halted CH_4 oxidation 3 h later (data not shown). At 20- to 30-min intervals for 3 h, 1-ml gas samples from 0-20 cm depth were taken for CH_4 determination (Fig. 4.2). The initial point (immediately after injecting CH_4) was omitted from calculations because $d[CH_4]/dz$ was nonlinear, and headspace CH_4 may not have equilibrated. CH_4 fluxes were estimated using the relaxation constant (Sparks 1989), obtained from first-order regression of $\ln([CH_4] - [CH_4]_{equilibrium})$ versus time. Flux estimation by linear regression at 20-100 min and 100-220 min (Fig. 4.2), or by difference at each time interval, gave results within 10% of the estimate by first-order regression. From J and $d[CH_4]/dz$, $D_{CH_4\ soil}$ could then be estimated from equation (4.1) at each sampling time. $D_{N_2O\ soil}$ was calculated from $D_{CH_4\ soil}$ assuming the same ratio as $D_{N_2O\ air} / D_{CH_4\ air}$ (0.734) (Campbell 1985; Striegl 1993).

As an alternative to the experimental determination of $D_{CH_4\ soil}$, the following estimate was used:

$$D_{CH_4\ soil} = D_{CH_4\ air} \times a\ \phi_g^{\ b} \qquad (4.2)$$

where $D_{CH_4\ air}$ is 1.95 $m^2\ d^{-1}$ at 22.5°C (Striegl 1993), ϕ_g is fractional gas-filled soil porosity, and a and b are factors to compensate for soil-dependent tortuosity. Suggested average values of a=0.9 and b=2.3 were used (Campbell 1985).

Gas-filled porosity (ϕ_g) was determined in cores using a plastic cap fitted with a 60-ml syringe and a pressure valve (0-60 mm Hg) as a gas pycnometer (Vomocil, 1965). After fitting the cap and ensuring a gas-tight seal, enough air to raise the pressure by about 30 mm Hg was injected into the core headspace. Core gas volume

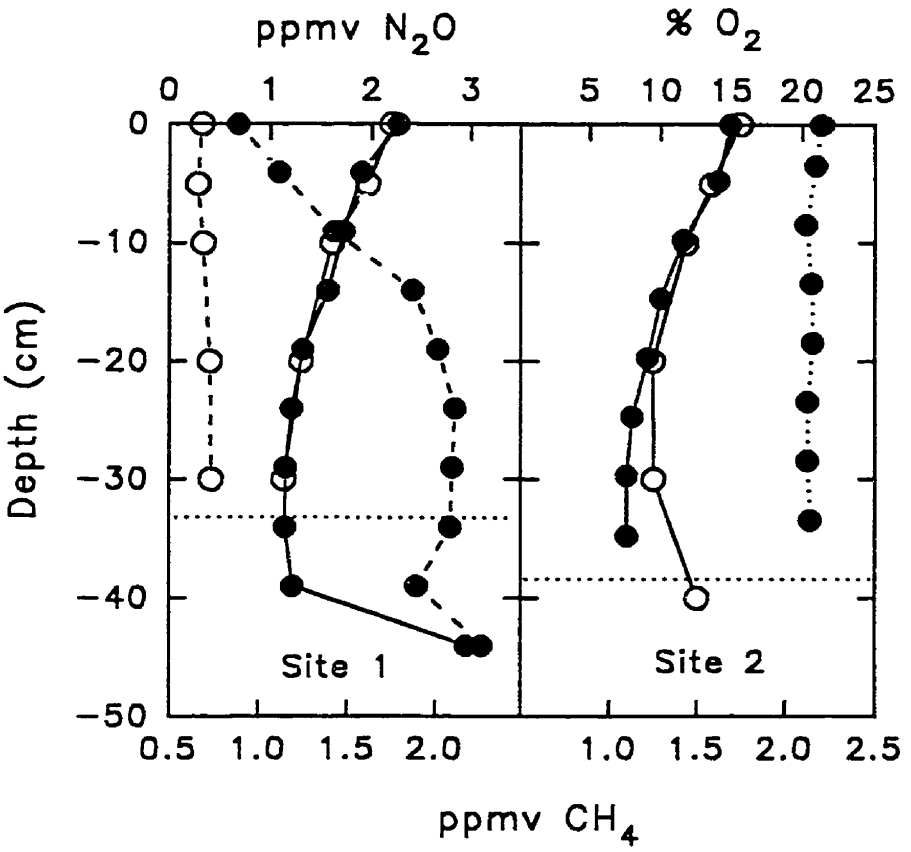

Fig. 4.1 Typical CH_4 (solid lines), N_2O (dashed lines), and O_2 (dotted lines) mixing ratio gradients measured in the field (○) and in soil cores (●) at two unfertilized sites on CD 201. Horizontal dotted lines indicate the position of the clay layer.

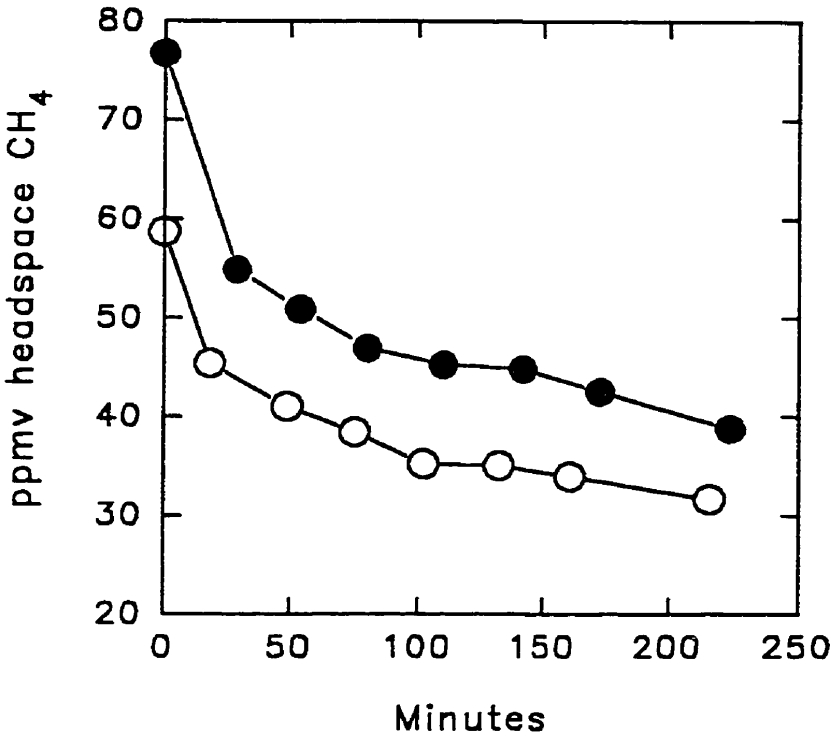

Fig. 4.2 Disappearance of added CH_4 from the headspace of two cores in which CH_4 oxidation was completely inhibited by CH_3F, used in the estimation of $D_{CH_4\ soil}$ on CD 201.

was determined from the equivalence of pressure × volume before and after injection. Cores were later harvested to determine the depth of the clay pan, and ϕ_g corrected by assuming a clay ϕ_g of zero. This assumption was found to be valid by comparing ϕ_g in deeper, 50-cm cores containing proportionally more clay. The ϕ_g of each core was considered separately in calculations involving that core or adjacent field sites on CD 201, while an average of 0.474 was used for field gradients on CD 222. The pycnometer procedure also served as a leak test for each core, and to estimate bulk density and soil density in cores dried at 50°C to constant weight.

4.3.4 The effect of H_2O on CH_4 consumption. A set of unfertilized soil cores was taken on CD 222. Three cores were kept as unwatered controls and three each were gradually saturated with H_2O in 50-ml or 150-ml increments, at 3- to 5-d intervals. One control and one core receiving 150-ml waterings were later discovered to be leaky and were discarded. Enclosure fluxes were measured at 5 h and 24 (±4) h after each H_2O addition, by sampling at 0.5-h intervals for 1.5 h. Gas mixing ratio gradients were measured after 24 (±4) h. Water penetration was slow on the first 3-4 waterings. To force water into the soil matrix at these times, and prevent percolation down the sides of cores, a 60-ml syringe was inserted into a subsurface port and a suction created. Cores were stored at 25°C, but flux measurements were done at room temperature varying from 23-27.5°C. All calculations accounted for temperature variation (Striegl 1993).

In a parallel set of cores, 1 ml of CH_3F was periodically injected into each side port and allowed to diffuse slowly out of the cores. These controls were intended to distinguish between H_2O effects on CH_4 oxidation through diffusion, and H_2O effects on methanogenesis. However, methanogenesis was shown to be insignificant without the need for these (see Results). Enclosure N_2O fluxes were measured at irregular intervals. CH_3F was assumed not to affect N_2O production, but this assumption is invalid if methanotrophs or nitrifiers contributed to N_2O flux and were inhibited by CH_3F.

4.4 Results

4.4.1 Field nitrogen fertilization. The field was a net CH_4 source until sometime between CD 194 and CD 201, when the moisture level declined from 130% to 95% (Fig. 4.3). Until late in the season, average CH_4 oxidation was then nearly constant between 20-25 μmol m^{-2} d^{-1}, despite a gradual decline in water content and, to a lesser extent, temperature. A repeated measures ANOVA of post-fertilization CH_4 flux showed no significant effects of fertilizer treatment (P=0.084), sampling day (P=0.21) or sampling day × fertilizer treatment (P=0.55).

N_2O fluxes were highly variable. On CD 173 and CD 186 (prior to fertilization) net fluxes were negative in 20 of 34, and 26 of 36 chambers, respectively. Although significance was not tested for each chamber, the binomial probability of obtaining 26 of 36 negative fluxes when the actual flux is nil, is only P(2-tail)=0.0064. The mean flux on CD 186 of -6.23 μmol N_2O m^{-2} d^{-1} (SEM=1.9), is significantly less than 0 at the P=0.01 level.

N_2O fluxes were log-transformed, and to avoid negative fluxes only data from CD 208 to CD 236 were used in the repeated measures ANOVA. N_2O flux on these days was significantly affected by treatment (P=0.015), and sampling day (P=0.002), but not by treatment × sampling day (P=0.30). Fluxes in control versus urea plots were not significantly different (P=0.11), but $NaNO_3$ plots produced significantly more N_2O than control plots (P=0.005). The stimulation by $NaNO_3$ was transitory (Fig. 4.3), and unless large flux episodes were missed by the sampling regime, accounted for a small fraction of the added fertilizer. Based on average fluxes between CD 201 and CD 222, 1.23 mmol m^{-2} of added NO_3^--N and 0.40 mmol m^{-2} of added urea-N (not significant) were lost as N_2O, or 0.17% and 0.056%, respectively, of the amounts added.

KCl-extractable NO_3^- profiles are shown in Fig. 4.4. No extra NH_4^+ was detected at any depth in urea plots compared to control plots on CD 201 or later (data not shown). This suggests that added urea, upon hydrolysis, was rapidly nitrified, volatilized, or assimilated by plants and microorganisms. Elevated NO_3^-

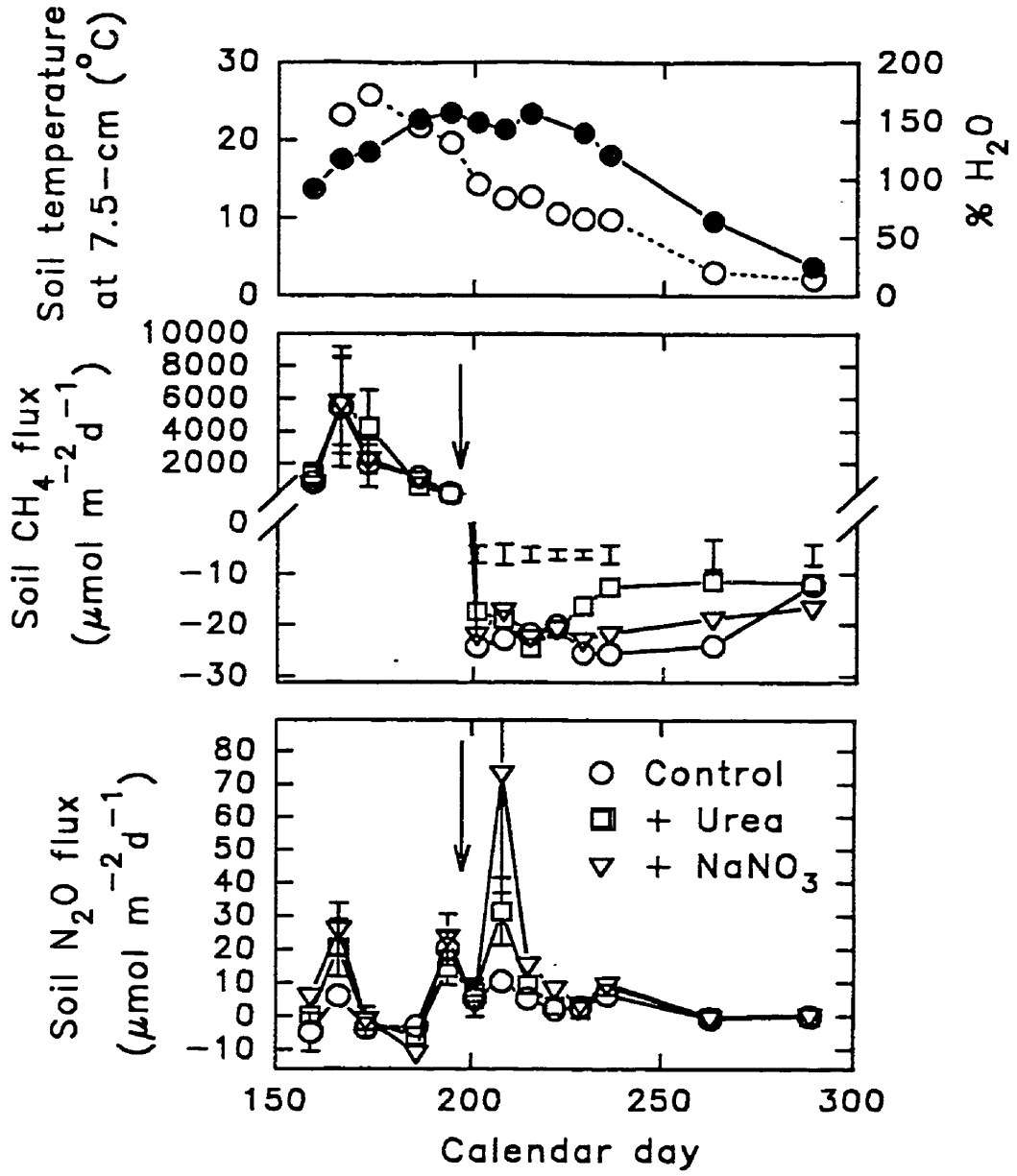

Fig. 4.3 Chamber CH_4 and N_2O fluxes in a humisol from 4 June to 16 October 1994, with corresponding soil moistures (% dry weight) (○) and soil temperatures at 7.5-cm (●). The arrow indicates the addition of 100 kg N ha^{-1} as urea or $NaNO_3$ on CD 195. Each point is the mean of four plots containing three chambers each, ±1 standard error of the mean (SEM) for each treatment. CH_4 flux error bars after CD 200 represent 1 SEM pooled for each day from the repeated measures ANOVA.

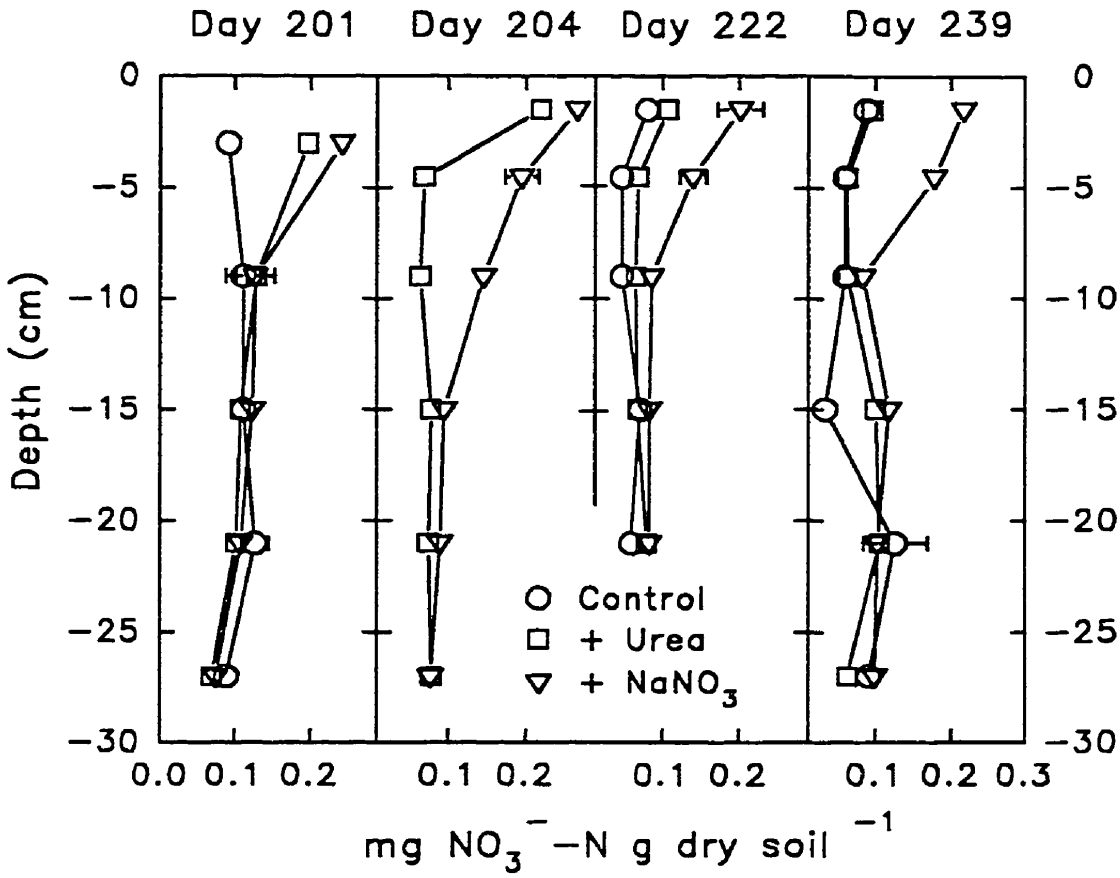

Fig. 4.4 2 *M* KCl-extractable NO_3^--N with depth and time, in control plots and plots receiving 100 kg N ha^{-1} as urea or $NaNO_3$ on CD 195. Data are means of 3 or 4 plots ± 1 SEM.

concentrations in urea plots on CD 201 implicates nitrification as the primary factor. Why NO_3^- in plots with added urea later declined while those in $NaNO_3$-fertilized plots did not is unclear, but may have been related to fertilization effects on plant growth and pH. Plant cover in $NaNO_3$-fertilized plots appeared chlorotic compared to other plots.

Typical depth profiles of potential CH_4 oxidation are shown in Fig. 4.5. A repeated measures ANOVA using sampling day (CD 215, CD 222, and CD 229), and depth (0-3, 3-6, and 6-12 cm) as repeat factors and fertilizer treatment as a grouping factor, showed neither significant fertilizer effects (P=0.53), nor significant interactive fertilizer effects (depth × fertilizer, P=0.96; sampling day × fertilizer, P=0.97; depth × day × fertilizer, P=0.23). The variability of CH_4 oxidation with depth was significant (P<0.001), as were sampling day (P=0.002) and depth × day effects (P=0.046).

4.4.2 Potential nitrification. Regardless of the amount of NH_4^+ added, 60-70% was not recoverable from the liquid phase of soil slurries and was assumed to be bound to exchange sites. This bound NH_4^+ was subject to nitrification, probably as it equilibrated with the solution. Average dissolved NH_4^+ concentrations (arithmetic means of concentrations at the beginning and end of the incubation) were 44 μM in control slurries and ranged from 91 μM to 2.1 mM in slurries with added NH_4^+. Nitrification, measured as appearance of NO_2^- and NO_3^-, was not affected by NH_4^+ concentrations over 91 μM (P=0.065; coefficient was a decrease of 3.6% per mM NH_4^+). The average nitrification rate in all vials with added NH_4^+ was 5.95 μmol N g dry soil^{-1} d^{-1}. An average of only 3.3% appeared as NO_2^- rather than NO_3^-.

4.4.3 Flux estimation methods comparison. Examples of field and core gas mixing ratio gradients on CD 201 are shown in Fig. 4.1. CH_4 mixing ratios declined linearly to 20-cm depth, but a slight horseshoe shape in many cases indicates two CH_4 sources, the atmosphere and a deep (>30-cm) soil layer. Soils were aerobic throughout the organic layer. Field and core CH_4 gradients were nearly identical at each sampling spot (e.g. Fig. 4.1), a fact reflected in the closeness of gradient-

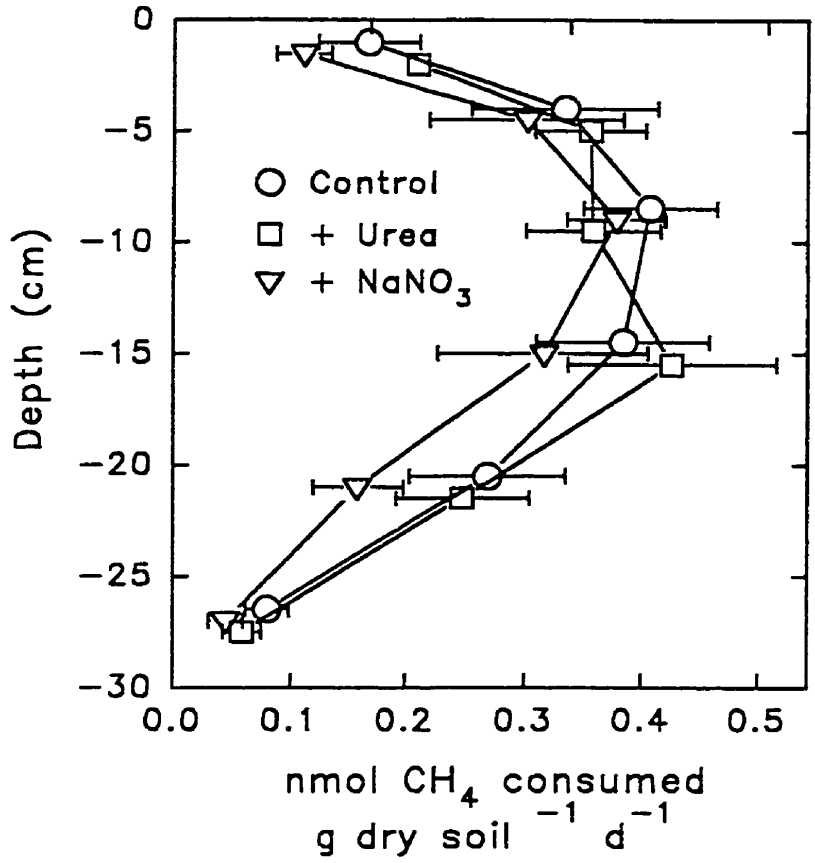

Fig. 4.5 Potential atmospheric (2 ppmv) CH_4 oxidation rates with depth on CD 215, of soil samples from control plots and plots receiving 100 kg N ha^{-1} as urea or $NaNO_3$ fertilizer on CD 195. Points are means of four samples ± 1 SEM.

calculated fluxes (Table 4.1). N_2O mixing ratio gradients were less consistent between the field and cores, although they were also linear to > 15-cm depth (Fig. 4.1).

Average ϕ_g was 0.395 on CD 201 and 0.474 on CD 222. Good agreement of CH_4 fluxes between field chambers and cores, and between gradient and enclosure estimates were obtained on both dates (Table 4.1). N_2O fluxes were much more variable (Table 4.2). N_2O flux comparisons are complicated by a non-normal distribution. Using a nonparametric Kruskal-Wallis test, core fluxes were significantly greater than chamber fluxes (P=0.003) on CD 201. However, $d[N_2O]/dz$ gradients in cores were not significantly greater than in adjacent field sites (Kruskal-Wallis test, P=0.076). The disparity between core and chamber fluxes could therefore result from non-random sampling (i.e. cores were taken from outside of the experimental area), combined with the large spatial variability of N_2O production, or it could result from some disturbance effect of coring. While core CH_4 fluxes can be extrapolated to the field, the same may not be true of N_2O. Applying Fick's law to N_2O mixing ratio gradients still agrees well with enclosure fluxes, if one considers field and core measurements separately (Table 4.2).

Equation (4.2) was used for empirical estimation of $D_{CH_4\ soil}$, due to a better agreement with chamber fluxes than other adjustments (Striegl 1993). Indeed, $D_{CH_4\ soil}$ determined in CH_3F-spiked cores on CD 201 (0.192 $m^2\ d^{-1}$) was close to the empirically estimated value from equation (4.2) (0.206 $m^2\ d^{-1}$). These values are similar to those reported for other soils (Koschorreck and Conrad 1993). The relaxation constant of added CH_4 in CH_3F-containing cores was -0.578 h^{-1}.

4.4.4 The effect of H_2O on CH_4 consumption. CH_4 oxidation increased nonlinearly with decreasing water content, exhibiting a plateau above about ϕ_g=0.2-0.25 (120-130% H_2O) (Fig. 4.6). An equation describing CH_4 oxidation as an exponential function of ϕ_g was fit to the cores watered in 50-ml increments, using the curve-fit function of SigmaPlot 5.1 (Jandel Scientific, San Rafael, CA, USA). This is unbiased over the entire range of ϕ_g tested and is of the form:

$$J\ (\mu mol\ m^{-2}\ d^{-1}) = 28.3 - 46.7\ e^{(-7.74\phi_g)}$$

Table 4.1 Fluxes of CH_4 (μmol m^{-2} d^{-1}) in a humisol determined by various methods on two dates, with standard errors of the mean (SEM) and coefficients of variation (CV). See text for details of methods and calculations.

Method	Calendar day 201				Calendar day 222			
	N	Flux	SEM	CV(%)	N	Flux	SEM	CV(%)
Enclosure methods								
Cores	11	-23.3	2.39	34.0	7	-28.9	2.51	22.9
Chambers	12	-23.9	4.16	60.3	12	-20.7	1.73	28.9
Gradient methods								
$D_{CH_4\ soil} = 0.9\ \phi_g^{2.3}\ D_{CH_4\ air}$								
Core gradients	11	-21.0	1.27	20.0	7	-23.2	1.79	20.4
Field gradients	11	-21.0	1.96	30.8	9	-20.2	6.28	92.6
$D_{CH_4\ soil} = 0.192\ m^2\ d^{-1}$								
Core gradients	11	-19.0	1.56	27.2				
Field gradients	11	-19.1	1.03	17.9				

Table 4.2 Fluxes of N_2O (μmol m^{-2} d^{-1}) in a humisol determined by various methods on calendar day 201, with standard errors of the mean (SEM) and coefficients of variation (CV).

Method	N	Flux	SEM	CV(%)
Enclosure methods				
Cores	5	43.5	9.33	47.9
Chambers	12	6.39	3.57	193
Gradient methods				
$D_{N2O\ soil} = 0.9\ \phi_g^{2.3}\ D_{N2O\ air}$				
Core gradients	5	36.4	13.3	81.9
Field gradients	5	10.3	7.35	159
$D_{N2O\ soil} = 0.141\ m^2\ d^{-1}$				
Core gradients	5	30.5	10.6	77.5
Field gradients	5	7.94	5.30	150

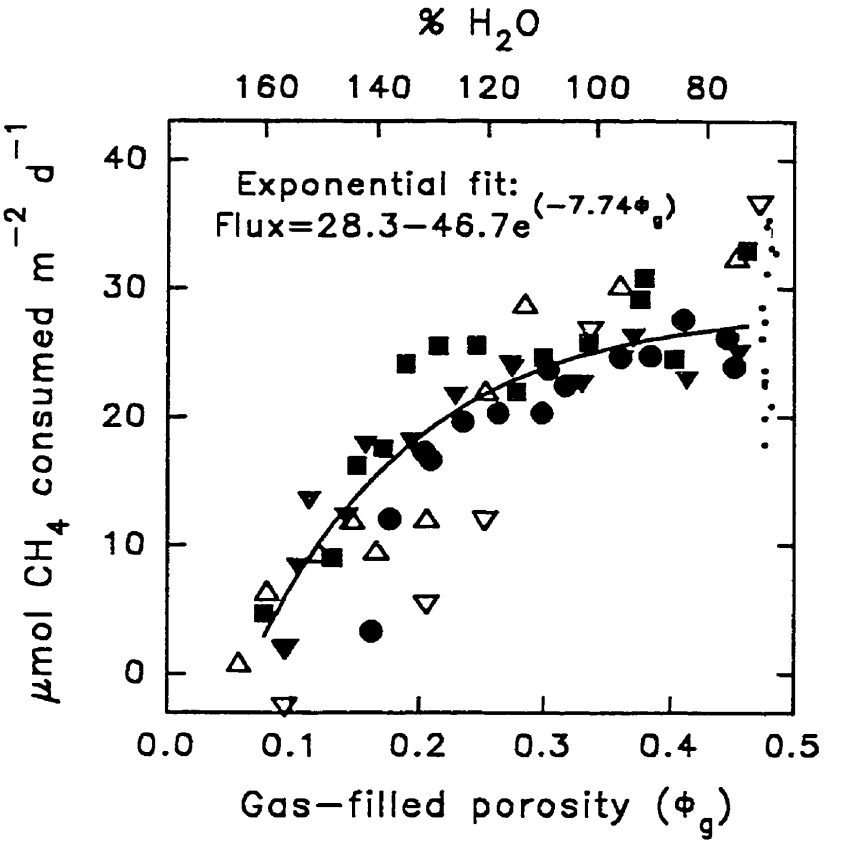

Fig. 4.6 Effect of H_2O content on CH_4 fluxes in soil cores. Each symbol type represents a separate core gradually saturated with H_2O in 50-ml (closed symbols) or 150-ml (open symbols) increments, at 3- to 5-d intervals. Fluxes are means of measurements taken 5 and 24 ($\pm$4) h after each H_2O addition. ϕ_g was estimated after each watering using a gas pycnometer; % H_2O from measurements at the start and end of the experiment only. Dots represent two cores unwatered throughout the experiment.

CH_4 oxidation in the two unwatered control cores decreased during the course of the experiment, by averages of 0.33 and 0.090 μmol m^{-2} d^{-2}. Core ϕ_g was experimentally decreased over time by watering, so any decline in soil CH_4-oxidizing capacity due to factors other than water content would introduce error into the experiment. Compensating for this decline by expressing fluxes as percentages of control cores on each day, and multiplying by the average control flux over the course of the experiment (23.5 μmol m^{-2} d^{-1}), gives a similar exponential relationship, with a sharper rise to the maximum:

$$J\ (\mu mol\ m^{-2}\ d^{-1}) = 23.3 - 78.9\ e^{(-16.7\phi_g)}$$

The decline does not affect the nature of the water relationship, only its magnitude.

Only cores watered in 50-ml increments were included in these calculations. In cores given 150-ml waterings, H_2O rather than gas was often sampled from the upper ports, and CH_4 gradients were often nonlinear, declining steeply in a 5-10 cm deep subsurface zone. The larger water addition may have had a piston effect, a front of infiltrating H_2O acting as a diffusion barrier. To show that this was not a problem in cores receiving 50-ml water additions, soil samples from 5-cm increments were taken at the end of the experiment and dried (50°C). There was no obvious increase or decrease of H_2O content with depth in these samples, it was constant within $\pm 12\%$ throughout the soil profile (data not shown).

Decreases in $d[CH_4]/dz$ (more negative values) as water content increased (Fig. 4.7) compensated in part for a concurrent decrease in $D_{CH_4\ soil}$, and flux remained constant over a wide ϕ_g range. Fig. 4.7 also suggests that the decline of CH_4 oxidation at low ϕ_g resulted from diffusion limitation rather than CH_4 production. $d[CH_4]/dz$ continued to decrease as long as H_2O was added, whereas an opposite trend would be expected if CH_4 production began. Also, CH_4 oxidation in cores watered in 50-ml increments was halted at the end of the experiment ($\phi_g \cong 0.11$) by injecting 1 ml CH_3F into each of the top four ports. No CH_4 flux, positive or negative, was then evident in cores (data not shown). The cores watered in 150-ml increments, when left at $\phi_g \cong 0.1$ for several days, did begin to produce CH_4.

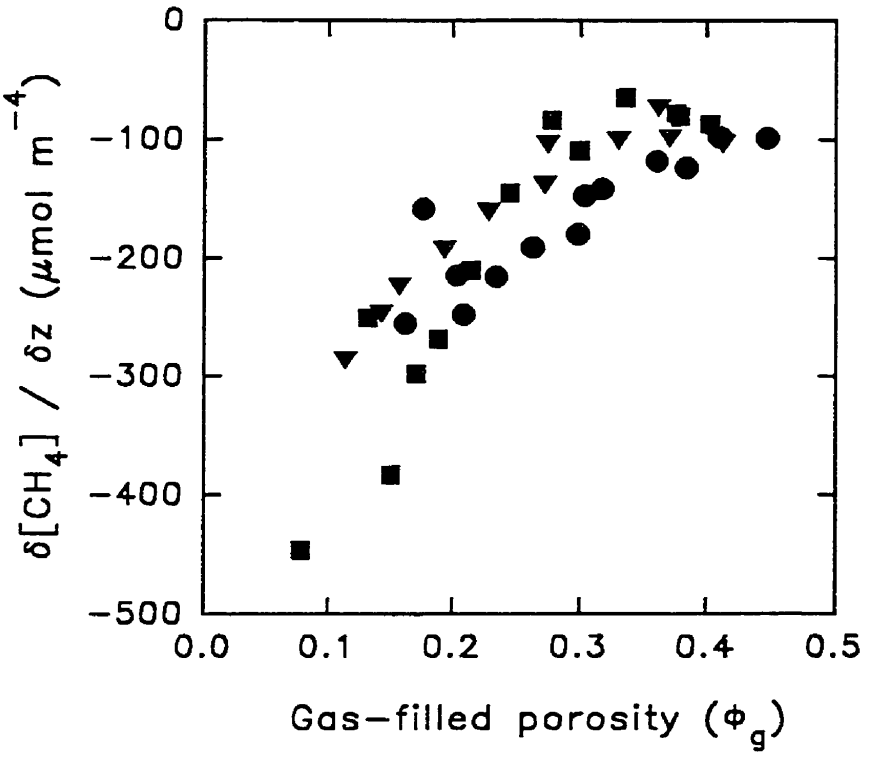

Fig. 4.7 Effect of gas-filled porosity (ϕ_g) on CH_4 mixing ratio gradients with depth (0-15 cm) in soil cores. Each symbol type represents a separate core gradually saturated with H_2O in 50-ml increments. Gradients were measured 24 ($\pm$4) h after each H_2O addition.

The coefficients a and b from equation (4.2) are empirical adjustments for soil tortuosity. These were estimated by fitting core enclosure flux, ϕ_g, and $d[CH_4]/dz$ to equations (4.1) and (4.2) for each 24-h sampling time of cores receiving 50-ml water additions. $d[CH_4]/dz$ was estimated for 0-15 cm, except on the last few dates when $[CH_4]$ reached a minimum before 15-cm depth. This gave a=0.67 and b=1.68, while a=0.9 and b=2.3 were average values suggested by Campbell (1985) and used in the methods comparison (Tables 4.1 and 4.2).

Gradient-based core CH_4 fluxes determined from equations (4.1) and (4.2) are unbiased in relation to ϕ_g (Fig. 4.8). Although using equation (4.2) with a=0.9 and b=2.3 (Campbell 1985) underestimates core enclosure fluxes, the underestimate is consistent over the entire range of ϕ_g. Our estimates of these factors (a=0.67, b=1.68) may not be ideal, since not enough low-flux points are present for even weighting. Nevertheless, they are also unbiased towards ϕ_g. The exponential nature of equation (4.2) seems valid, introducing no skew at extremes of ϕ_g.

N_2O fluxes in cores gradually saturated with H_2O are shown in Fig. 4.9. Due to extreme variability, the same ϕ_g versus flux relationships could not be calculated as with CH_4. Cores often showed a burst of N_2O production after water addition, which then gradually trailed off. Absolute water content also affected flux, as revealed by the gradually increasing size of the flux bursts at each watering time.

4.5 Discussion

4.5.1 Field nitrogen fertilization. Fertilization with 100 kg N ha^{-1}, or 1 mg N cm^{-2}, assuming penetration to only 5-cm depth, amounts to 0.48 mg N g dry $soil^{-1}$, about 34 m*M* N at 100% H_2O. This level of $NaNO_3$ inhibited CH_4 consumption in slurries by only 19% (Dunfield and Knowles, unpublished data), and such inhibition would occur in the upper 5 cm only. However, less than 500 μM NH_4Cl was needed to completely halt CH_4 oxidation in slurries (Dunfield and Knowles, unpublished data). Variations in penetration depth or moisture content have very little bearing on the conclusion that, assuming slurry studies were correct, 100 kg urea-N ha^{-1} should have

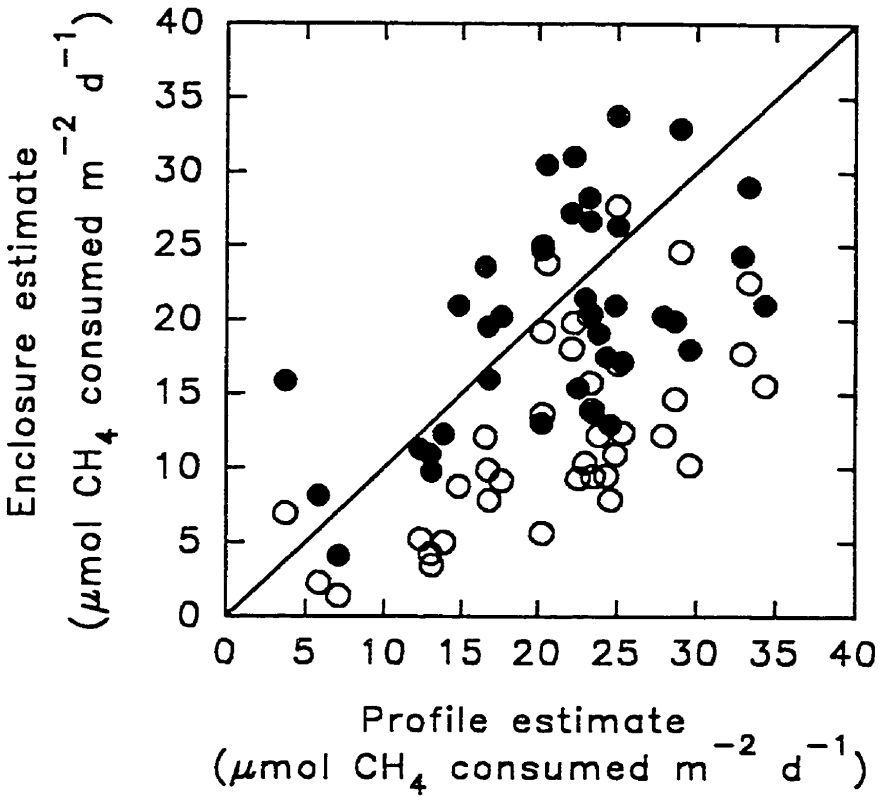

Fig. 4.8 Comparison of CH_4 fluxes in cores estimated either by measuring enclosed headspace depletion, or by applying Fick's law in the form $J = D_{CH_4\,air} \times a\,\phi_g^{\,b} \times d[CH_4]/dz$; where a=0.9 and b=2.3 (○), or a=0.67 and b=1.68 (●). A range of ϕ_g was created by gradually saturating cores with H_2O in 50-ml increments. The line at x=y is included as a visual aid only.

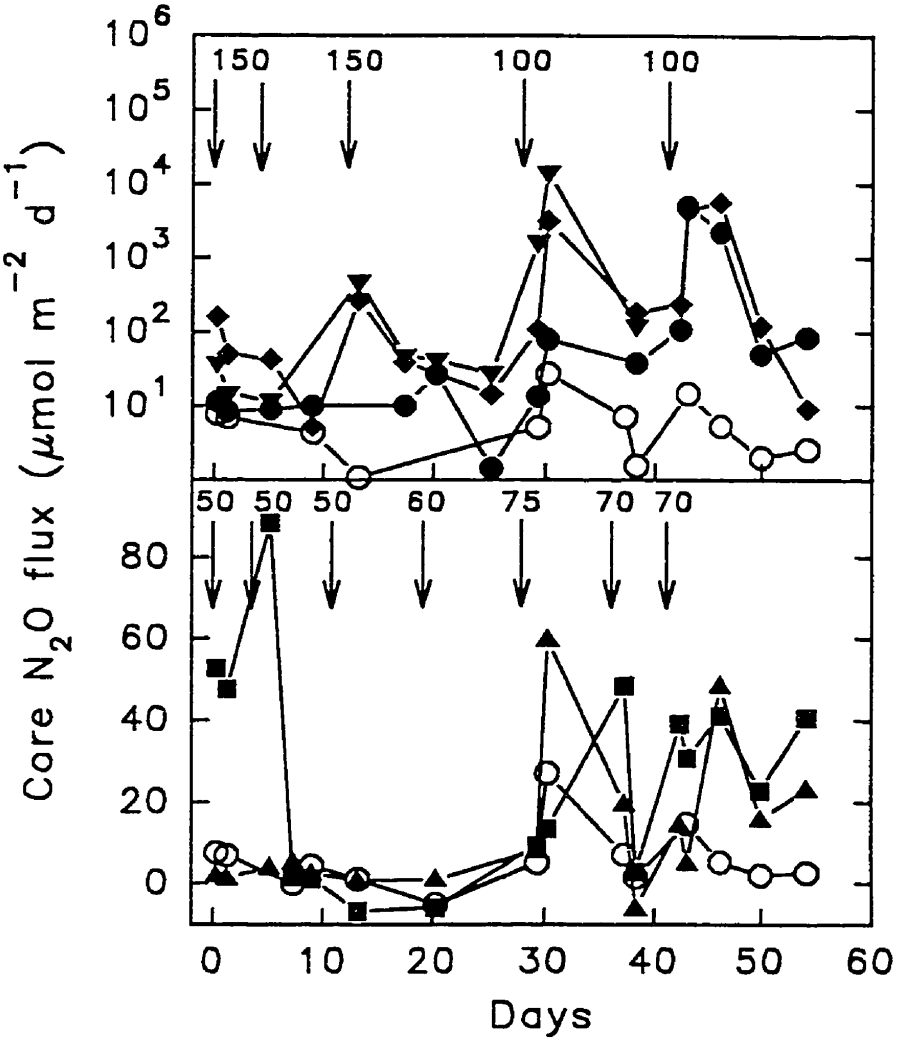

Fig. 4.9 Soil core N_2O fluxes in response to water additions. Arrows indicate the timing and amounts (in ml) of H_2O additions. Each symbol type represents a separate core, except (○) which represents average flux in three cores unwatered throughout the experiment.

inhibited field methanotrophic activity.

We suggest two hypotheses to explain the lack of such an effect in this study. (i) Most of the NH_4^+ released upon urea hydrolysis was immobilized on cation exchange sites in the upper few cm of the soil. The field showed a subsurface maximum of potential CH_4 oxidation similar to forest soils (Adamsen and King 1993; Koschorreck and Conrad 1993), although CH_4 oxidation was evident throughout the humic layer. Since CH_4 oxidation was not diffusion-limited, surface-localized NH_4^+ should have little effect on overall CH_4 flux. CH_4 oxidation would simply occur in deeper soil. However, incubations of soil at three, four, and five weeks following fertilizer application showed no inhibition of CH_4 oxidation at any depth, and no extra NH_4^+ was detectable in the upper few cm of urea-fertilized plots.

While surface-immobilization could have been a factor immediately after fertilization, a more likely explanation is: (ii) NH_4^+ was rapidly oxidized, volatilized as NH_3, or assimilated as urea was hydrolysed (Gould et al. 1986). Measurements of soil nitrogen implicate oxidation as a major factor. In comparison to control plots, no extra KCl-extractable NH_4^+ was recovered from urea plots six days after fertilization, but NO_3^- levels were elevated. The estimated nitrification rate of 5.95 μmol N g dry soil^{-1} d^{-1}, or 83.3 μg N g dry soil^{-1} d^{-1}, even without considering the possibilities of population growth and enzyme production, could consume 100 kg NH_4^+-N ha^{-1} in 5.8 days (assuming an active depth of 5 cm). This nitrification potential is higher than in most other soils (Malhi and McGill 1982, and references therein; Megraw and Knowles 1987a). As a comparison, CH_4 oxidation in another drained peat soil was inhibited by urea or NH_4Cl addition three weeks after fertilization, but NH_4^+ also remained elevated at this time (Crill et al. 1994). Bronson and Mosier (1993) observed inhibition of CH_4 oxidation from 218 kg urea-N ha^{-1} only immediately after fertilization. Cessation of inhibition corresponded to loss of soil NH_4^+.

Unlike some arable soils, humisols may have considerable methanogenic potential (Megraw and Knowles 1987b; Glenn et al. 1993). Our humisol exhibited net CH_4 production early in the season when flooded, and even after becoming a net CH_4 sink, a subsurface CH_4 source was indicated by CH_4 gradients. The deep CH_4

source occurred in the clay layer or humisol-clay interface, where O_2 diffusion may have been limited and reducing microsites occurred. Soil CH_4 profiles were not measured throughout the year, so the dynamics of this phenomenon are unclear. An endogenous dissolved CH_4 supply, even if transitory, could help maintain soil methanotrophs. Inhibition of CH_4 oxidation through long-term fertilization might then occur as elevated NO_3^- levels raise the soil redox potential and reduce methanogenesis. The CH_4 oxidation rate in this field (20-25 μmol m^{-2} d^{-1}) is at the low end of the range found in natural grasslands and forests (Steudler et al. 1989; Born et al. 1990; Ojima et al. 1993), but similar to levels in other cultivated soils (Mosier et al. 1991; Bronson and Mosier 1993; Hansen et al. 1993; Lessard et al. 1994). The endogenous CH_4 source may or may not mitigate net CH_4 flux, depending on its magnitude and on CH_4 diffusion rates.

The negative N_2O flux on CD 186 is unusual, but not without precedent (e.g. Ryden 1983). The soil at this time was still flooded, and under these reducing conditions denitrifying organisms may have been limited by the supply of oxidized nitrogen compounds. Complete reduction of NO_2^- and NO_3^- to N_2 would therefore be advantageous (Davidson 1991), as would the ability to scavenge ambient N_2O. N_2O fluxes are strongly affected by H_2O content, because this influences availability of O_2, NO_3^-, NO_2^-, and growth substrates. Bursts of N_2O production, as observed in our cores, often follow water addition (Davidson 1992; Bronson and Mosier 1993; Hansen et al. 1993). However, the CH_3F in these cores may complicate the results by inhibiting nitrifiers.

About 0.17% of added NO_3^--N, and 0.056% (not significant) of added urea-N were lost as N_2O in this experiment. The NO_3^--N loss as N_2O is within the ranges found in other experiments, while the urea-N loss is somewhat less (Eichner 1990). Stimulation of N_2O production by $NaNO_3$ suggests a denitrification source of N_2O. However, if nitrification of urea-N did occur as predicted in less than 6 days, the sampling regime could have missed an episodic, nitrification-related N_2O burst. On the initial post-fertilization sampling date (6 days) N_2O fluxes were small. This apparent delay probably attests to the number of temporally-variable factors

controlling N_2O fluxes (Davidson 1991).

The fact that $NaNO_3$ stimulated N_2O flux even though NO_3^- concentrations were also high (>1 m*M*) in control plots suggests that the ratio of products rather than the absolute denitrification rate was altered by fertilization. With more NO_3^- available, and perhaps limiting energy sources, reduction of N_2O would become less advantageous for denitrifiers. Inhibition of soil N_2O-reducing activity has been shown to result from NO_3^- addition (Blackmer and Bremner 1978). The transitory nature of N_2O production from $NaNO_3$, even while NO_3^- levels remained elevated, probably resulted from increasing aeration as water content declined. N_2O profiles late in the season indicated a subsurface N_2O source, perhaps because denitrification at this time was confined to anaerobic microsites in deep soil.

4.5.2 Diffusion. Application of Fick's law to mixing ratio gradients produced similar flux estimates as enclosure methods, over a wide range of ϕ_g. The usefulness of diffusion-based CH_4 flux estimation has been previously demonstrated by Koschorreck and Conrad (1993), using experimentally determined soil diffusion coefficients. Whalen et al. (1992) bypassed estimation of the diffusion coefficient in a forest soil by applying equation (4.2), but with less success than in our study. Vertical zonation and horizon structure introduce problems, although not with the humisol used in our study. Whalen et al. (1992) also pointed to the difficulty in applying average tortuosity adjustment values of a=0.9 and b=2.3 (Campbell 1985) to different soils. Accounting for tortuosity is a problem in any such study, and several correction factors have been suggested (Striegl 1993). Equation (4.2) worked well here, giving a $D_{CH_4\,soil}$ estimate similar to that determined in cores containing CH_3F, and causing no skew in flux estimation at extremes of ϕ_g. The suggested average values a=0.9 and b=2.3 were very close to those calculated from cores.

The use of soil cores for further study was justified by the agreement of field and core-derived CH_4 fluxes, whether by enclosure or diffusion-based estimates. Compared to chambers, the small surface area of cores and the restriction of lateral gas diffusion should result in higher variability, but this was not evident. Longer core incubation times (2-3 h versus 30 min) may have compensated by allowing more

precise measurements. Cores certainly are not perfect mimics of the field. The temperature variability with depth is much less, for example. However, other studies stress a relatively minor influence of temperature on CH_4 oxidation (Steudler et al. 1989; Born et al. 1990; Dörr et al. 1993; Dunfield et al. 1993; Koschorreck and Conrad 1993; Crill et al. 1994).

Since CH_4 relaxation constants are much larger than first-order rate constants for CH_4 consumption in tundra soil, movement of CH_4 through soil matrix air should not limit CH_4 oxidization (Whalen and Reeburgh 1990). However, diffusion in our humisol was much slower, perhaps due to small particle size and lack of macropores. In cores, the average relaxation constant on CD 201 (ϕ_g=0.4) was -0.58 h^{-1}, and the average first-order rate constant for CH_4 consumption was -0.11 h^{-1}. Although diffusion is the faster process, the figures are in the same order of magnitude. Gas transport could be a limiting factor in this soil, especially at higher water content.

The relationship between CH_4 oxidation and ϕ_g in soil cores was an exponential rise to a maximum. The exponential nature of the relationship is expected from the theoretical exponential dependence of the diffusion coefficient on ϕ_g (Campbell, 1985). At ϕ_g values above 0.2 (<130% H_2O) the plateau region of the relationship is reached, where the diffusion rate is very fast in relation to the microbial oxidation rate. Further increases in ϕ_g above 0.2 had little effect on CH_4 oxidation, either in cores or in the field. Soil CH_4 consumption acts as its own buffer, causing decreases in $d[CH_4]/dz$ (i.e. steeper mixing ratio gradients) which partially compensate for decreasing $D_{CH_4\,soil}$ as ϕ_g decreases. Microbial CH_4 consumption is concentration dependent, but at high ϕ_g, CH_4 gradients are shallow (CH_4 declined by <0.3 ppmv over 15 cm at ϕ_g>0.4), and relatively large changes in $d[CH_4]/dz$ can arise with small changes in absolute soil CH_4 mixing ratios.

Diffusion of CH_4 in soil water rather than soil air may limit methanotrophic activity, and explain the commonly observed dependence of CH_4 consumption on moisture content (Koschorreck and Conrad 1993). However, this hypothesis is not consistent with our data. Even as CH_4 oxidation in the humisol declined with H_2O contents above 130%, $d[CH_4]/dz$ (of soil matrix air) continued to steepen. If CH_4

diffusion through water films from gas-filled pores to methanotrophs was limiting, such a trend should not have occurred. In fact, the opposite would be expected. Rather, our data are consistent with the idea that at high H_2O content, CH_4 delivery from the atmosphere to subsurface methanotrophs is limited by its diffusion rate through soil matrix air. As diffusion rates decline in relation to CH_4 oxidation rates, subsurface CH_4 concentrations decrease and, since it is a first-order process, so does CH_4 oxidation. Another important point is that, although our humisol was diffusion-limited only at extremely high H_2O contents, limitation would occur over a greater range of H_2O content if potential CH_4 oxidation was much higher. Therefore, in soils with similar diffusion characteristics but higher CH_4 oxidation potentials (e.g. fine-textured forest soils), diffusion limitation of CH_4 oxidation could be evident at commonly occurring moisture contents.

Soil cores wetted in 50-ml increments had constant moisture content with depth, and no methanogenic activity, so this was purely a study of diffusion control. In the field, of course, this is not always the case. The exponential relationship did not hold in cores watered in 150-ml increments (equivalent to 3-cm rainfall), as diffusion in these cores may have been limited by a penetrating water front. Adamsen and King (1993) also noted an effect of the amount of water added to cores. In our humisol cores receiving 150-ml H_2O additions, methanogenesis began at $\phi_g \cong 0.1$, after a lag phase (data not shown). Methanogenesis as a control of flux was not investigated thoroughly here, but is probably a complex relationship involving total soil moisture, the timing and amount of rainfall events, and substrate availability.

That immediate inhibition of methanotrophs and methane monooxygenase from nitrogen fertilization can occur is well proven by laboratory soil studies (Nesbit and Breitenbeck 1992; Adamsen and King 1993; Bosse et al. 1993; Bronson and Mosier 1994). However the extent to which this potential inhibition manifests itself in the field is mitigated by the four-dimensional nature of soil. Temporal changes in soil nitrogen through processes such as nitrification may be critical. Physical soil factors controlling the downward movement of fertilizer must be considered in relation to the depth at which CH_4 oxidation does or can occur, and to soil porosity and water

content as determining the severity of diffusion limitation.

4.6 Acknowledgements

Thanks to P. Rochette for chambers, and to R. Carrier, M. Kuhl, and C. Tauchner for laboratory assistance.

PREFACE TO SECTION 5

Although no significant inhibition of methane oxidation resulted from nitrogen fertilization in the field, NH_4^+, NO_2^-, and NO_3^- are known inhibitors of CH_4 oxidation. The mechanisms of inhibition are poorly understood. The examination of the kinetic mechanisms of inhibition presented in Section 5 forms a companion study to Section 4, where temporal and spatial factors explained the lack of any field effect due to the inhibitory mechanisms described here.

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Dunfield PF, Knowles R (1995) Kinetics of inhibition of methane oxidation by nitrate, nitrite, and ammonium in a humisol. Appl Environ Microbiol 61:3129-3135

SECTION 5. KINETICS OF INHIBITION BY NITRATE, NITRITE, AND AMMONIUM, OF METHANE OXIDATION IN A HUMISOL

5.1 Abstract

The kinetics of inhibition of CH_4 oxidation by NH_4^+, NO_2^-, and NO_3^- in a humisol was investigated. Soil slurries exhibited nearly standard Michaelis-Menton kinetics, with half-saturation constant ($K_{m(app)}$) values for CH_4 of 50 to 200 parts per million of volume (ppmv) and V_{max} values of 1.1 to 2.5 nmol of CH_4 g of dry soil^{-1} h^{-1}. With one soil sample, NH_4^+ acted as a simple competitive inhibitor, with an estimated K_i of 8 μM NH_4^+ (18 nM NH_3). With another soil sample, the response to NH_4^+ addition was more complex and the inhibitory effect of NH_4^+ was greater than predicted by a simple competitive model at low CH_4 mixing ratios ($<$50 ppmv). This was probably due to NO_2^- produced through NH_4^+ oxidation. Added NO_2^- was inherently more inhibitory of CH_4 oxidation at low CH_4 mixing ratios, and more NO_2^- was produced as the CH_4-to-NH_4^+ ratio decreased and the competitive balance shifted. $NaNO_3$ was a noncompetitive inhibitor of CH_4 oxidation, but inhibition was evident only at $>$10 mM concentrations, which also altered soil pHs. Similar concentrations of NaCl were also inhibitory of CH_4 oxidation, so there may be no special inhibitory mechanism of nitrate *per se*.

5.2 Introduction

Methane (CH_4) is an important greenhouse gas that is increasing in atmospheric mixing ratio (Duxbury and Mosier 1993). Much interest has focused on the role of aerobic soils as a sink for CH_4 and the ecological and land-use practices that affect its magnitude (Ojima et al. 1993). Field studies have shown that fertilization with nitrogen, especially in the form of ammonium (NH_4^+) or urea, can reduce CH_4 oxidation rates in soils (Steudler et al. 1989; Mosier et al. 1991; Bronson and Mosier 1993; Hansen et al. 1993; Crill et al. 1994; King and Schnell 1994a; Schnell and King 1994) and sediments (Bosse et al. 1993). In some cases, this is a long-term

effect of repeated fertilizer applications rather than an immediate inhibition of methanotrophic bacteria or the methane monooxygenase (MMO) enzyme (Mosier et al. 1991; Hütsch et al. 1993). However, fertilization can also have an immediate effect on CH_4 oxidation in the field (Steudler et al. 1989; Bosse et al. 1993; Crill et al. 1994; King and Schnell 1994a). In laboratory incubations of soils and sediments, inhibition is caused by NH_4^+ (Conrad and Rothfuss 1991; Nesbit and Breitenbeck 1992; Adamsen and King 1993; Bronson and Mosier 1994; King and Schnell 1994a; Schnell and King 1994), nitrite (NO_2^-) (King and Schnell 1994a; Schnell and King 1994), and high (>10 m*M*) concentrations of nitrate (NO_3^-) (Nesbit and Breitenbeck 1992; Adamsen and King 1993).

MMO can oxidize a variety of substrates besides CH_4; these should therefore compete with CH_4 for the active site of this enzyme. One such cosubstrate is ammonia (NH_3), which is oxidized to NO_2^- via hydroxylamine (Dalton 1977). Pure culture studies with *Methylococcus capsulatus* (Carlsen et al. 1991), *Methylomonas methanica* (Ferenci et al. 1975), and *Methylosinus trichosporium* (O'Neill and Wilkinson 1977) have shown that NH_3 acts as a competitive inhibitor of CH_4 oxidation. Two of these studies (O'Neill and Wilkinson 1977; Carlsen et al. 1991) noted a significant pH effect on the K_i measured as the NH_4^+ concentration, but the K_i was more constant if NH_3 rather than NH_4^+ was considered to be the inhibitor. The competition between NH_3 and CH_4 for the active sites of MMO in methanotrophs, and of ammonia monooxygenase in nitrifiers has led to speculation regarding the contribution of nitrifiers to CH_4 oxidation and of methanotrophs to nitrification in natural environments (Ward 1987; Bédard and Knowles 1989; Ward and Kilpatrick 1990).

Inhibition patterns that are more complex than simple enzymatic competition between CH_4 and NH_3 are occasionally evident in methanotrophs, for example, in low-copper *Methylococcus capsulatus* cells (Carlsen et al. 1991). The requirement of MMO for cosubstrates oxygen and NADH can complicate the interpretation of NH_3 inhibition (Bédard and Knowles 1989). The addition of CH_4 may stimulate rather

than inhibit NH_3 oxidation by methanotrophs (O'Neill and Wilkinson 1977; Knowles and Topp 1988; Megraw and Knowles 1989; King and Schnell 1994b; Schnell and King 1994), presumably because of alleviation of NADH limitation. Hydroxylamine (Hubley et al. 1975) and NO_2^- (O'Neill and Wilkinson 1977; Jolliе and Lipscomb 1991; King and Schnell 1994b) produced through methanotroph oxidation of NH_3 are themselves inhibitors of methanotrophic activity. In a forest soil, the inhibitory effect of NO_2^- was shown to be greater and more enduring than the direct effect of NH_3 (King and Schnell 1994a; Schnell and King 1994).

The half-saturation constant ($K_{m(app)}$) for soil CH_4 oxidation of 30 to 50 nM (Bender and Conrad 1992) is several orders of magnitude lower than the values (1 to 66 μM) for pure cultures of methanotrophs (Bédard and Knowles 1989). Known methanotrophs should not be capable of surviving solely on atmospheric CH_4 (Conrad 1984). Those methanotrophs active in aerobic soils might therefore employ a CH_4-oxidizing system unlike that of known methanotrophs. Two forms of MMO, one particulate and one soluble, are known. Although these exhibit different substrate affinities, neither approaches the $K_{m(app)}$ measured in soils (Bédard and Knowles 1989; Bender and Conrad 1992). This paper presents some experiments on the kinetics of CH_4 oxidation in a humisol designed to examine the competitive strength of NH_3 and to determine the amounts of NH_4^+, NO_2^-, and NO_3^- needed to cause inhibition of CH_4 oxidation.

5.3 Materials and methods

The study site, a humisol on the Central Experimental Farm of Agriculture Canada in Ottawa, has been described previously (Dunfield et al. 1995). Soil samples were taken from a depth of 0 to 20 cm on 17 August 1993 and 30 June 1994 and stored at 12°C. The 1993 soil sample was stored for up to ten months before experiments were performed. The 1994 soil sample was stored for no more than 40 days. Although NH_3 rather than NH_4^+ is thought to be an inhibitor of MMO, we shall refer primarily to measured NH_4^+ concentrations. When given, NH_3 concentrations have been calculated on the basis of average pH values of 6.6 and 6.9 for 1993 and 1994 soil

samples, respectively.

To investigate NH_4^+ inhibition of CH_4 oxidation, aggregates were broken by hand and a 1:3 ratio of field moist soil (81% H_2O, 1993 sample; 136% H_2O, 1994 sample) to distilled deionized H_2O was homogenized 30 minutes on a magnetic stirrer. Fifteen-millilitre portions (2.5 to 3.1 g of dry soil) were distributed into 60-ml serum vials, and NH_4Cl solutions were added to give final volumes of 20 ml. Of the NH_4^+ added, 60 to 70% was not recoverable from the slurry liquid phase shortly after addition and was assumed to be held on exchange sites. CH_4 mixing ratios of approximately 2, 30, 75, 100, 150, 200, 300, 400, 500, and 700 parts per million of volume (ppmv) were added to duplicate vials and incubated on a gyratory shaker at 250 rpm and 25°C. For each experiment, a complete set of CH_4 mixing ratios was run for control soil samples (no added NH_4^+) and up to three levels of added NH_4^+. Specific details of each experiment are given in Table 5.1.

Three more rigorous experiments (no. 6, 7, and 8) with the 1994 soil sample used initial CH_4 mixing ratios of approximately 2, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 125, 150, 175, 200, 250, 300, 350, 400 and 500 ppmv (the two highest levels were omitted in experiment 8). Slurries were shaken for 20 to 24 h before NH_4Cl and CH_4 addition in an attempt to alleviate two problems noted in earlier trials. First, slurrying caused transiently elevated NO_2^- levels which could be depleted through nitrification in a pre-incubation. Second, initial net CH_4 production rather than consumption was occasionally observed at 2 ppmv CH_4. This soil has considerable methanogenic capacity (Dunfield et al. 1995), and although no methanogenesis should occur in aerobic slurries, a short period of net CH_4 production may result from equilibration of CH_4 already in the soil, perhaps hydrophobically bound to organic matter, with the gas phase of vials.

CH_4 was measured at 1 to 1.5 and 5.5 to 6 h after the addition of NH_4Cl and CH_4 by injections of 2-ml headspace volumes into a Shimadzu gas chromatograph with flame ionization detector equipped with a 1.8-m Porapak Q column and 0.5-ml sample loop. At the same times, 1-ml slurry samples were taken into microcentrifuge tubes and frozen immediately. In calculating CH_4 oxidation rates, compensation was

Table 5.1 Effects of NH_4Cl additions on the kinetic coefficients of CH_4 oxidation in humisol slurries. CH_4 mixing ratios, $K_{m(app)}$, and K_s* values are in ppmv, V_{max} rates are in nmol g^{-1} h^{-1}.

Experiment	NH_4^+ (μM)[a]	NO_2^- (μM)[a]	Hyperbolic Model[b]				Hill Model[b]		
			CH_4 range	levels	$K_{m(app)}$	V_{max}	n_{app}	K_s*	V_{max}
1993 soil									
1	12.7	NT[c]	15-730	9	189	1.65	1.06	150	1.47
	20.8[d]	NT	35-730	8	349	2.06	1.19	174	1.42
	34.3	NT	40-730	8	413	1.81	1.22	340	1.21
	94.2	NT	70-730	7	1016	2.01	1.20	464	1.25
2	16.5	NT	15-730	9	184	2.79	1.05	144	2.22
	35.2[d]	NT	15-730	9	377	2.99	1.12	178	1.89
	47.9	NT	65-730	7	579	3.36	1.14	328	2.47
	121	NT	65-730	7	982	2.89	1.37	409	1.83
3	26.6	29.8	45-750	9	111	1.38	0.91	101	1.31
	57.5	51.6	45-750	9	275	1.35	1.07	256	1.29
	136	65.4	45-750	9	991	2.03	1.23	623	1.17
1994 soil									
4[e]	20.4	20.9	30-450	8	88.7	1.51			
	54.6	54.2	30-450	8	316	1.29			
	138	78.1	50-450	7	370	0.71			
5[e]	27.7	28.0	40-550	9	60.2	1.31			
	52.2	52.7	40-550	9	173	1.27			
	96.2	53.2	70-550	8	153	0.79			
6	24.1	30.8	15-550	16	86.3	1.61	1.16	60.2	1.37
	70.3	48.4	40-550	13	172	1.13	1.39	97.6	0.85
7	19.5	15.8	15-550	14	107	1.64	1.27	56.1	1.22
	48.8	42.3	40-550	11	144	1.16	1.62	68.7	0.80
8	3.4	4.1	15-350	13	86.5	1.48	1.12	63.5	1.28
	94.4	42.2	60-350	10	370	1.52	1.53	119	0.77

[a] NH_4^+ and NO_2^- concentrations given are averages of concentrations at the start and end of each incubation.

[b] Kinetic constants were estimated by using the linear portion of Lineweaver-Burk plots for the Michaelis-Menton hyperbolic model or by fitting the data to the Hill cooperative model (Segel 1975).

[c] NT, not tested.

[d] Added NH_4^+ was completely consumed by the end of the experiment and was not used in K_i calculations or statistics.

[e] The Hill model could not be applied to experiments 4 and 5 because of high data variability.

made for removal of gas during sampling. Vials containing 20 ml of H_2O and several CH_4 mixing ratios were included as checks on standardization and leakage. In the event of non-zero CH_4 oxidation rates calculated in these vials, linear regression of the calculated rate versus CH_4 mixing ratio was used to correct soil CH_4 oxidation rates.

Inhibition by $NaNO_3$ or $NaNO_2$ was tested by essentially the same procedure. Incubations for experiments with NO_2^- and NH_4^+ supplements were limited to 5 to 6 h because nitrification rapidly depleted these ions, but NO_3^- concentrations remained constant and thus incubation times could be longer. The possibility of phase transfer limitation was examined with triplicate 20-ml slurries containing 1, 2, 3, or 4 g of 1993 soil, at 100 ppmv CH_4. To test the reversibility of NO_2^- and NH_4^+ inhibition, vials from representative NH_4^+ and NO_2^- experiments were incubated until all added NO_2^- or NH_4^+ was oxidized and then CH_4 oxidation was retested.

For nitrogen analyses, slurry samples in microcentrifuge tubes were thawed by centrifugation for 5 min at 13000 × g, resuspended by manual shaking, and centrifuged again for 15 minutes. NO_3^-, NO_2^-, and NH_4^+ levels in the supernatant were measured colorimetrically by using an automated analysis system (ChemLab Instruments, Hornchurch, Essex, England) (Megraw and Knowles 1989). Initial concentrations were estimated from 2-ppmv CH_4 vials only (this was valid since CH_4 mixing ratio did not affect the nitrification rate [see results]). Final concentrations were the averages of at least six vials. pH values were not affected by experimental NH_4^+ additions.

The CH_4, NH_4^+, NO_3^-, and NO_2^- concentrations presented and used in kinetic calculations are the arithmetic means of measurements at the beginning and end of each incubation. The arithmetic mean is an overestimate of the actual average CH_4 mixing ratio over this time, but since maximum CH_4 depletion is 17%, the error incurred should be minor (Segel 1975). All kinetic and inhibition coefficients were estimated as described by Segel (1975). Statistical procedures were performed with SYSTAT (SYSTAT, Inc., Evanston, Ill.). Except when noted, statistical analyses were multivariate analyses of variance (MANOVAs) with soil sample and inhibitor

(NH_4^+, NO_2^-, NO_3^-) concentrations as independent variables and kinetic coefficients as dependent variables. Variance assumptions were checked by using residual plots, and data occasionally log-transformed to satisfy these. Multiple comparisons are Bonferroni contrasts.

5.4 Results

5.4.1 Phase transfer limitation and initial rates. CH_4 oxidation rates increased nearly linearly with increasing soil contents in slurries (Fig. 5.1), indicating that slurries were not limited by phase transfer and diffusion of CH_4. The slight curvature of the plot may result from dilution alterations of slurry pH and NH_3 concentrations.

Slurries used in inhibition experiments were never incubated for more than 24 h (usually <7 h) with added CH_4. During these periods, kinetic coefficients were nearly constant in slurries without added NH_4^+ (Table 5.2). Methanotrophic activity was stimulated by several orders of magnitude by exposure to 10% CH_4 (data not shown), suggesting that the growth of methanotrophs is not nitrogen-limited and should not be affected by NH_4^+ additions. The rates presented in this paper are therefore based on initial enzyme concentrations, and are true initial rates with one potential exception. When significant accumulations of NO_2^- occurred during incubations through oxidation of added NH_4^+, measured rates may have gradually decreased over time. We have attempted to compensate for this in our calculations (see below).

5.4.2 NO_2^- inhibition. The addition of $NaNO_2$ inhibited CH_4 oxidation in both soil samples (Fig. 5.2). Although each graph contains curves from two separate experiments, in each case the two control curves are similar. Progressively higher $NaNO_2$ additions resulted in increasingly sigmoidal kinetics, with the highest relative inhibition of CH_4 oxidation occurring at the lowest CH_4 mixing ratios.

5.4.3 NH_4^+ inhibition. The experiments comparing CH_4 oxidation kinetics with and without added NH_4^+ are summarized in Table 5.1. The CH_4 oxidation rates in soil slurries without added NH_4^+ agreed well with a Michaelis-Menton hyperbolic model (Fig. 5.3). However, when NH_4^+ was added, kinetic curves often became sigmoidal,

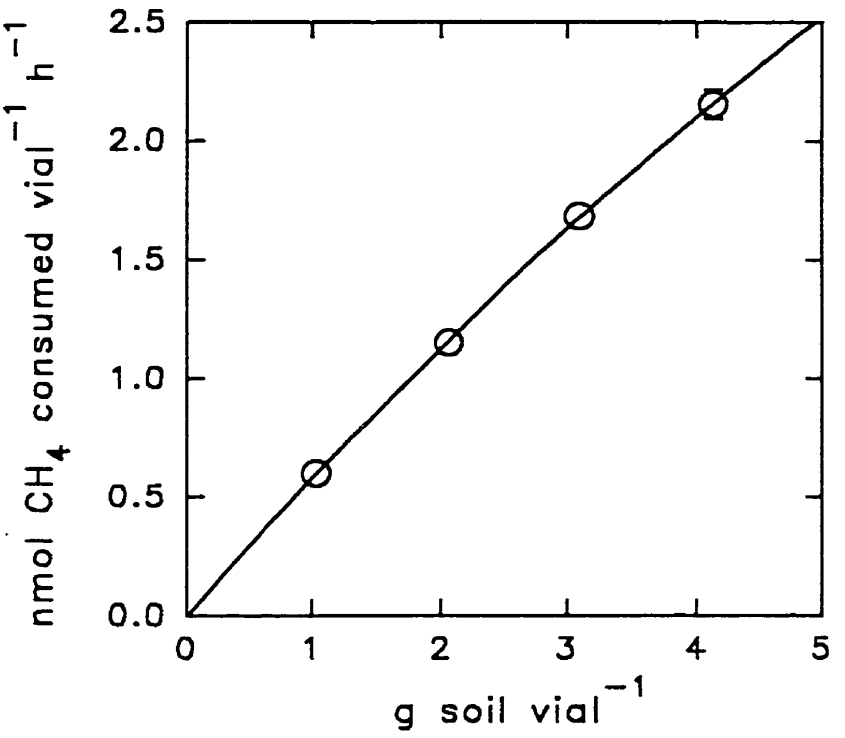

Fig. 5.1 Oxidation rates of 100 ppmv CH_4 in 20-ml slurries containing different amounts of 1993 soil. Each point is the mean of triplicate vials ± 1 standard error of the mean. When error bars are not visible, they are contained within the symbol.

Table 5.2 Kinetic coefficients calculated at various times after the addition of CH_4 to soil slurries.

Soil sample	Incubation time (h)	V_{max} (nmol g^{-1} h^{-1})	$K_{m(app)}$ (ppmv)
1994 soil[a]	3.5 - 9.0	1.50	68
	9.0 - 22.5	1.54	92
	22.5 - 28.5	1.73	92
1994 soil			
+ 74 m*M* NO_3[a]	3.5 - 9.0	0.86	43
	9.0 - 22.5	1.00	77
	22.5 - 28.5	1.11	60
1993 soil[b]	1.5 - 7.5	1.12	
	7.5 - 27.0	1.09	
	27.0 - 32.0	1.49	

[a] For more details, see results for experiment 11 in Table 5.3.

[b] Based on duplicate flasks at >400 ppmv CH_4.

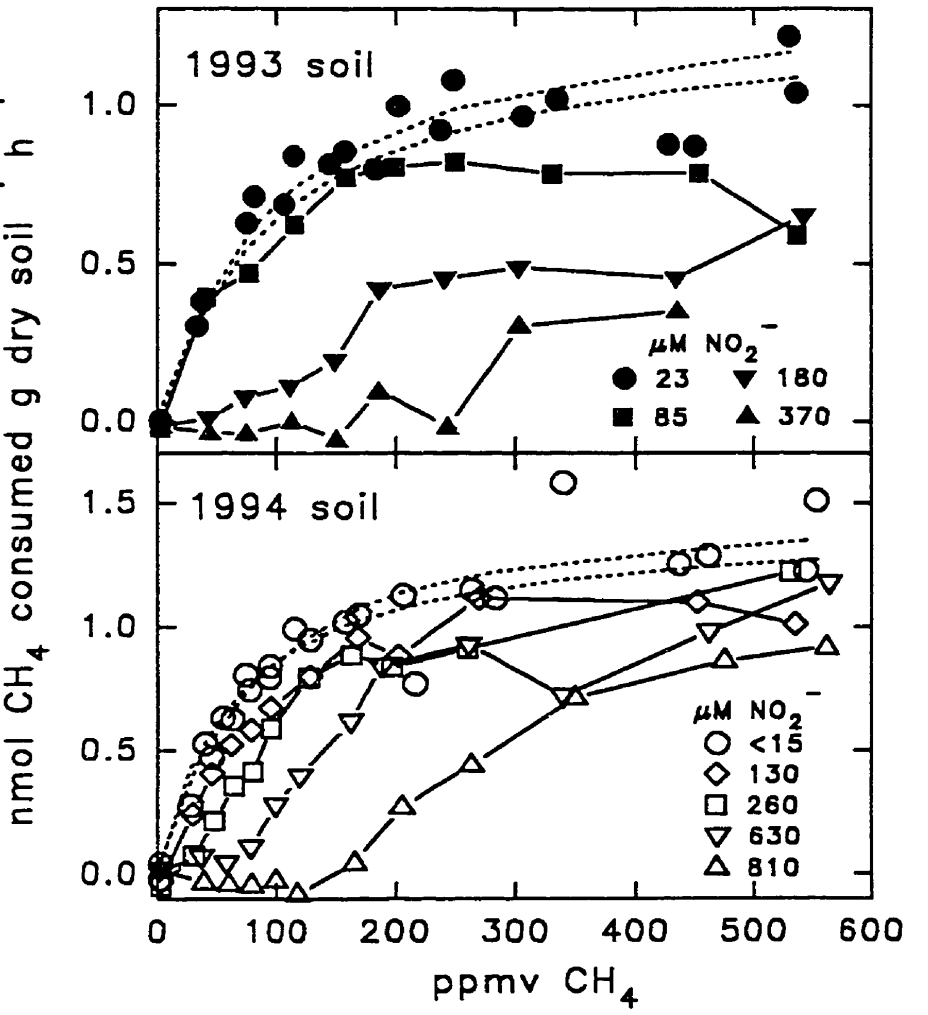

Fig. 5.2 Effects of $NaNO_2$ additions on the kinetic curves of CH_4 oxidation in the two soil samples. Each graph includes curves for two trials. Circles represent control rates. The average NO_2^- concentrations during incubations are indicated.

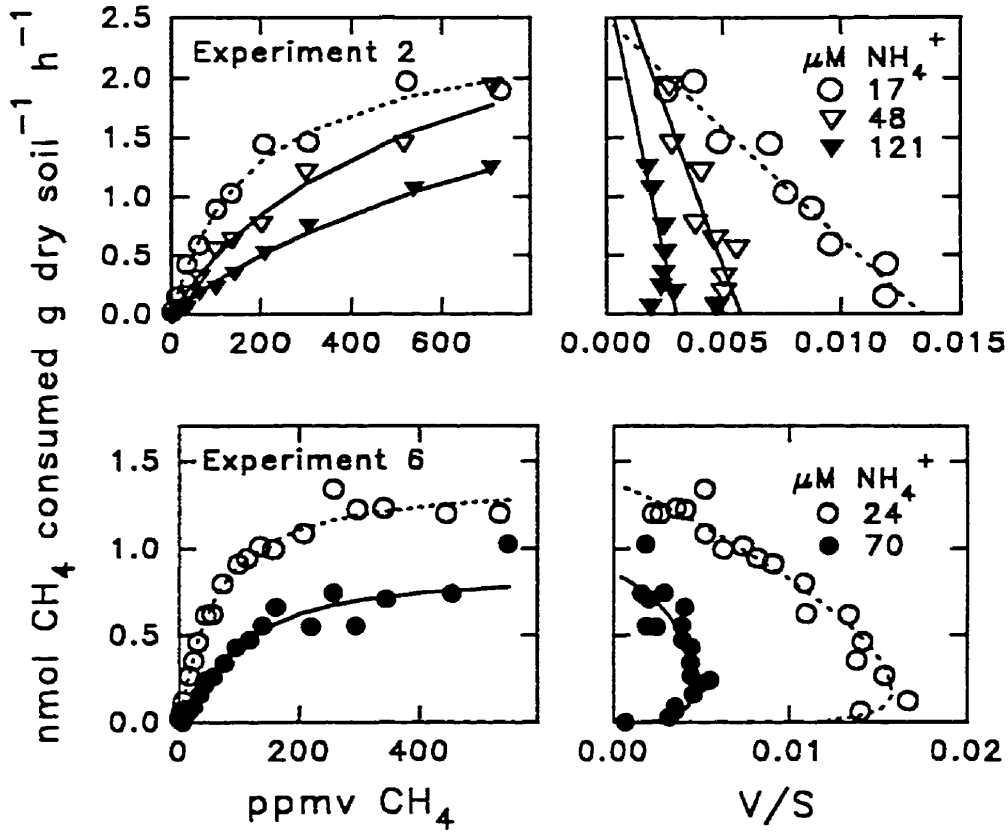

Fig. 5.3 Representative kinetic curves of CH_4 oxidation in 1993 (experiment 2) and 1994 (experiment 6) soil samples with and without NH_4Cl additions as well as Eadie-Hofstee replots to show deviations from true hyperbolic curves at low CH_4 oxidation rates for 1994 soil. Lines were fit to the curves by using a Michaelis-Menton hyperbolic model for 1993 soil and a Hill cooperative model for 1994 soil. Points are means of duplicate vials. The average liquid-phase NH_4^+ contents are indicated.

and curvature of Lineweaver-Burk or Eadie-Hofstee plots was evident at low velocities, especially for the 1994 soil sample (Fig. 5.3). Replots used to estimate the Michaelis-Menton kinetic coefficients in Table 5.1 were linearized by deleting the lowest substrate concentrations. This procedure gives valid estimates when curves of v plotted against s are only slightly sigmoidal, as was the case with the 1993 soil sample. However, it is not an ideal treatment of sigmoidal kinetics (Segel 1975), and gives biased estimates for 1994 soil sample. Therefore, $1/v$-versus-$1/s$ plots were also fit to the Hill cooperative model (Segel 1975), by using the curve-fit function of SigmaPlot 5.1 (Jandel Scientific, San Raphael, Calif). Because of weighting toward low-velocity points, the lowest were deleted until an unbiased agreement with the equation was obtained (in practice, this usually meant deleting any velocity that was <0.1). Representative experiments showing a good agreement with the hyperbolic model for the 1993 soil sample, and agreement with the Hill model for the 1994 soil sample are given in Fig. 5.3.

Ammonium acted as a simple competitive inhibitor in the 1993 soil sample. The $K_{m(app)}$ values for CH_4 increased with increases in the NH_4^+ concentration ($P<0.001$; log-transformed data), but V_{max} values were unaffected ($P=0.003$; log-transformed data). Plot curvature was minor, as shown by low n_{app} values (Table 5.1). Replots of $K_{m(app)}$ versus NH_4^+ concentration were used to estimate the K_i of NH_4^+ and the true K_m of CH_4 oxidation in the absence of NH_4^+ (Fig. 5.4). These estimates were a K_i of 8.3 μM NH_4^+ (18.5 nM NH_3) and a K_m of 63.5 ppmv CH_4 (88.2 nM dissolved).

The 1994 sample shows a more complex response to NH_4^+ addition. The V_{max} decreased, whether estimated with the hyperbolic or Hill model ($P=0.004$), while the $K_{m(app)}$ (and the K_s*) again increased ($P<0.001$) with increasing NH_4^+ concentrations (Table 5.1). Unlike those for the 1993 soil sample, the $K_{m(app)}$ estimates for the 1994 soil sample are not particularly reliable because of sigmoidal v-versus-s plots. These estimates would give a K_i of 25.2 μM NH_4^+ (112 μM NH_3) and a K_m of 56.7 ppmv CH_4 (78.7 nM dissolved). Methanotrophs in the 1994 soil sample seemed to have a

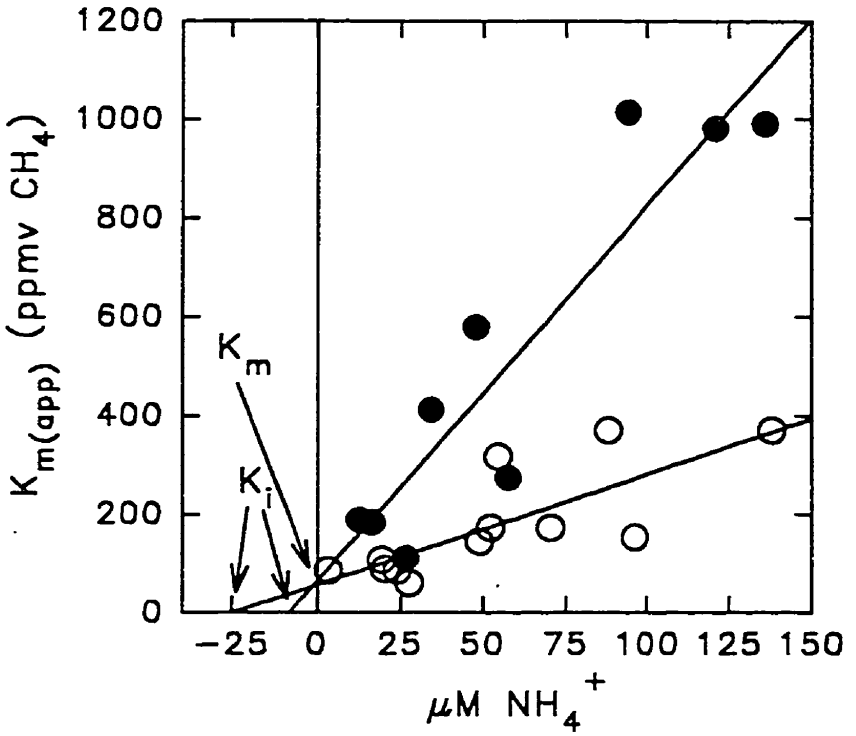

Fig. 5.4 $K_{m(app)}$ values of CH_4 oxidation, estimated by using Lineweaver-Burk replots, plotted against average liquid-phase NH_4^+ concentrations in 1993 (●) and 1994 (○) humisol slurries. The K_m of CH_4 oxidation in an NH_4^+-free system, and the K_i of NH_4^+ for CH_4 oxidation are estimated from axis intercepts.

lower affinity for NH_4^+ than those in the 1993 soil sample.

At levels below about 50 ppmv CH_4, measured CH_4 oxidation rates were less than predicted by a strictly Michaelis-Menton hyperbolic model, resulting in sigmoidal kinetics. n_{app} increased with increasing NH_4^+ concentration (Table 5.1), especially in the 1994 soil sample, whose kinetic plots were often strikingly sigmoidal (Fig. 5.3). The competitive model of NH_4^+ inhibition is therefore conservative, underestimating inhibition at low CH_4 mixing ratios. The n_{app} parameter should probably be considered simply a measure of plot curvature rather than in its correct sense as a measure of cooperativity, since we hypothesize that the sigmoidal curves result from NO_2^- toxicity. While the data could result from an allosteric enzyme, the Hill model might fit because (i) NO_2^- is more inhibitory of CH_4 oxidation at low CH_4 mixing ratios and (ii) if NH_4^+ is competitively oxidized by MMO, NO_2^- production decreases with increasing CH_4 mixing ratios. This system may thereby mimic a cooperative system whereby increasing CH_4 mixing ratios result in optimal oxidation.

5.4.4 Nitrification. The maximum nitrification rate, measured as the production of NO_3^- and NO_2^- in slurries with NH_4^+ added (>30 μM), was 0.18 to 0.25 μmol g^{-1} h^{-1} for the 1994 soil sample (experiments 5 through 8), and 0.11 to 0.25 μmol g^{-1} h^{-1} for the 1993 soil sample [experiments 1 and 2 and data from Dunfield et al. (1995)]. The control 1993 soil sample also accumulated NO_3^- and NO_2^- at 0.03 to 0.15 μmol g^{-1} h^{-1}, while this was undetectable in control 1994 soil (<0.03 μmol g^{-1} h^{-1}), indicating a lower rate of mineralization in the latter. The net NH_4^+ depletion rate in the 1993 soil sample with added NH_4^+ was only 0.08 to 0.10 μmol g^{-1} d^{-1}. Nitrification was rapid enough to consume a large proportion of the added NH_4^+ during incubation, introducing error into the determination of its K_i for CH_4 oxidation. However, the arithmetic mean should be a valid estimate of the slurry NH_4^+ concentration since measured nitrification rates were nearly constant regardless of the amount of NH_4^+ added [i.e., the $K_{m(app)}$ of soil nitrification was probably lower than 30 μM (data not shown)].

The effects of CH_4 mixing ratios on nitrification were examined by measuring

postincubation NO_3^- and NO_2^- concentrations in all vials from experiment 3, and the 94.4 μM NH_4^+ treatment of experiment 8. A two-way MANOVA with the CH_4 mixing ratio as a regression factor and NH_4^+ addition as a categorical factor revealed no significant effects of CH_4 mixing ratio on total nitrification (final NO_2^- + NO_3^- concentration, P=0.16), but the effects on net NO_2^- production were significant (P=0.014; overall Pillai Trace P=0.025). This difference probably simply reflects the more precise measurement of NO_2^- than of NO_3^- (NO_2^- was typically <50 μM, while the pool of NO_3^- was >800 μM). While statistically more NO_2^- was produced in slurries with low CH_4 concentrations, the difference was small compared with absolute NO_2^- concentrations. The average regression slope was only -0.015 μM NO_2^- ppmv CH_4^{-1}, and the effect of CH_4 on total nitrification was therefore minute.

5.4.5 $NaNO_3$ inhibition. Three experiments investigated $NaNO_3$ inhibition (Table 5.3), one of them is shown in Fig. 5.5. A noncompetitive mechanism of inhibition (the $K_{m(app)}$ was unaffected, and the V_{max} decreased) is indicated. Statistical tests were not performed because of the paucity of curves, but the 1993 soil sample appeared more sensitive to $NaNO_3$ inhibition than the 1994 soil sample was. An increase in plot curvature (n_{app}) also accompanied $NaNO_3$ addition, probably because of NO_2^- contained as a contaminant or produced through NO_3^- reduction (Table 5.3).

This noncompetitive inhibition is not necessarily due to NO_3^- *per se*. Slurry pH values were altered by $NaNO_3$ additions, although greater than 40 mM increases continued to decrease the V_{max} values without further affecting the pH values (Table 5.3). Similar concentrations of NaCl also inhibited CH_4 oxidation. In experiment 9, the CH_4 oxidation rates (at 160 ppmv CH_4) in slurries supplemented with 35 or 55 mM NaCl were not significantly different from those in slurries with 38 or 59 mM $NaNO_3$, respectively (two-way analysis of variance [ANOVA] with four Bonferroni contrasts, P=0.009). The pH of slurries with added NaCl (6.49) was nearly the same as that of slurries with added $NaNO_3$. Smaller NaCl additions (up to 10 mM) did not significantly affect CH_4 oxidation at 2-, 8-, 32-, and 120-ppmv CH_4 in the

Table 5.3 Effects of $NaNO_3$ additions on kinetic coefficients of CH_4 oxidation in humisol slurries. CH_4 mixing ratios, $K_{m(app)}$, and K_s* values are in ppmv, V_{max} rates are in nmol g^{-1} h^{-1}.

Trial	Incubation time (h)	NO_3^- (m*M*)	NO_2^- (μM)	pH	Hyperbolic Model				Hill Model		
					CH_4 range	levels	$K_{m(app)}$	V_{max}	n_{app}	K_s*	V_{max}
1993 soil											
9	5.5	1.17	26.0	6.70	30-500	8	158	1.57	1.46	61.0	0.99
		37.8	72.5	6.47	70-550	7	141	0.80	1.38	86.7	0.64
		58.7	105.	6.46	70-550	7	195	0.53	2.27	86.7	0.34
1994 soil											
10	7.0	0.31	<3.	NT[a]	30-350	9	65.3	1.21	1.25	52.3	1.07
		18.2	11.9	NT	30-350	9	77.2	1.24	1.49	48.4	0.98
		33.9	29.6	NT	30-350	9	71.4	1.07	1.57	44.8	0.85
11	13.5	0.44	<3.	7.14	25-500	9	91.7	1.54	1.11	74.5	1.41
		74.2	37.4	6.74	25-550	9	82.0	1.07	1.18	61.6	0.96
		102.	57.8	6.70	45-550	8	73.1	0.64	1.73	52.0	0.53

[a] NT=not tested.

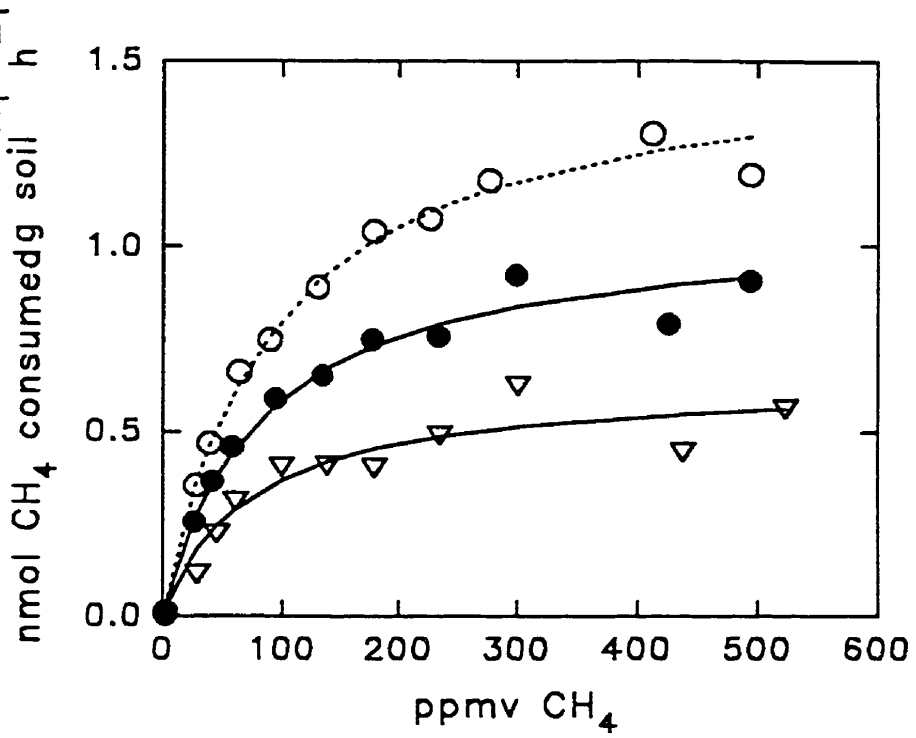

Fig. 5.5 Effects of $NaNO_3$ additions on the kinetics of CH_4 oxidation in the 1994 soil sample (experiment 11 [Table 5.3]). The average NO_3^- concentrations are 0.4 (○), 74 (●), and 102 (▽) m*M*.

1994 soil sample (two-way ANOVA of log-transformed data, P=0.61); therefore, dissolved salts should not have affected the results of NH_4Cl or $NaNO_2$ inhibition experiments.

5.4.6 Reversibility of inhibition. After 70 h, 0.7 or 1 mM of added NO_2^- (1994 soil) reached background, control levels. In 7-h incubations at 2-, 50-, 85-, 200-, and 380-ppmv CH_4, these slurries then had the same CH_4 oxidation rate as slurries that had never received NO_2^- additions (two-way ANOVA, P=0.70). In experiment 5, the effects of NH_4^+ additions on the CH_4 oxidation rate were also not significant after the NH_4^+ was nitrified in 5-h incubations at 2-, 120-, 275-, and 450-ppmv CH_4 (two-way ANOVA of log-transformed data, P=0.30). Inhibition by both NH_4^+ and NO_2^- at these concentrations was fully reversible.

5.5 Discussion

It should be stressed that the statistically significant effects of NH_4^+ and NO_3^- on kinetic coefficients of CH_4 oxidation do not justify the basic assumption that the kinetic response of MMO is being measured. Measuring kinetic coefficients on a preparation as crude and ecologically complex as soil is problematic. However, the CH_4 oxidation rates measured in control soil slurries were true initial rates, there was no diffusion limitation, and v-versus-s curves usually fit a hyperbolic model well. CH_4 oxidation is the initial step in an oxidative pathway, and CH_4 is freely diffusible across the cell membrane. This system may therefore be relatively simple and allow for a kinetic interpretation. If this is accepted, then we can make some conclusions on the mechanisms of inhibition and the strengths of various inhibitors.

Ammonium acted as a competitive inhibitor of CH_4 oxidation in the 1993 soil sample. Our kinetic plots cannot distinguish between simple competitive and partial competitive inhibition (Segel 1975). However, since the CH_4 oxidation rate could be driven to zero with very high levels of NH_4^+ additions ($>$ 1 mM, data not shown), the mechanism is probably simple competition. The two samples produced similar K_m estimates (about 60 ppmv CH_4). Nevertheless, while the results of the 1993 soil

sample were easily interpretable as simple competition between CH_4 and NH_4^+, the results of the 1994 soil sample were more complex, with decreased V_{max} values and strongly sigmoidal kinetics at high NH_4^+ concentrations. NH_4^+ probably acted as a competitive inhibitor in this sample as well, and although accurate estimation of the K_i was impossible, this soil seemed less sensitive to NH_4^+.

Explaining the differences between the two samples is difficult. There were differences in storage time and experimental protocol (experiments 6-8 included 24 h of pre-shaking and more intense sampling at lower CH_4 values). Sampling variations may have influenced the microbial flora present, and if measured $K_{m(app)}$ values resulted from the activities of several enzymes rather than the activity of one, their ratios could have varied between samples. The more rapid NH_4^+ depletion rate of the 1994 soil sample might also have protected methanotrophs from inhibitory effects of NH_4^+. Considering NH_3 to be the inhibitor instead of NH_4^+ increases the disparities in K_i values, but pH could certainly affect the enzyme system in ways that are distinct from its effects on the NH_4^+-to-NH_3 ratio.

Errors in determining $K_{m(app)}$ values mean that the K_i of 8 to 25 μM NH_4^+ (18-112 nM NH_3) should be considered a rough estimate only, but it does suggest that *in situ* soil NH_4^+ concentrations inhibit CH_4 oxidation. Similar to the finding that $K_{m(app)}$ values for soil CH_4 oxidation are much lower than those for pure cultures of methanotrophs (Bédard and Knowles 1989; Bender and Conrad 1992), our soil K_i is lower than the K_i values of 1.7-18 mM NH_4^+ at pH 6-7 (8-56 μM NH_3) determined for pure cultures (Ferenci et al. 1975; O'Neill and Wilkinson 1977; Bédard and Knowles 1989; Carlsen et al. 1991). However, our results also agree with those of King and Schnell (1994a; Schnell and King 1994) that the inhibition caused by the addition of NH_4^+ may not be simply competitive dilution, but may also be the result of production of toxic NO_2^-.

Kinetic curves became increasingly sigmoidal with increased NH_4^+ or NO_2^- additions. This pattern would result if the MMO exhibited cooperative behaviour which was influenced by NO_2^- or NH_4^+. It could also result from NO_2^- toxicity. The

relative inhibition caused by NO_2^- was highest at low CH_4 mixing ratios, a pattern also noted for another soil (Schnell and King 1994). This pattern, combined with the shift in competitive balance of MMO as the ratio of NH_4^+ to CH_4 increases, could contribute to the sigmoidal CH_4 oxidation curves of soils with NH_4^+ added. In other words, more NO_2^- is produced in soil at low CH_4 concentrations, at which it is also inherently more inhibitory of CH_4 oxidation. Nitrite is an inhibitor of formate dehydrogenase and can contribute to NADH limitation (Jollie and Lipscomb 1991), explaining its greater influence on CH_4-limited cells. A high metabolic rate might also be necessary to export toxic, cellular NO_2^-. The NO_2^- concentrations measured in NH_4^+ inhibition experiments were slightly lower ($< 100\ \mu M$) than the levels of exogenously added NO_2^- required to cause inhibition of CH_4 oxidation, but if methanotrophs themselves produce NO_2^-, intracellular concentrations would be higher.

In this soil, as in many other soils (McCarty and Bremner 1991) nitrification was not affected or was only slightly affected by up to 800 ppmv CH_4. Methanotrophs can occasionally contribute to soil nitrification (Bédard and Knowles 1989), and a methane-dependent nitrifying consortium was isolated from a similar humisol (Megraw and Knowles 1989), but methanotrophs probably contributed little to nitrification in our humisol. Slightly elevated soil NO_2^- concentrations were noted as CH_4 mixing ratios decreased. This indicates a competitive effect of CH_4 on NH_4^+ oxidation by nitrifiers or methanotrophs, but the effect was too small to greatly affect the overall nitrification rate.

The competitive nature of NH_4^+ inhibition in our soil, whereby inhibition decreased at high CH_4 levels because of competitive dilution, is the complete opposite of a trend found in a forest soil (Schnell and King 1994). The same authors also showed that NO_2^- production from NH_4^+ in methanotroph cultures increased with increasing CH_4 mixing ratios (King and Schnell 1994b), and advanced this as an explanation of the ammonium inhibition pattern observed in soil (Schnell and King 1994). Other reports (O'Neill and Wilkinson 1977; Knowles and Topp 1988; Megraw and Knowles 1989) also note that while large amounts of added CH_4 may inhibit NH_4^+ oxidation by methanotrophs, small amounts of added CH_4 may actually

stimulate it. The former effect is presumably competitive, with the latter stemming from energetic limitation of methanotrophs at low CH_4 concentrations. Increased NH_4^+ oxidation and NO_2^- production can result from increased MMO activity, through the alleviation of NADH limitation or induction of enzyme production and population growth. In the study of forest soil noted above (Schnell and King 1994), the authors make no mention of the linearity of their rates over time at high CH_4 levels; it seems likely that the methanotrophic population was stimulated. Our short incubations provide a look at initial rates based on initial enzyme concentrations, a "snapshot" indicating that NH_4^+ or NH_3 probably acts as a competitive inhibitor. It is perhaps also noteworthy that the initial CH_4 concentrations in our soil were greater than expected from ambient-air CH_4 equilibration, presumably because of methanogenesis in anaerobic soil microsites, so methanotrophs may not have suffered from NADH limitation. The slurrying of soil, as well as other disturbances (such as sonication), results in transient CH_4 efflux.

There is a discrepancy between the complete reversibility of NO_2^- and NH_4^+ inhibition in our humisol and the persistence noted in other studies (Nesbit and Breitenbeck 1992; King and Schnell 1994a). Perhaps the absolute concentration and time of exposure affect the ability of the methanotrophs to recover from NH_4^+ and NO_2^- inhibition, and perhaps the extremely high natural nitrification rate of the humisol shields methanotrophs from NO_2^- and NH_4^+. The levels of our NH_4^+ additions were much lower than those used in other studies (Nesbit and Breitenbeck 1992; King and Schnell 1994a), although in a parallel field study, we also found no effect of 100 kg urea-N ha^{-1} addition on CH_4 oxidation (Dunfield et al. 1995).

These studies demonstrate the competitive nature of NH_4^+ inhibition of soil CH_4 oxidation and further implicate NO_2^- as a significant inhibitor, as previously noted (King and Schnell 1994b; Schnell and King 1994). Methanotrophic activity in soil has a higher affinity for NH_4^+ than noted in pure methanotroph cultures. As found in other studies (Nesbit and Breitenbeck 1992; Adamsen and King 1993), inhibition by $NaNO_3$ was evident only at concentrations at which salts had a similar inhibitory effect. This inhibition was not purely attributable to pH, and only the

maximum catalytic rate of the enzyme was affected.

5.6 Acknowledgements

Thanks to Chris Tauchner for nitrogen analyses.

PREFACE TO SECTION 6

The high nitrification rate measured in Chapter 4 suggested that large gaseous N-oxide fluxes might result from application of NH_4^+ fertilizer to the humisol. This hypothesis was tested using intact cores, and the results presented in Section 8. Sections 6 and 7 precede this work, providing a context to explain NO fluxes as net results of both production and consumption processes. The studies presented in Section 6 were inspired by observations of NO depth profiles made during core flux measurements. These profiles clearly showed that NO consumption as well as NO production occurred in aerobic fertilized soil. Previous to this work, only denitrification of NO was considered a significant NO sink in soil, although during the publication of Section 6, similar findings were published by Baumgärtner et al. (1996).

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SECTION 6. BIOLOGICAL OXIDATION OF NITROGEN MONOXIDE IN A HUMISOL

6.1 Abstract

Consumption of nitrogen monoxide (NO) in a humisol was studied at 0.1-2 parts per million of volume (ppmv) NO, a range representative of NO mixing ratios in ammonium-fertilized soil. Denitrification was not a major sink for NO. The principal NO-consuming reaction was a biological oxidation, leading ultimately to nitrate (NO_3^-). Nitrogen dioxide (NO_2) and nitrite (NO_2^-) may have been intermediates in this pathway. An abiological reaction accounted for about 25% of the NO-consuming activity in soil at 90% H_2O (d.w.) and 25°C, but contributed relatively more to total NO consumption at higher temperatures. Biological NO-consuming activity was highest at 25°C, while the abiological activity increased exponentially with temperature. The product of the abiological activity was neither NO_3^-, NO_2^-, nor nitrous oxide (N_2O), and the activity did not require O_2.

6.2 Introduction

Nitrogen monoxide is a highly reactive trace gas with diverse effects on the chemistry of the troposphere. Nitrogen monoxide and nitrogen dioxide (collectively called NO_x) participate in reactions involving O_3, CO, and OH. By influencing the mixing ratios of tropospheric oxidants, NO_x indirectly affects the destruction rates of greenhouse gases, including methane and other hydrocarbons. NO_x can be converted to nitric acid, a component of acid precipitation (Logan 1983).

Soil NO_x emissions probably contribute less to the total tropospheric NO_x budget than do anthropogenic emissions. However, because NO_x is short-lived and not transported far from its sources, soils control the tropospheric mixing ratio over a much greater geographical area (Slemr and Seiler 1991). Inhibitors specific for autotrophic microbial nitrification have been used to show that this process is the dominant source of NO in most soils (Remde and Conrad 1991c; Davidson 1992; Davidson et al. 1993; Hutchinson et al. 1993; Skiba et al. 1993). Part of the NO

produced during nitrification arises through chemodenitrification of NO_2^- (Remde et al. 1989; Davidson 1992). NO is also produced during denitrification (Remde and Conrad 1991c; Cárdenas et al. 1993).

Soil NO emissions are attenuated by NO-consuming processes, and some soils act occasionally as net sinks for tropospheric NO (Slemr and Seiler 1991; Skiba et al. 1994). Consumption involves chemical transformations as well as surface sorption (Voldner et al. 1986). High mixing ratios of NO (> 1000 ppmv) are oxidized to NO_3^- and NO_2^- in soil (Prather et al. 1973; Stark and Firestone 1995), but the fate of NO at naturally-occurring mixing ratios cannot be inferred from such experiments. NO at mixing ratios > 1000 ppmv is rapidly oxidized by O_2 to NO_2, which then disproportionates in H_2O to form nitric and nitrous acids. However, each of these reactions is second-order in NO_x, and they proceed slowly at tropospheric NO_x mixing ratios (Galbally and Roy 1978, Schwartz 1984). Organic peroxyl radicals, hydroperoxyl radicals, and ozone are the main tropospheric oxidants of NO (Logan 1983). Antioxidants such as apoplastic ascorbate may be important in reducing NO_2 to NO_2^- in plants (Ramge et al. 1993).

Reduction of NO to N_2O by denitrifiers often contributes to soil NO turnover (e.g. Remde et al. 1989; Schuster and Conrad 1992). NO reduction by anaerobically-grown denitrifiers is not completely inhibited by O_2, so the process could conceivably occur in aerobic or partially aerobic environments (Remde and Conrad 1991b). However, NO consumption occurs in soils with little denitrifying activity (Hutchinson et al. 1993). *Nitrobacter* spp. produce NADH while oxidizing NO to NO_2^- (Freitag and Bock 1990). The potential importance of this metabolic process is suggested by a positive correlation between NO uptake rates and the number of NO_2^- oxidizers in a cambisol (Baumgärtner and Conrad 1992a). NO undergoes a variety of other reactions in biological systems (Fontecave and Pierre 1994), so the soil microflora may contribute in still more ways to NO turnover.

Organic soils and soil horizons may have exceptionally high NO-consuming capacities (Baumgärtner and Conrad 1992a). We therefore conducted experiments on a cultivated humisol (Dunfield et al. 1995) to examine the properties of NO

consumption, and to determine the relative importance of biological versus chemical and oxidative versus reductive processes of NO consumption.

6.3 Materials and methods

6.3.1 Study site and sampling. Four soil cores in polyvinyl chloride tubing (7.8 cm diameter, 35 cm depth) were taken from a cultivated humisol in Ottawa, Canada (Dunfield et al. 1995) on 5 September and 13 October 1995. The site comprises an organic layer (pH 7.1, loss on ignition 70%) underlain by a clay pan at 30-35 cm depth. Cores were capped at the bottom, wetted to gas-filled porosities of approximately 0.22 (Sept.) or 0.37 (Oct.), and stored at 20°C. Profiles of NO mixing ratio with depth were determined by syringe sampling of soil-matrix air through ports drilled at 1-cm intervals in the polyvinyl chloride tubing (Dunfield et al. 1995). Cores were fertilized with 100 kg N ha^{-1} as NH_4Cl in 80 ml H_2O, and NO profiles determined at intervals for two weeks.

Soil from the organic layer (500 cm^2, 0-20 cm depth from three spots) was sampled on 30 June (81% H_2O d.w.) and 13 October (96% H_2O d.w.) and stored at 8°C. Experiments used homogenized subsamples of this soil, incubated in gas-tight stoppered flasks or in a continuous-flow system. Except where otherwise noted, all experiments were done at 25-27°C.

6.3.2 Closed-system experiments. In closed-system experiments, Erlenmeyer flasks or serum vials containing soil were capped air-tight with SubaSeals (William Freeman Company, Barnsley, England) or butyl rubber stoppers. NO was injected to give 1-3 ppmv, and its decline followed at 2- to 30-min intervals, depending on the rate, until it was exhausted. First-order rate constants were estimated by logarithmic regression of NO mixing ratios between about 2000 and 120 ppbv versus time. The lower limit was chosen because NO production, even when 5 Pa C_2H_2 was added to halt nitrification, caused the NO mixing ratio to approach a non-zero threshold (compensation point). This compensation point was, however, much lower than 100 ppbv, and could be considered 0 in the range used. Experiments performed at >50°C were an exception, as NO production was greatly stimulated. In these cases

data were fit to a function incorporating a first-order NO consumption rate and a zero-order NO production rate (Remde et al. 1989), using the curve-fit utility of SigmaPlot (Jandel Scientific, San Raphael, CA).

As NO consumption was first order with respect to soil mixing ratio (data not shown), rates could be expressed as ml min^{-1} (g dry soil)$^{-1}$ to standardise across experimental variations in soil weights and flask volume. Flasks containing H_2O or illite clay were used as controls. Rates in these controls were not first-order, but were calculated as such over a limited NO range (generally 1-2 ppmv) to show that the reaction of O_2 with NO was much slower than the NO consumption rate of soil.

To determine whether soil NO consumption was a biological phenomenon, soil was sterilized using several procedures: (i) 20.0-g amounts of soil in 150-ml Erlenmeyer flasks were autoclaved for 1 h on each of three consecutive days; (ii) 2 ml of 50 mg HgCl ml^{-1} was added to 5-g amounts of soil in 50-ml flasks and incubated 2 d (Wolf and Skipper 1994); (iii) 12.5 ml of 7.5% glutaraldehyde was added to 25.0-g amounts of soil in 150-ml flasks, incubated for 2 days, heated to 85°C for 30 min, then flasks were twice evacuated for 15 min and backfilled with air before testing NO consumption (Labeda et al. 1975); (iv) 3.0-g amounts of soil in 50-ml flasks were fumigated with chloroform in 1-l Mason jars for 21.5 h (Horwath and Paul 1994); (v) 1.5-g soil samples + 0.2 ml H_2O in 14-ml serum vials were gamma-irradiated at 35 kGy over 2 days. Sterility was tested by inoculation of soil into Difco nutrient broth. In a separate experiment, heterotrophic activity was stimulated by adding 0.5 ml of 25 m*M* D-glucose to 2.5-g amounts of soil in 60-ml serum vials and testing NO consumption after 90 min. When solutions were added in these experiments, control soil received equal volumes of H_2O.

To determine the kinetics of NO consumption, 60-ml serum vials containing 5.0-g amounts of soil or 150-ml flasks containing 10.0-g amounts of autoclaved soil were supplemented with 5 Pa C_2H_2 and up to 14 ppmv NO. The effect of temperature was studied using 2.5-g amounts of fresh soil (+5 Pa C_2H_2) or 5.0-g amounts of autoclaved soil in 50-ml Erlenmeyer flasks. These were incubated for 1 day at temperatures between -8 and 67°C before testing NO consumption. Soil

moisture was varied by adding different amounts of H_2O to 2.5-g amounts of soil in 50-ml flasks. The effect of O_2 on NO consumption was tested in autoclaved soil, but could not be determined in fresh soil because NO production was too greatly stimulated under anaerobic conditions. An anaerobic atmosphere was created by six times evacuating for 15 min and refilling with N_2 gas.

6.3.3 Continuous-flow experiments. A flow-through system was used to examine the products of soil NO consumption. Compressed air was humidified by bubbling through H_2O in two 1-l flasks (each containing 200-300 ml H_2O). In one of these flasks it was mixed with N_2, in the other an equal flow rate of 1.75-ppmv NO in N_2. All gases were supplied by Matheson Gas Products, Ville St. Laurent, Quebec. Gas delivery rates were adjusted with flow controllers. C_2H_2 could be added to the air inlet line with a syringe pump. The air flow rate was always much greater than N_2 or NO in N_2 flow rates, so all experiments were aerobic (>15% O_2). The gas mixture from each 1-l flask was fed through three 250-ml Erlenmeyer flasks containing 20.0-g amounts of sieved (1 mm) soil.

Each continuous-flow experiment therefore consisted of three control sample flasks and three with an elevated NO mixing ratio. Gas-sampling ports allowed measurement of N_2O and NO in each sample flask. At least three, 1.50-g soil samples flask^{-1} were taken after 100-250 h of incubation, frozen, and later extracted in 3 ml of 2 *M* KCl for NO_2^- and NO_3^- analyses. Production of N_2O, NO_2^-, and NO_3^- from NO were calculated from the concentration difference in control versus elevated-NO flasks. These values were compared to the total NO uptake rate, estimated using separate, stoppered flasks as described above.

NO was determined using a Sievers chemiluminescence analyzer equipped with an injection port, N_2O using a Perkin-Elmer gas chromatograph with a ^{63}Ni electron capture detector (3-m Porapak Q column, oven temperature 50°C, detector temperature 275°C). An automated Greiss-Ilosvay method was used to measure NO_2^-, and NO_3^- after reduction to NO_2^- with hydrazine-copper reagent (Megraw and Knowles 1987a). The Greiss-Ilosvay procedure detects NO_2 as well as NO_2^- (National Research Council 1977). However, only NO_3^- (rather than $NO_2^-+NO_2$) accumulation

from NO was detected in soil extracts (see "Results"), suggesting that there was little NO_2 present.

6.4 Results

5 Pa C_2H_2 was effective at blocking microbial nitrification (data not shown), and was used to facilitate measurement of NO consumption rates. This level was chosen because C_2H_2 at high mixing ratios reacted rapidly with NO (Fig. 6.1). This reaction required O_2 (data not shown).

A major problem with measuring gas transformations in closed systems is that products accumulate and may alter reaction rates. NO consumption in this soil was pseudo first-order, even after repeated NO additions (Fig. 6.2), indicating that end-product inhibition was not a problem. The curve tailed off at <10 ppbv and approached a non-zero compensation point, due to NO produced through chemodenitrification or denitrification. In autoclaved soil, NO consumption was first-order for a limited time only, suggesting that end-product inhibition or conversion of products back to gaseous NO occurred (Fig. 6.2). Initial linear rates could still be calculated.

After fertilizing soil cores, the NO mixing ratios rose to above 1 ppmv of the soil matrix air, and remained elevated even 1-2 weeks later (Fig. 6.3). The NO levels used in our experiments are therefore directly representative of NH_4^+-fertilized soil. Unfertilized soil cores contained only about 5 ppbv NO, and the compensation points in closed-flask experiments were generally <20 ppbv. Nevertheless, the first-order nature of the reaction over a mixing ratio range of 0 to 14 ppmv (Fig. 6.2) suggests that NO consumption rate constants calculated at starting mixing ratios of 2 ppmv can predict rates at these lower mixing ratios.

The NO mixing ratios in NH_4Cl-fertilized cores were highest near the surface where most of the NH_4^+ would be immobilized (Fig. 6.3). The decrease with depth indicates that NO production and consumption occurred simultaneously. A rough calculation based on the 48-h profiles, assuming steady-state conditions and NO consumption rate constants of 2-3 ml min^{-1} g^{-1} (see below) gives estimated areal

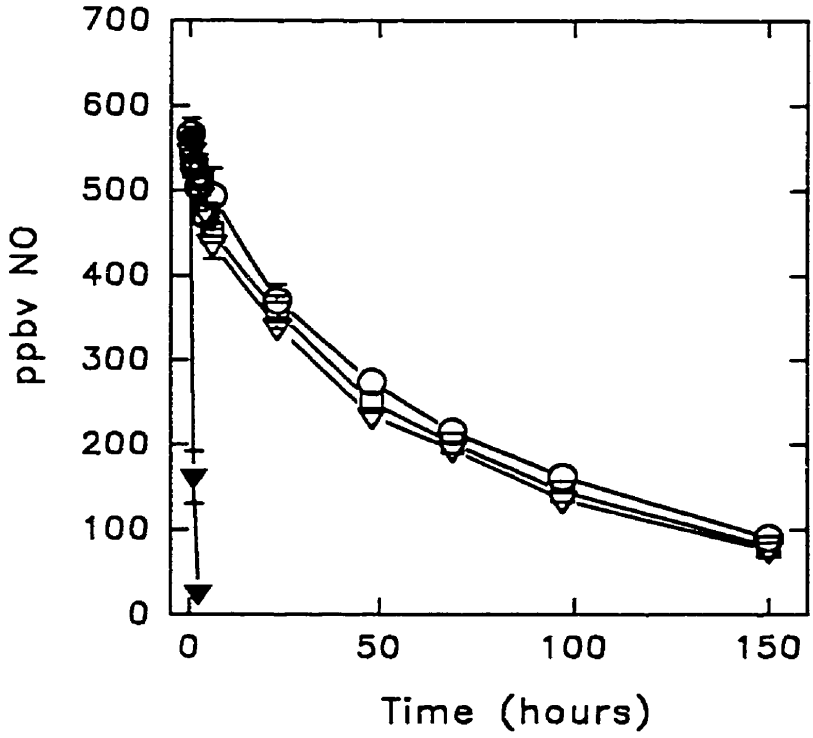

Fig. 6.1 Effect of C_2H_2 on NO consumption measured in 60-ml serum vials containing 10 ml H_2O only. C_2H_2 levels are: 0 Pa (○), 5 Pa (□), 70 Pa (▽), and 2 kPa (▼). Data are means of triplicates ±1 standard error of the mean (SEM). Where error bars are not visible they are contained within the symbol.

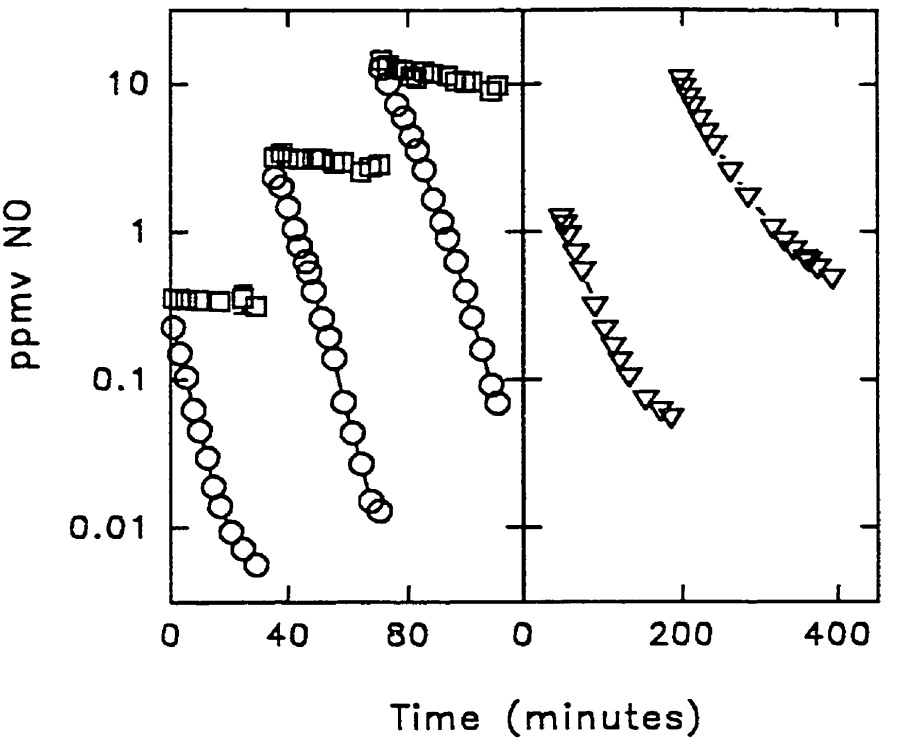

Fig. 6.2 NO consumption by 5.0-g amounts of soil in 60-ml vials (○), 5.0-g amounts of illite in 60-ml vials (□), or 10.0-g amounts of autoclaved soil in 150-ml flasks (▽). Data are means of triplicates. Discontinuities in the curves result from successive NO additions.

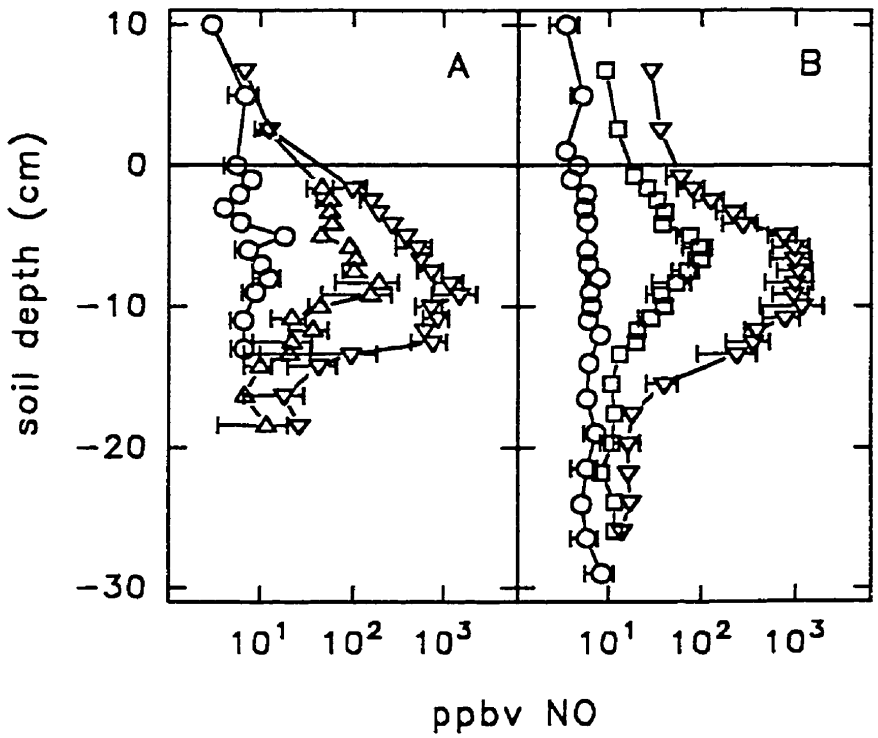

Fig. 6.3 Mixing ratios of NO with depth in soil cores at gas-filled porosities of 0.22 (*panel A*) or 0.37 (*panel B*), before (○), 2 days after (▽), 6 days after (△), or 10 days after (□) fertilization with 100 kg NH_4Cl-N ha^{-1}. Data are means of quadruplicate cores ±1 SEM.

consumption rates of 4-6 mmol NO m^{-2} day^{-1}. This is nearly 1% day^{-1} of the added fertilizer N. NO fluxes calculated from NO gradients in the upper 5 cm of soil using diffusion relationships from Dunfield et al. (1995), or by measuring initial linear increases in headspace NO mixing ratios in capped fertilized soil cores at similar gas-filled porosities to those in Fig. 6.3 (Dunfield and Knowles, unpublished data) are never greater than 15% of these estimated consumption rates. Although these are rough calculations, most of the NO produced in soil appears to be consumed before it can escape to the atmosphere.

Soil NO-consuming capacity was reduced 60-80% by most sterilization procedures (Table 6.1). Most of the activity was therefore a result of biological, probably microbial, processes. The 300% stimulation of NO consumption in irradiated soil may have resulted from the creation of highly reactive free radicals (Choudhry 1984). Addition of 15 m*M* glucose stimulated soil NO consumption (from 2.93 $\pm$0.21 to 6.17 $\pm$0.11 ml min^{-1} g^{-1} after a 90-min incubation), without any increase in N_2O production. NO consumption may therefore result from heterotrophic microbial activity other than denitrification.

N_2O production from NO in fresh soil was monitored in the aerobic continuous-flow system at 50-1500 ppbv NO. This pathway usually accounted for $<2\%$, and never $>8\%$, of the total NO consumed (data not shown). Soil supplemented with 450 ppmv N_2O also exhibited no detectable N_2O-consuming capacity, so denitrification to N_2O or N_2 was not a significant sink for NO. The major product of NO consumption in fresh soil was NO_3^-, regardless of the gas flow rate (Table 6.2). We attempted to discern whether NO_2^- was an intermediate in the oxidation of NO by adding 10 m*M* chlorate, an inhibitor of NO_2^- oxidation (Belser and Mays 1980). However, chlorate was not an effective inhibitor of NO_2^- oxidation at the low (<50 μM) NO_2^- concentrations which might have arisen from NO oxidation in these experiments (data not shown).

The product of NO consumption in autoclaved soil was neither N_2O (measured in stoppered flasks with added NO, data not shown), NO_2^-, nor NO_3^- (Table 6.2). The NH_4^+ pool was too large to discern any increase or decrease. At the conclusion

Table 6.1 Effects of various sterilizing agents on soil NO consumption rate constants. Data are in ml min^{-1} g^{-1}, and are means ± 1 standard error of the mean. The NO consumption rate in flasks containing H_2O only has been subtracted from reported rates. Details of each technique are given in the text.

Sterilizing agent	n	Control soil		Sterilized soil		Growth in nutrient broth	Inhibition (%)
Autoclaving	3	2.10	±0.14	0.548	±0.013	-	74
Fumigation	3	3.22	±0.034	0.581	±0.0067	+	82
HgCl	3	1.77	±0.097	0.724	±0.0097	-[a]	59
Glutaraldehyde	3	0.389	±0.038	0.153	±0.018[b]	-[a]	61
Irradiation	4	0.751	±0.15	2.14	±0.047	-	

[a] One of three replicates showed weak growth in nutrient broth.

[b] This value is NO consumption by soil less NO consumption by a glutaraldehyde solution.

Table 6.2 Percentage estimated NO consumed recovered as $NO_3^-+NO_2^-$ in soil incubated in a continuous-flow system. Recovery is based on postincubation [$NO_3^-+NO_2^-$] in elevated-NO soil versus [$NO_3^-+NO_2^-$] in control soil, except for experiments 6 and 7 which are based on [$NO_3^-+NO_2^-$] change in measurements made at the beginning and end of the incubation period. Data are means of three replicates ±1 standard error of the mean (SEM).

Experiment	mean residence time of flush gas (s)	[NO] (ppbv)	time (h)	% as $NO_3^-+NO_2^-$ (Mean ±1 SEM)
1 Soil[a]	218	428	139	96.4 ±13.3
2 Soil[a]	122	420	120	108. ±19.7
3 Soil[ab]	21.5	324	110	129. ±17.0
4 Soil + 100 ppmv C_2H_2[a]	239	326	119	80.4 ±8.2
5 Autoclaved soil	230	569	240	15.4 ±9.7
6 Autoclaved soil[c]	189	1075	240	-1.2 ±3.4
7 Soil + HgCl	550	350	169	14.6 ±18.2

[a] No increase in NO_2^- was detected.

[b] This experiment used 6.0-g amounts of soil in 50-ml Erlenmeyer flasks. All others used 25.0-g amounts in 250-ml flasks.

[c] n=2 replicates.

of NO feed experiments 1, 5, and 7 (Table 6.2), NO consumption in soil from the elevated-NO treatments (Expt. 1: 3.09 $\pm$0.12; Expt. 5: 0.522 $\pm$0.0067; Expt. 7: 0.295 $\pm$0.0044 ml min^{-1} g^{-1}) was not significantly decreased compared to control soil (Expt. 1: 3.04 $\pm$0.12; Expt. 5: 0.576 $\pm$0.139; Expt. 7: 0.311 $\pm$0.018 ml min^{-1} g^{-1}). The product or products of NO consumption in autoclaved soil, which inhibited NO consumption in stoppered flasks (Fig. 6.2), are therefore probably flushed out of the system as gases. NO consumption in autoclaved soil was not O_2-dependent (Table 6.3).

The NO-consuming process in sterile soil, which represented about 25% of the total activity, did not result in NO_3^- accumulation. Yet on average 100% of the NO consumed by fresh soil was recovered as NO_3^- (Table 6.2). The product of the abiological NO-consuming process, perhaps NO_2, may therefore have been oxidized microbially to NO_3^-. The NO source also contained 7.5% of the NO mixing ratio as contaminating NO_2 (Matheson Gas Products, Ville St. Laurent, Quebec). If all this NO_2 in the feed was oxidized to NO_3^-, it could account for 20% of the NO_3^- produced in experiments 1 and 4, 34% in experiment 2, and up to 81% in experiment 3. Microbial NO_2 metabolism could explain why an NO oxidation product accumulated and inhibited NO oxidation in autoclaved but not fresh soil (Fig. 6.2). An alternate explanation for >75% NO_3^- recovery is that NO consumption rates estimated in closed flasks could be lower than actual rates in the continuous-flow system. Despite the gas humidifier, soil dried out slightly during some experiments, especially experiment 3 (from 97% to 83% H_2O d.w.). Increased NO_3^- production would occur in drier soil because NO consumption is diffusion-limited. NO consumption decreased with increasing soil moisture, both in fresh and autoclaved samples (Fig. 6.4). However, H_2O content changes of <15% are probably not great enough to account for the increased NO_3^- production in the continuous-flow system.

The NO consumption rate rose to a peak at 25°C and remained almost constant from 25 to 70°C (Fig. 6.5). Abiological NO consumption was exponentially related to temperature, and accounted for an increasing proportion of the total activity with increasing temperature. If the biological activity is represented as the difference in

Table 6.3 NO consumption rate constants in autoclaved soil and nonsterile illite in the presence and absence of O_2. Data are in ml min^{-1} g^{-1}, and are means of two replicates ± 1 SEM.

Trial		Aerobic		Anaerobic	
1	Soil	0.505	±0.0156	0.507	±0.021
	Illite	0.0336	±0.0011	0.00591	±0.0004
2	Soil	0.378	±0.018	0.420	±0.018
	Illite	0.0449	±0.0042	0.0224	±0.0097

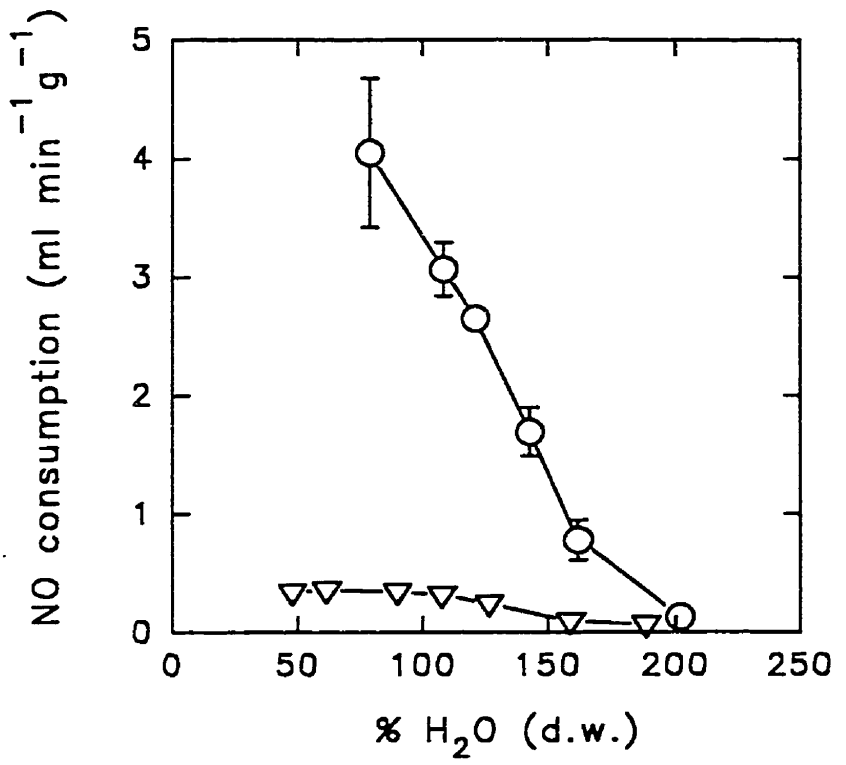

Fig. 6.4 Effect of H_2O content on NO consumption rate constants in fresh (○), and autoclaved (▽) soil. Data are means of duplicates ±1 SEM, and have been corrected for the NO consumption rate of vials containing H_2O only. The water-holding capacity of the soil is 230% d.w. Where error bars are not visible they are contained within the symbol.

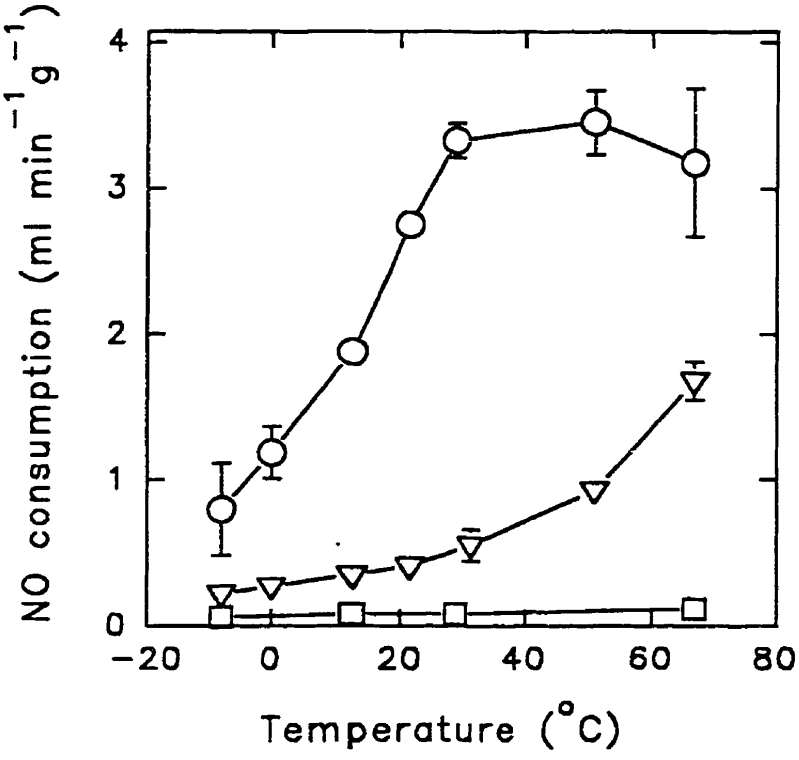

Fig. 6.5 Effect of temperature on NO consumption rate constants in fresh soil (○), autoclaved soil (▽), or illite clay (2.0 g + 0.5 ml H_2O) (□). Data are means of duplicates or triplicates ±1 SEM. Where error bars are not visible they are contained within the symbol.

the two curves in Fig. 6.5, then it is bell shaped and peaks at 25°C.

6.5 Discussion

Autoclaving eliminated 75% of the NO-consuming activity in the humisol. This was not due to the destruction of reactive chemical compounds or some other artifact of autoclaving. Several sterilization procedures agreed that the majority of the activity was biological under the conditions used, although the proportion changed with temperature. Two pathways for NO consumption are also indicated by the temperature response, which could be explained as the sum of a chemical reaction exponentially related to temperature and a microbial process with a bell-shaped response peaking at 20-30°C. In some mineral soils, NO consumption exhibits bell-shaped temperature responses with optima of 25-30°C (Saad and Conrad 1993a), and autoclaving completely halts the activity (Remde et al. 1989). Abiological consumption of NO at sub-ppmv mixing ratios may be peculiar to certain soils such as this humisol.

In autoclaved soil, NO consumption apparently slowed as the reaction proceeded in closed flasks. NO uptake by sterile soil could involve physical sorption, the rate tending to zero as a sorption-desorption equilibrium is approached. Alternatively, it could be a chemical transformation, perhaps to NO_2, where accumulated product can reconvert to NO. The process was independent of O_2 and therefore not a catalysis of the reaction of NO with O_2. Remde et al. (1989) observed no oxidation of NO to NO_2 in an autoclaved soil with only 2-3% organic carbon, but the chemical smorgasbord of organic matter contains many potentially reactive compounds. Humic material is rich in carbonyl groups and organic free radicals (Choudhry 1984), which are involved in NO oxidation (National Research Council 1977). The stimulatory effect of gamma irradiation is suggestive of NO consumption by free radicals. Nitrosation of soil phenolic compounds during nitrification, attributed to NO_2^- (Azhar et al. 1986), could occur through intermediates of NO reaction pathways (Fontecave and Pierre 1994), and account for a portion of soil NO-consuming activity.

The main pathway for NO consumption was a biological oxidation, leading ultimately to NO_3^-. Denitrification accounted for little activity. N_2O is the major product of NO consumption in anaerobic soil (Baumgärtner and Conrad 1992b), but NO reductase is repressed in aerobically-grown denitrifier cells (Remde and Conrad 1991b). NO consumption kinetics vary between aerobic and anaerobic soil incubations. In our humisol and another aerobic soil (Schuster and Conrad 1992), the rate was first-order to above 15 ppmv. Denitrifiers and anaerobic soils typically show a hyperbolic kinetic response, with $K_{m(app)}$ values of 0.3-5 ppmv (Remde and Conrad 1991a; Baumgärtner and Conrad 1992b; Schuster and Conrad 1992).

NO oxidation by O_2 is an exothermic process under standard conditions. Oxidation of NO to NO_2^- is coupled to NADH production in *Nitrobacter* spp. (Freitag and Bock 1990). These organisms may contribute to the activity in our humisol, but the stimulatory effect of glucose implies a process of heterotrophic NO oxidation. Such a process could involve spurious reactions with enzyme active sites, or reactions with cellular materials and metabolites produced during the degradation of humic substances. Organic free radicals and reactive oxygen species are transiently produced during enzymatic degradations. NO also undergoes redox reactions with transition metals and metal-containing proteins (Wellburn 1990; Fontecave and Pierre 1994). Chemical reactions with cellular materials and metabolites, rather than enzymatic NO oxidation, might explain the relatively high NO-consuming activity in soil below 0°C, and even account for part of the activity in sterile soil.

The pathway of NO oxidation could involve a variety of intermediates, including NO_2 and NO_2^-. Nitrite would be readily oxidized to NO_3^- by *Nitrobacter* spp. For NO_2 to be a free intermediate, the stoichiometric recovery of NO as NO_3^- in our continuous-flow experiments requires that NO_2 deposition (or conversion to NO_2^- or NO_3^-) be much faster than oxidation of NO to NO_2, a reasonable proposition (Voldner et al. 1986; Baumgärtner et al. 1992). In fresh soil NO_3^- accumulation accounted for more than the 60-80% of NO-consuming activity assumed to be microbial, suggesting that NO_2 formed through chemical NO oxidation or contained as a contaminant in the feed was also converted to NO_2^- and NO_3^-. No accumulation of

$NO_2^-+NO_3^-$ occurred in sterile soil, so any conversion of NO_2 to these ions would have to be a biological process, perhaps involving antioxidants (Ramge et al. 1993). This proposition contrasts with the observation that NO_2 is converted chemically to NO_3^- and NO_2^- in soil (Ghiorse and Alexander 1976), but up to 1000-fold lower NO_x mixing ratios were used in our study. Rate constants of chemical NO and NO_2 disproportionation are second-order (Schwartz 1984).

Increasing moisture content typically decreases NO production (Shepherd et al. 1991; Slemr and Seiler 1991; Drury et al. 1992; Cárdenas et al. 1993; Davidson et al. 1993; Skiba et al. 1994; Yamulki et al. 1995), and the ratio of NO to NO_3^- produced in soil (Hutchinson et al. 1993). It has been suggested that slowed diffusion in wet soil facilitates the reduction of NO to N_2O before it can escape to the atmosphere (Davidson et al. 1993; Yamulki et al. 1995), but a similar argument applies to oxidative NO consumption. In the humisol, NO consumption apparently showed the opposite trend, decreasing with increasing H_2O content. There is, however, a fundamental difference between gaseous NO diffusing into solution, and NO produced in solution by nitrifiers diffusing into the atmosphere. In the first instance the diffusion path to the organisms (or reaction sites) is increased by high moisture content, in the second the diffusion path away from them is increased. The implication of our result is simply that NO oxidation is diffusion-limited.

We did not use high levels (>2 kPa) of C_2H_2 to inhibit nitrification in these experiments because, as noted previously (Paul et al. 1993), C_2H_2 at such partial pressures reacts with NO. Klemedtsson et al. (1988b) advocate the use of 5 Pa C_2H_2 to inhibit nitrification in experiments where N_2O is being measured, as this partial pressure has little effect on N_2O reductase. We suggest that this level also be used for NO experiments, as the reaction of NO with C_2H_2 becomes negligible at <50 Pa. Incubating soil with >2 kPa C_2H_2 and replacing the headspace atmosphere before adding NO is also appropriate (Davidson 1992; Schuster and Conrad 1992), but if this is not done (Parsons and Keller 1995), conclusions about the contribution of nitrifiers to NO flux cannot be made. The reaction of NO with C_2H_2 might also compete with NO reductase in studies of potential denitrification, although only when molecular

oxygen, required by the reaction, is present. In this way aerobic denitrification could potentially be inhibited by C_2H_2, and thereby overlooked in some environmental studies.

We have demonstrated here that a biological pathway of NO oxidation is an ecologically significant component of soil NO turnover. It should be stressed that our sampling scheme was not intended to be temporally or spatially exhaustive, so periods and hotspots of intense denitrification may also be important in NO turnover. The rate constants of oxidative NO uptake in this humisol, up to 4 ml min^{-1} g^{-1}, are similar to values of 0.3-6 ml min^{-1} g^{-1} attributed to soil denitrification in other studies (Remde et al. 1989; Krämer and Conrad 1991; Schuster and Conrad 1992). The intermediates of the process were not determined, but microbial NO_2 oxidation may also occur. The relative importance of NO_x metabolism by *Nitrobacter* spp., and reaction pathways mediated by soil heterotrophs deserves further attention.

6.6 Acknowledgements We thank Dr. Francois Lepine at L'Institut Armand-Frappier for gamma-irradiation of samples.

6.7 Note added in proof

Baumgärtner et al. (1996) also reported oxidative consumption of NO by heterotrophic bacteria in soil, and McKenney et al. (1996) reported a C_2H_2 - NO reaction during denitrification in soil.

PREFACE TO SECTION 7

Section 7 continues the investigation of NO consumption. The NO uptake rate constants measured in Section 6 were extremely high compared to other aerobic soils. Other data suggest that this may be a property common to organic soils (Baumgärtner and Conrad 1992a). In Section 6, aerobic NO oxidation was proposed to result from heterotrophic activity, providing a possible explanation for the high rates in organic soils.

This section, and a portion of section 8, diverge somewhat from the humisol theme of the thesis. They do so only to provide a context for the humisol within a range of soil types, and to make some inferences about the roles of organic matter and soil heterotrophs in NO flux regulation. NO uptake was tested in a set of soils incorporating a wide range of organic contents. A humic gleysol with only 7% organic matter was also used in an experiment to examine the effects of organic amendments on NO cycling. In Section 8 the same gleysol is compared to the humisol, this time to assess the relative importance of NO oxidation in controlling NO fluxes in organic versus mineral soils.

Section 7 has been accepted for publication and is reproduced with kind permission from Blackwell Science Ltd.

Dunfield PF, Knowles R. Organic matter, heterotrophic activity, and $NO^{\bullet}$ consumption in soils. Global Change Biol (in press)

SECTION 7. ORGANIC MATTER, HETEROTROPHIC ACTIVITY, AND NO CONSUMPTION IN SOILS

7.1 Abstract

When the effect of water content was minimized, soil CO_2 evolution and soil organic matter content were good predictors of aerobic NO uptake rate constants across a wide range of soil types. Field manure application to a gleysol stimulated NO uptake rate constants and lowered NO compensation points compared to unfertilized or NH_4NO_3-fertilized soil. This effect lasted for months after manure application. In a laboratory experiment, addition of manure reduced the NO efflux associated with nitrification of NH_4Cl fertilizer, and manured soils had a greater capacity to remove NO from polluted air. Evidence is presented that these observations result from NO oxidation during heterotrophic microbial activity in soil.

7.2 Introduction

The trace gas nitrogen monoxide (NO) is of biogeochemical interest because of its role in soil fertilizer loss, acid precipitation, and tropospheric chemistry. The atmospheric chemistry of NO is closely tied to other oxidants, notably ozone, and it is therefore a major factor in many atmospheric processes of present concern, including the turnover of greenhouse gases. Recent reviews have dealt with the biochemistry (Fontecave and Pierre 1994, Wink et al. 1996), soil microbiology (Williams, Hutchinson et al. 1992; Conrad 1996a), and atmospheric chemistry (Logan 1983) of NO.

Nitrogen monoxide emissions from soils account for an estimated 15-40% of the total global NO source strength (Williams, Hutchinson et al. 1992; Conrad 1996a). Soil NO production results primarily from enzymatic or chemical reductions of nitrite (NO_2^-) (Remde et al. 1989; Remde and Conrad 1990; Davidson, 1992) during autotrophic nitrification (Remde and Conrad 1991c; Davidson 1992; Hutchinson et al. 1993; Skiba et al. 1993), but denitrification, heterotrophic nitrification, nitrate respiration, and chemical processes may also contribute in some

situations (reviewed in Conrad 1996a).

Although usually NO sources, soils act occasionally as net sinks (Slemr and Seiler 1991; Skiba et al. 1994). Nitrogen monoxide production is virtually zero-order, but consumption is concentration-dependent and exerts more control on net flux as the NO mixing ratio rises (Remde et al. 1989). A compensation point therefore exists where production and consumption processes are in balance, and the direction of soil flux depends on the ambient NO mixing ratio. This ranges from <0.05 parts per billion of volume (ppbv) in clean rural air, to over 100 ppbv in polluted urban air (Logan 1983). Soil compensation points overlap this range, with measurements varying from 1-1500 ppbv (Johansson and Galbally 1984; Johansson and Granat 1984; Remde et al. 1989; Baumgärtner and Conrad 1992a; Kim et al. 1994).

However, the importance of soil NO consumption is not limited to polluted areas. The flux of NO across a soil surface is a balance of productive and consumptive processes occurring within the soil column. Where production is rapid compared to diffusion in soil water and air, the NO mixing ratio in soil is higher than in the (unpolluted) overlying atmosphere. A model based on measured NO diffusion, NO production, and NO consumption rates in a loam soil predicted that compensation NO mixing ratios were reached within <3 cm of the soil surface (Galbally and Johansson 1989). Indeed, during nitrification and denitrification of fertilizer nitrogen, NO mixing ratios in soil air reach values several orders of magnitude higher than in ambient air (Rudolph and Conrad 1996; Dunfield and Knowles 1997), and most of the NO produced can be consumed before escaping to the atmosphere (Dunfield and Knowles 1997).

Although NO adsorbs both chemically and physically to soil particles (Mortland 1965), soil NO uptake is drastically reduced by sterilization, and therefore principally a result of microbial activity (Johansson and Galbally 1984; Remde et al. 1989; Baumgärtner et al. 1996; Dunfield and Knowles 1997). Denitrification may be important in some situations (Remde et al. 1989; Schuster and Conrad 1992), perhaps even under aerobic conditions (Remde and Conrad 1991b), but recent evidence suggests that oxidation to NO_2^- and nitrate (NO_3^-) represents the major NO sink in

aerobic soils (Baumgärtner et al. 1996; Dunfield and Knowles 1997). This process may be mediated by soil heterotrophs (Baumgärtner et al. 1996; Koschorreck et al. 1996; Dunfield and Knowles 1997), although *Nitrobacter* spp. couple NO oxidation to NADH generation (Freitag and Bock 1990). Since the major NO-producing process, nitrification, is aerobic, these oxidative reactions are probably of greater overall importance than microbial denitrification.

Nitrogen monoxide chemistry in biological systems has been well studied because of the roles NO plays in signal transduction and pathogenicity. The primary reactions leading to NO_2^- and NO_3^- production are: (i) the reaction of NO with the superoxide anion (O_2^-) to give oxoperoxonitrate ($ONOO^-$), which decomposes to NO_3^- when protonated (Wink et al. 1996), or possibly is oxidized to NO_2^- through metal-catalysed reactions (Fontecave and Pierre 1994); (ii) reactions with carbon-centred, -oxyl, or -peroxyl radicals (Padmaja and Huie 1993; Wink et al. 1996) which are transient products of enzymatic functions; and (iii) formation of metal-nitrosyl adducts through NO binding on enzyme cofactors, especially ferrous ions. Once bound to metals, NO may react with O_2 or oxygen-containing compounds (Kanner 1996; Wink et al. 1996).

While soil NO production should be controlled by the same factors controlling the magnitude of nitrogen cycle processes, especially nitrification, controls of NO consumption are less clear. The vital factors may be those which influence heterotrophic activity. We therefore investigated NO uptake in a variety of soils, and attempted to relate observed differences to total carbon content and heterotrophic activity.

7.3 Materials and methods

7.3.1 NO uptake in various soils. Samples of various soil types were collected primarily from southern Ontario and Quebec, Canada (Table 7.1). Soils were stored at 8-10°C, but all experiments performed at 25-27°C. The following measurements were made on duplicate subsamples of each soil: loss on ignition (LOI) (420°C, 90 min), gravimetric water-holding capacity (WHC), pH (at 300% WHC), potential NO_2^-

Table 7.1 Selected properties of soils used in this study. All are surface (0-20 cm) samples from agricultural fields except where noted. Soils are classified based on the Canadian system (Canada Soil Survey Committee 1978).

Soil series/description (Order)	Site	Sampling date	pH	LOI[a] (%)	Ref.[b]
1 Clay pan underlying humisol, >35-cm depth	Ottawa, Ont	08/93	7.6	1.6	1
2 Rubicon sandy loam (Podzol)	Ottawa	08/96	8.0	3.2	1
3 Subsurface clay, >35-cm depth	Napierville, QC	08/94	4.4	3.4	2
4 Granby sandy loam (Gleysol)	Ottawa	08/96	7.8	3.8	1
5 Chicot sandy loam (Podzol)	Montreal, QC	11/94	7.2	4.3	3
6 St. Bernard sandy loam (Brunisol)	Montreal	11/94	7.3	4.5	3
7 Grenville loam (Brunisol)	Ottawa	08/96	7.3	4.8	1
8 Matilda loam (Brunisol)	Ottawa	08/96	7.8	5.0	1
9 Ormstown silty clay loam (Gleysol)	Ormstown, QC	08/94	5.7	5.2	4
10 Manotick sandy loam (Brunisol)	Ottawa	08/96	7.3	5.4	1
11 Rideau clay (Brunisol)	Ottawa	08/96	7.4	5.7	1
12 Grenville loam (Brunisol)	Ottawa	10/94	7.0	5.8	1
13 Kars gravelly sandy loam (Brunisol)	Ottawa	08/96	7.2	6.1	1
14 St. Bernard loam (Brunisol)	Montreal	11/94	7.1	7.1	3
15 North Gower clay loam (Gleysol)	Ottawa	06/95	6.1	7.2	1,6
16 Rideau clay, eroded (Brunisol)	Ottawa	10/94	5.7	7.2	1
17 Dalhousie clay (Gleysol)	Montreal	11/94	6.6	8.6	3
18 North Gower clay loam, manured (Gleysol)	Ottawa	06/95	6.8	9.4	1,6
19 St. Zotique silt loam (Podzol)	Montreal	11/94	7.3	9.7	3
20 North Gower clay loam (Gleysol)	Ottawa	10/94	6.8	14.6	1
21 North Gower clay loam (Gleysol)	Ottawa	08/96	7.0	15.7	1
22 Morristown silt loam (Podzol)	Karsdale, NS	10/94	6.2	19.5	5
23 North Gower clay loam (Gleysol)	Ottawa	08/96	7.0	28.0	1
24 (Humisol)	Montreal	08/96	7.3	31.6	3
25 Mixed hardwood forest H horizon	Durham, NH	07/94	4.4	35.9	7
26 (Humisol)	Napierville	08/94	6.6	52.0	2
27 Aspen forest H horizon	Thompson, Man	07/94	5.1	56.4	
28 (Humisol)	Ottawa	08/96	7.2	58.2	1,8
29 (Humisol)	Ottawa	09/94	6.9	61.6	1,8
30 (Humisol)	Napierville	08/94	6.2	73.7	2
31 (Humisol)	Ste. Clothilde, QC	08/94	6.1	78.8	2

[a] LOI, loss-on-ignition.

[b] References: 1 (Schut and Wilson 1987); 2 (Glenn et al. 1993); 3 (Lajoie and Baril 1954); 4 (Fan and MacKenzie 1994); 5 (MacDougall and Nowland 1972); 6 (Topp et al. 1996); 7 (Crill 1991); 8 (Dunfield et al. 1995; Dunfield and Knowles 1997).

oxidation, CO_2 production, NO consumption rate, and N_2O formation from NO.

To determine CO_2 production and NO uptake rates, 5.0-g (fresh weight) amounts of each soil in 60-ml serum vials were wetted to 70% WHC, and vials sealed with butyl rubber stoppers. CO_2 production was estimated by linear regression of headspace mixing ratios measured at 1-day intervals for 3 days. The pseudo first-order NO uptake rate constant (k_{NO}) was estimated by logarithmic regression of the disappearance of 2-3 parts per million of volume (ppmv) added NO (Dunfield and Knowles 1997). N_2O was measured before NO addition and after complete (>90%) removal of added NO from the gas phase. Nitrogen monoxide was measured with a Sievers chemiluminescence analyzer and N_2O by gas chromatography (GC) with a ^{63}Ni electron capture detector (Dunfield and Knowles 1997). CO_2 was measured by GC with a Gow-Mac thermal conductivity detector equipped with a 3 m × 3 mm Porapak Q column.

NO_2^- oxidation was determined by adding 16 ml of 60 μM $NaNO_2$ to 5.0 g of fresh or sterile soil (autoclaved for 1 h on each of three consecutive days) in 60-ml serum vials (shaken 200 rpm). The gas phase of sealed vials was supplemented with 100 ppmv C_2H_2 to halt NO_2^- production, and 1-ml slurry samples taken at intervals for 48 h. These were centrifuged (13000 × g, 15 min) and the supernatant frozen for later colorimetric NO_2^- determination (Dunfield and Knowles 1997). The difference in NO_2^- disappearance between fresh and autoclaved samples was assumed to be microbial NO_2^- oxidation.

7.3.2 Field manuring experiment. Agronomic details of this experiment are described elsewhere (Topp et al. 1996). Briefly, continuous corn plots (9.1 m × 7.6 m) on a humic gleysol (samples 15, 18 in Table 7.1) in a randomized-block (3 blocks) design were annually treated with: (i) no nitrogen fertilizer; (ii) 100 kg N ha^{-1} as NH_4NO_3 (banded); or (iii,iv) 100 Mg ha^{-1} of stockpiled or composted dairy manure applied with a spreader and uniformly mixed with a mouldboard plow to 15-cm depth. Based on average 1994-1995 values, the stockpiled manure was 77% moisture and contained per kg (d.w.): 420 g total organic C, 31 g soluble organic C, 21 g total N, 3.2 g NH_4^+, and 0.35 g NO_3^-. The composted manure was 65% moisture, and

contained per kg: 370 g total organic C, 19 g soluble organic C, 23 g total N, 1.1 g NH_4^+, and 3.0 g NO_3^-. Treatments had been maintained since 1992. During the course of our sampling, inorganic fertilizer was applied in mid-May and manure in the first week of November 1994 and 1995, and on 19-20 Nov 1996.

Surface samples (0-15 cm depth) pooled from 3 spots within each plot were taken at several dates, and 5.0-g subsamples placed in sealed 60-ml serum vials. The equilibrium NO mixing ratios after >36 h were assumed to represent the compensation points. NO uptake rate constants were estimated as previously described, with 100 ppmv C_2H_2 often added to halt nitrification (Dunfield and Knowles 1997). Soil cores 20 to 25 cm deep in 7.8 cm diameter, 50 cm long polyvinyl chloride tubes (Dunfield et al. 1995) were taken, one per plot, on several sampling dates. These were stored at 20°C, and NO flux determined by capping gas-tight in the lab (25-27°C) and measuring headspace NO at 2- to 3-minute intervals for 15-20 minutes. During these short incubations the increase in headspace NO was linear with time.

The final samples were taken on 22 Nov 1996, two days after manure application. Because of the variable surface of the plowed soil, cores were not taken directly. Instead, loose soil was passed through a 1.9-cm sieve and packed 20-cm deep into cores. The bulk density of these artificial cores was slightly less than intact cores taken in August (1.05 vs 1.34 g cm^{-3}). After 7 days, these cores were fertilized with 25 kg N ha^{-1} as NH_4Cl in 40 ml of water.

7.3.3 Mixed-culture experiments. For most experiments, 10^{-5} dilutions of an organic soil (sample 29 in Table 7.1) in 100 ml tryptic soy broth (TSB) (Difco) were grown for 24-48 h on a rotary shaker at 200 rpm. Culture aliquots were then distributed into 60-ml serum vials and treated as described in Table 7.2. Shaking was continued during determination of NO uptake rates. Culture density (based on MPN counts) was usually 10^9-10^{10} cells ml^{-1}. Exact counts are not given since all results are comparative to untreated controls, and NO uptake rates per cell are not extremely meaningful due to the mixed nature of the inocula and diffusion limitation. Exceptions to the above protocol are given below.

Table 7.2 Gaseous NO consumption rate constants in mixed heterotroph cultures from an organic soil (sample 29 in Table 7.1) grown in TSB. Data are means of triplicates ±1 standard error of the mean. Significant differences from control treatments (Bonferroni contrasts, $P<0.05$) are indicated by letters (a,b).

Treatment	NO consumption (ml gas [ml culture]$^{-1}$ min^{-1})		
Exp. 1:			
control culture	0.462	±0.094	a
culture grown with 4.5 ppmv NO	0.415	±0.097	a
Exp. 2:			
control culture	0.563	±0.052	a
uninoculated TSB	0.144	±0.0038	b
autoclaved culture (30 min)	0.175	±0.0096	b
supernatant (cells removed)[a]	0.202	±0.0049	b
Exp. 3:[b]			
control culture	0.708	±0.043	a
culture + 10 m*M* $NaNO_3$	0.763	±0.069	a
1 h anaerobic culture + 2.5 ml C_2H_2	0.447	±0.016	b
Exp. 4:[c]			
control culture	0.947	±0.092	a
+ 1.6 m*M* reduced glutathione	0.963	±0.0064	a
+ 0.8 m*M* ascorbate	0.946	±0.048	a
Exp. 5:[c]			
control culture	0.954	±0.019	a
+ 0.8 m*M* uric acid (suspension)	0.820	±0.057	a
Exp. 6:[d]			
control culture	0.692	±0.082	a
+ 2 m*M* paraquat	0.954	±0.043	b

[a] Centrifuged 15 min at 13000 × g.
[b] All values are less NO uptake in autoclaved culture.
[c] NO uptake tested after a 2-hour incubation with added reagents.
[d] The value given is the aerobic rate less the anaerobic rate.

For experiment 1, 0.25 ml of a 10^{-2} soil dilution was added to 7.5 ml TSB in 50-ml Erlenmeyer flasks. Triplicate flasks were flushed continuously for 21 h with 10 ml min^{-1} of NO-free air or air containing 3-6 ppmv of NO. These were then aired for 2 h before testing NO uptake.

Experiment 3 was intended to ensure that NO uptake occurred in cultures with no denitrifying enzymes present. Cells were grown for 23 h in 1 l TSB with magnetic stirring and continuous sparging with 160 ml air min^{-1}. 2.0-ml culture aliquots were then treated $\pm 5\%$ C_2H_2 and ± 10 m*M* $NaNO_3$. After a further 8-h incubation (shaken 200 rpm), the amount of N_2O formed during the consumption of 3 ppmv NO was measured. Because C_2H_2 reacts rapidly with O_2 and NO (Dunfield and Knowles 1997), the $+C_2H_2$ treatments were evacuated and backfilled (3 times, 15 min) with He, then C_2H_2 readded, before testing NO uptake.

7.3.4 Statistical analyses. Analyses of variance (ANOVAs) were performed using SYSTAT (Systat, Inc., Evanston Illinois). Multiple comparisons are Bonferroni contrasts. Bonferroni adjustments were also made to compensate for multiple dependent variables, and thereby maintain experiment-wide significance levels at $P=0.05$. Data were often log-transformed to satisfy parametric assumptions. For analyses of manuring experiments, multivariate repeated measures ANOVAs were used. Because the effects of stockpiled and composted manure were indistinguishable, these treatments were pooled to increase the power of multiple comparisons.

7.4 Results

7.4.1 NO uptake in various soils. For simplicity, we measured NO uptake rates and compensation points in closed rather than in flow-through systems. The advantage of flow-through systems is that gaseous reactants or products are not allowed to accumulate and thereby alter reaction rates. However, if the NO sink is situated in the microbial cytosol rather than in the gas phase, the composition of the gaseous environment is only important insofar as it affects microbial activity. Control experiments with sample 29 showed that NO uptake rate constants measured within 10 min of completely flushing the headspace atmosphere did not vary significantly from

those measured after a lengthy (18 h) closed incubation (2.09 $\pm$0.16 versus 1.96 $\pm$0.038 ml g^{-1} min^{-1}), and the rate constant was little affected by turning off the laboratory lights (2.06 $\pm$0.020 ml g^{-1} min^{-1}). Mass-balance (input versus output NO mixing ratio) calculations on soil samples in 150-ml flasks flushed continuously with 40 ml min^{-1} of air containing 300 ppbv NO gave the same rate constants as estimated in closed flasks (2.12 $\pm$0.073 versus 1.99 $\pm$0.076 ml g^{-1} min^{-1}).

A strong significant relationship existed between NO uptake and CO_2-evolution rates across soil types (Fig. 7.1). Because of covarying soil properties, strong correlations of log-transforms of CO_2 production (0.82), loss-on-ignition (0.79), and NO_2^- oxidation rate (0.73) with NO uptake were evident. There was no correlation (-0.16) with pH, but addition of pH to a multivariate analysis with LOI did significantly improve the model. The relationships of NO uptake rate constants with CO_2 evolution and LOI were: $\log(k_{NO}) = 0.945 \times \log(CO_2) - 2.26$, or $\log(k_{NO}) = 0.931 \times \log(\%LOI) - 1.63$. Since diffusion limitation should increase with increasing uptake rate constants, these numerical relationships apply only for gaseous NO uptake (at 70% WHC). Slopes for dissolved NO uptake would be higher.

These soils were sampled in dry, aerobic conditions where little denitrification activity was expected, but NO consumption rates were measured in wetter conditions (70% WHC). N_2O formed during NO consumption was usually undetectable, and never accounted for >5% of the NO consumed in any soil (data not shown). Denitrification was therefore unlikely, although complete reduction of NO to N_2 was not impossible. As a further control, samples 6, 9, 16, 18, 19, 20, 27, 29, 30, and 31 were adjusted to 20 m*M* $NaNO_3$ (70% WHC). This was intended to stimulate denitrification and inhibit N_2O reduction (Blackmer and Bremner 1978). After a 20-h incubation, still no more than 6% of consumed NO was converted to N_2O (data not shown).

7.4.2 Field manuring experiment. As expected, the highest NO fluxes, NO production rates, and NO compensation points occurred in NH_4NO_3-fertilized soil, almost certainly due to nitrification (Fig. 7.2). This stimulation lasted for months after fertilizer application. Organic fertilizers caused no significant overall

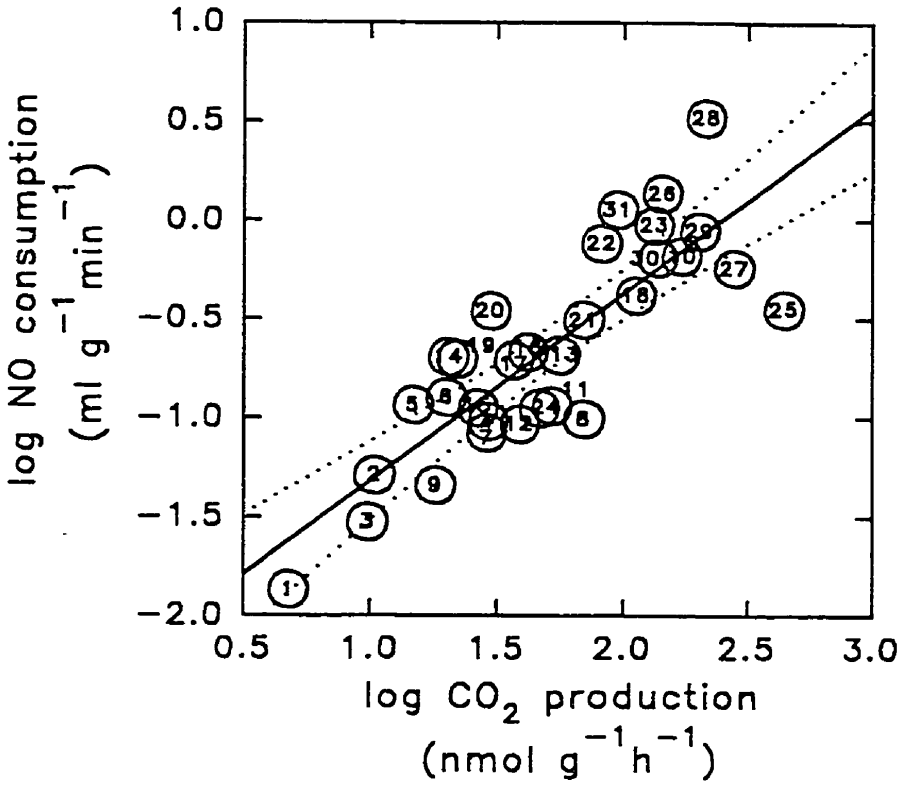

Fig. 7.1 Correlation of gaseous NO uptake rate constants with gaseous CO_2 evolution rates in various soils. The line represents a least-squares regression with 95% confidence limits. Numbers refer to samples in Table 7.1.

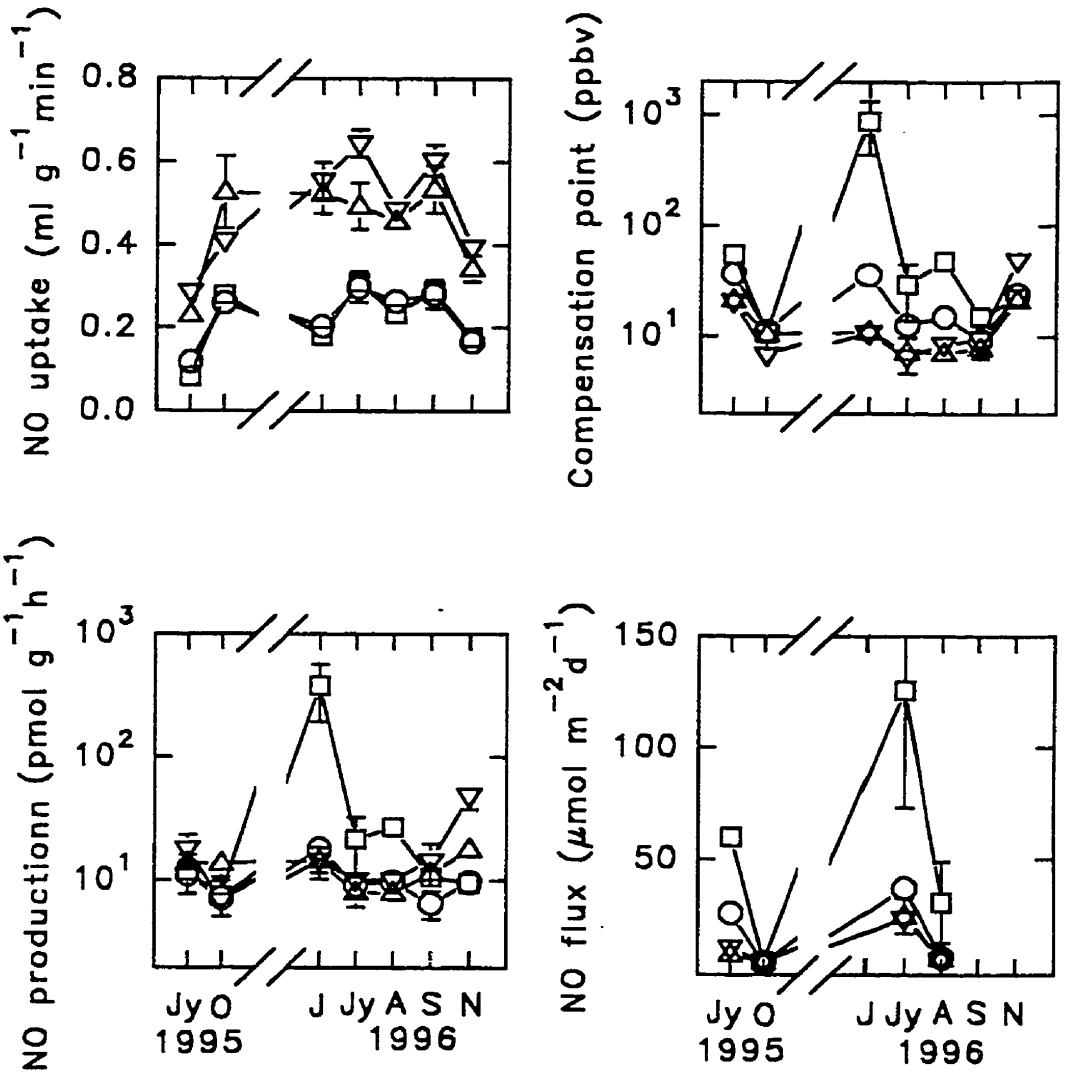

Fig. 7.2 Summary of NO dynamics in a gleysol (sample 15 in Table 7.1) treated annually with no N fertilizer (○), inorganic NH_4NO_3 (□), composted dairy manure (△), or stockpiled dairy manure (▽). NO consumption rate constants and compensation points were measured in closed flasks, and used to calculate NO production. NO fluxes were measured in cores. Bars represent ±1 standard error of the mean. The x-axis is chronological but not to scale. Sampling times were: 21 July 95, 13 Oct 95, 29 June 96, 23 July 96, 22 Aug 96, 11 Sept 96, and 22 Nov 96.

stimulation of NO production. In fact, considered together, manure and composted manure application significantly decreased compensation points compared to untreated soil across the entire sampling period ($P<0.05$). Fig. 7.2 clearly shows that this resulted from increased NO consumption rather that decreased NO production. Soils receiving organic amendments consistently had significantly higher NO consumption rate constants than either unfertilized or NH_4NO_3-fertilized soil. N_2O production during NO consumption was undetectable even in the wettest samples taken 22 Nov 1996, immediately after manure application (data not shown).

Due probably to high variability of soil cores and insufficient sampling, the differences in NO consumption did not translate into significant overall core-flux differences between the two manure treatments versus unfertilized soil (Fig. 7.2), although the difference was significant at the first sample point even after Bonferroni adjustment for multiple sampling ($P<0.0004$).

Table 7.3 gives a more detailed picture of one sampling point in Fig. 7.2. Manuring affected a variety of soil properties, including pH and total microbial activity. Although most of the NO-consuming activity was microbial, some activity remained in sterile soil (Table 7.3). This activity was also increased by manuring, and may have resulted from surface-adsorption.

An overall NO budget comparing inorganic versus organic fertilizers cannot be made here. However, soil given composted manure never had higher NO fluxes or compensation points than untreated soil, even immediately after application (Fig. 7.2). Transiently increased NO production did follow the addition of stockpiled manure (Figs. 7.2 and 7.3), probably due to its higher NH_4^+ content.

NO fluxes after NH_4Cl fertilization were significantly lower in soil cores amended with manure or composted manure compared to unmanured treatments (Fig. 7.3). Several factors might have contributed to this effect, including microbial N immobilization and slowed diffusion in the wetter manured soils. The latter factor would allow more NO to be consumed before it could escape the soil environment. Average gas-filled porosity for soil from unfertilized plots was 0.327, from inorganic fertilizer plots 0.284, manure plots 0.214, and composted manure plots 0.234.

Table 7.3 Selected soil properties on 29 June 1996 from a manuring experiment on a humic gleysol (sample 15 in Table 7.1), with pooled standard errors of the mean (SEM). Significant treatment differences (Bonferroni contrasts, $P<0.05$) for NO parameters are indicated by letters (a,b,c). In the cases of variables log-transformed to satisfy parametric variance assumptions, standard errors are expressed as exponents of e.

Property	Treatment				
	Control	+NH_4NO_3	+Manure	+Compost	SEM
% H_2O d.w.	21.4	19.2	25.7	29.0	3.4
pH	6.3	5.7	7.0	7.1	0.08
CO_2 production (nmol g^{-1} h^{-1})	23.3	21.7	88.9	116	22.0
NO_2^- oxidation (nmol g^{-1} h^{-1})	64.3	66.2	239	191	17.9
NO consumption (ml g^{-1} min^{-1})	0.203 a	0.181 a	0.556 b	0.523 b	0.034
Compensation point (ppbv)	35.6 a	882 b	10.6 a	10.8 a	$e^{0.41}$
NO production (pmol g^{-1} h^{-1})	18.0 a	379 b	14.5 a	14.4 a	$e^{0.42}$
NO consumption autoclaved soil (ml g^{-1} min^{-1})	0.023 a	0.011 a	0.053 b	0.058 b	0.0034

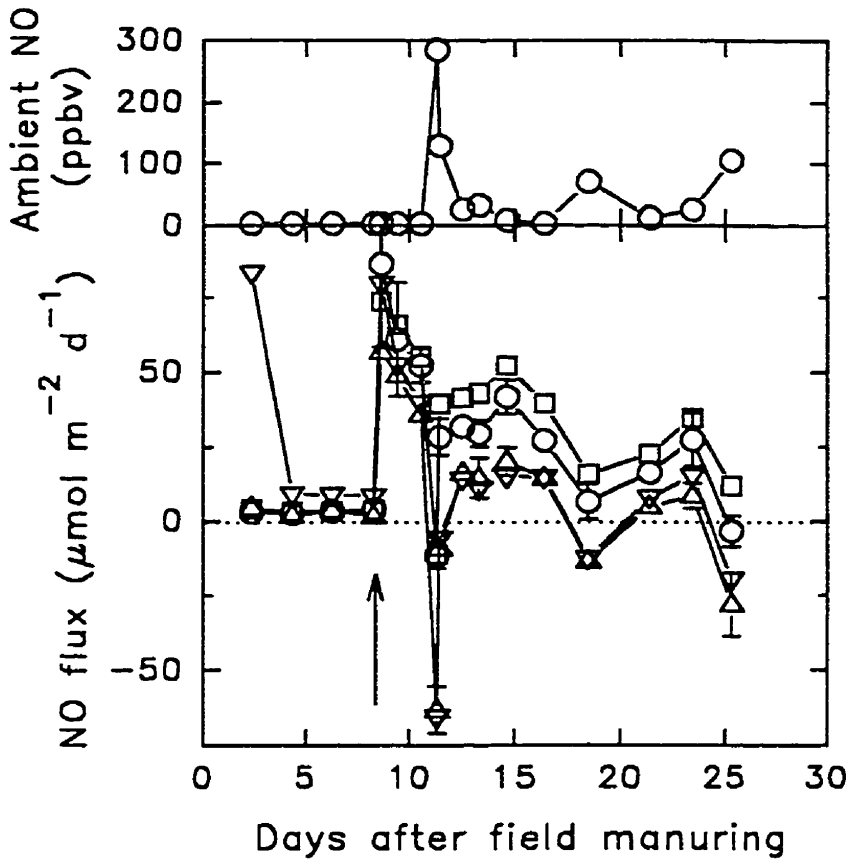

Fig. 7.3 NO fluxes in cores of a gleysol (sample 15 in Table 1) receiving field applications of composted dairy manure (△), stockpiled dairy manure (▽), or no manure addition (○ and □), on 20 Nov 1996 (day 0). Symbols represent soils from the same field treatments as in Fig. 7.2. The arrow indicates the addition of 25 kg NH_4Cl-N ha-1 to cores in the laboratory. The top panel indicates initial ambient NO mixing ratios during flux determinations. Standard error bars are shown for unfertilized (○) and composted manure (▽) treatments only.

However, gas-filled porosity was not a significant factor when included as a covariate in the ANOVA. That the increased NO consumption potential of manured soils was an important factor in flux regulation was revealed by measurements taken during NO-polluted conditions. At these times treatment differences were maximized and manured soils often exhibited negative NO fluxes (Fig. 7.3). NO pollution did not result from laboratory activities, but was representative of actual external air conditions in suburban Montreal.

7.4.3 Mixed-culture experiments. The NO uptake rate constant of a mixed soil heterotroph culture was not affected by the NO mixing ratio under which cells were grown, suggesting that the activity is not inducible (Table 7.2, Exp. 1). The activity was associated primarily with live cells, not with the growth medium or with autoclaved cells, and was decreased by depriving cells of oxygen (Table 7.2, Exp. 2,3). In experiment 3, no (<3%) N_2O production during NO consumption was observed, even in cultures with 10 m*M* $NaNO_3$ added and incubated anaerobically with 5% C_2H_2 to inhibit N_2O reductase (data not shown), so NO consumption clearly occurs independently of denitrification. The free radical scavengers ascorbate, glutathione and uric acid had no significant effects (Exp. 4,5), suggesting that NO uptake was not mediated by small free radicals, but paraquat (ICN Biochemicals), which leads to superoxide formation in living cells (Hassan 1984) did significantly ($P<0.05$) stimulate NO uptake (Exp. 6). In summary, the data suggest an enzymatic co-oxidation mechanism for NO uptake by heterotrophs, but the nature of the oxidant(s) is uncertain.

7.5 Discussion

Several studies have attempted to relate soil NO fluxes to environmental parameters. Interpretation of the results is difficult not only because there are several potential NO sources in soil, but also because surface fluxes are the net result of combined productive and consumptive processes, which are differentially regulated. A more useful approach may be to characterize these processes separately.

A major factor controlling NO fluxes is soil H_2O content, although the nature

of the effect varies over different soils. Watering a dry soil may lead to a sudden burst of mineralization and nitrification (Davidson 1992; Williams, Hutchinson et al. 1992). Positive correlations of NO flux with soil H_2O content (Slemr and Seiler 1991; Williams, Hutchinson et al. 1992) probably result from inhibition of microbial nitrification under dry conditions, but negative correlations have also frequently been noted (Shepherd et al. 1991; Hutchinson et al. 1993; Skiba et al. 1994; Yamulki et al. 1995). These would arise if nitrification becomes O_2-limited at high soil moisture, but another explanation is suggested by the observation that the NO/NO_3^- product ratio of nitrification decreases with water addition (Hutchinson et al. 1993). Slowed diffusion at high H_2O content may allow more of the NO produced to be oxidized before it can escape the soil environment. Such data implicate NO uptake as a strong regulator of net efflux.

Gaseous NO uptake is diffusion-limited and controlled by soil H_2O (Baumgärtner and Conrad 1992; Dunfield and Knowles 1997). To minimize this effect, we adjusted all soils to 70% WHC. This did not eliminate diffusion differences across soil types, but should have resulted in similar % water- versus air-filled pore space, which is a critical control of diffusion (Williams, Hutchinson et al. 1992). The result was a strong correlation between heterotrophic microbial activity, indexed as CO_2 evolution, and NO uptake. This is expected for denitrification, but we consistently found no evidence of denitrification as an NO sink. Unfortunately, C_2H_2 could not be used to inhibit soil N_2O reductase, because of the reaction of C_2H_2 with NO under aerobic conditions (Dunfield and Knowles 1997). However, we did show that NO uptake occurred independently of denitrification in soil heterotrophs.

The correlation of soil NO uptake rate constants with CO_2 production rates therefore supports the hypothesis that NO uptake results from co-oxidation during aerobic heterotrophic microbial activity (Baumgärtner et al. 1996; Dunfield and Knowles 1997), but naturally such a correlation is not proof of causality. CO_2 evolution is closely correlated with organic carbon content, to which many other soil properties are correlated. For example, the correlation with NO_2^- oxidation may indicate a role for *Nitrobacter* (Freitag and Bock 1990). Whether causal or not, soil

organic matter content and/or CO_2 evolution provide useful predictive indices of NO uptake.

The stimulation of NO uptake by manure also agrees with a heterotroph co-oxidation hypothesis. Although carbon addition can stimulate soil denitrification and lead to N_2O evolution, it has an ameliorating affect on NO emissions. The manured gleysol, compared to NH_4NO_3-treated or even to unfertilized soil, consistently had higher NO uptake rate constants and lower NO compensation points. Manured soils should therefore more efficiently recycle soil-produced NO and have a greater capacity to remove NO from polluted air. Both factors were apparent in our experiment combining manure and NH_4Cl additions. The increased NO uptake rate constants of soil with manure or compost not only attenuated NO release, but also led to negative NO fluxes under NO-polluted air.

Overall NO budgets for the inorganic and organic fertilizers used in this study could not be made. NO production through nitrification of manure NH_4^+ (Paul et al. 1993) was evident with stockpiled but not composted manure in our study, probably due to the higher NH_4^+ content of the stockpiled manure. Typically 1-4% of NH_4^+-N is released from soil as NO, although values from 0.1-10% have been noted (Williams, Hutchinson et al. 1992). NO released from several types of manure was estimated as 0.26% of manure N in one experiment (Paul et al. 1993). NO production was a minor, short-term effect of manure in our study, although the analysis ignores NO release during storage and composting.

The mechanism of NO uptake during heterotrophic metabolism is uncertain. The positive effect of paraquat on NO uptake suggests that O_2^- can be involved, but no effect of adding free-radical scavengers was observed. The O_2^- concentration in growing *E. coli* cells has been estimated as 20-40 p*M*, rising to 300 p*M* in superoxide dismutase-incompetent cells (Gardner and Fridovich 1991). Using a reaction constant for NO and O_2^- of 4×10^7 to 7×10^9 M^{-1} s^{-1} (Fontecave and Pierre 1994), the generous assumption that the soil volume is 5% microbial and the very generous assumption that measured gaseous NO uptake rates are not underestimates due to diffusion limitation (which is clearly not true, see Dunfield and Knowles 1997),

microbial O_2^- concentrations of at least 4-800 n*M* would be needed to achieve the NO uptake rates observed in a humisol (Dunfield and Knowles 1997). If the O_2^- concentration measured in *E. coli* is representative of microbes in general, O_2^- is an unlikely agent for NO uptake.

Concentrations of O_2^- and other oxygen radicals can be unusually high in certain situations. Photosynthetic organisms produce O_2^- (Halliwell and Gutteridge 1989), but we measured the same NO uptake rates in dark and light conditions. White rot fungi produce superoxide in order to break down recalcitrant polymers (Cross and Jones 1991). Certain microbial enzymes, such as peroxidases and oxygenases, also leak small amounts of O_2^- (Halliwell and Gutteridge 1989). However, one need not postulate superoxide or other oxygen radicals free in solution to react with NO. The small NO molecule could attack oxidizing species formed transiently in enzyme active sites, or even react directly with enzyme cofactors. Such a mechanism might explain the NO consumption observed in methanotrophs (Krämer et al. 1990), since powerful oxidizing species are produced by monooxygenase enzymes such as methane monooxygenase (Halliwell and Gutteridge 1989).

Nitrogen monoxide is consumed through aerobic co-oxidation reactions in heterotrophic bacteria. Such reactions are suspected to be the primary NO sink in aerobic soils (Baumgärtner et al. 1996; Koschorreck et al. 1996; Dunfield and Knowles 1997). While the exact reaction mechanism was not determined in the present study, the data support this view by revealing a strong correlation between NO uptake rate constants and aerobic heterotrophic activity across various soils. Organic carbon additions to soil can therefore decrease NO efflux.

7.6 Acknowledgements

For permission and/or assistance in sampling field sites we would like to thank Guy Beauduin at Les Distributeurs des Legumes du Quebec, Dr. Michel Fortin, Dr. E. Topp, and Dr. A.F. MacKenzie. We thank especially Dr. E.G. Gregorich for access to and data about the manuring experiment.

PREFACE TO SECTION 8

Section 8 follows up on the suggestion made in Section 4 that rapid nitrification in the humisol could lead to large, transient gaseous nitrogen oxide emissions after fertilization. This may have caused inaccurate field flux estimations in Section 4, as measurements were not intensely replicated in time. Section 8 attempts to estimate the magnitudes of these transient fluxes, and determine the processes controlling them under different water regimes.

Fluxes across a soil surface are a balance of both productive and consumptive processes occurring within the soil. The balance between production and consumption of NO and N_2O by denitrifiers tends to be very complex and difficult to predict. However, initial data from studies presented in Section 8 showed that NO production was only due to nitrification, and Section 6 described a nondenitrification NO consumption process. Attempts were therefore made to simultaneously analyze both NO production and NO consumption, and investigate the effects of fertilization on both processes.

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Dunfield PF, Knowles R. Processes of NO and N_2O production and consumption in an organic soil.

SECTION 8. PROCESSES OF GASEOUS NITROGEN OXIDE PRODUCTION AND CONSUMPTION IN AN ORGANIC SOIL

8.1 Abstract

We examined the processes controlling NO and N_2O fluxes in a humisol. Addition of the nitrification inhibitor C_2H_2 (2-5 Pa) to intact soil cores showed nitrification to be the major source of NO emitted to the atmosphere, regardless of whether cores were dry (gas-filled porosity, ϕ_g=0.27) or nearly saturated (ϕ_g=0.065). N_2O was produced through both nitrification and denitrification, but N_2O losses from nitrification of ammonium fertilizer were small compared to potential losses from denitrification. NO and N_2O losses through nitrification were stimulated by NO_3^- as well as by NH_4^+. Net NO efflux from the soil surface accounted for 0.2-1% of ammonium fertilizer N added to intact cores, but the gross NO production was much higher. Up to 26% of the NH_4^+ oxidized was converted to NO when air-dried samples were rewetted. In aerobic humisol cores (ϕ_g=0.44) the majority (95%) of the NO produced was consumed before escaping to the atmosphere. In cores of a humic gleysol with a lower NO uptake capacity, only an estimated 38% of the produced NO was reconsumed within the soil core.

8.2 Introduction

Transformations of soil nitrogen through the +1 to +2 oxidation states often produce nitrous oxide (N_2O) and nitrogen monoxide (or nitric oxide, NO). Nitrous oxide contributes to global warming and stratospheric ozone depletion (Davidson 1991). Soil-produced NO is active only in the troposphere, where it regulates ozone generation and thereby indirectly influences other processes such as oxidations of greenhouse gases (Williams, Hutchinson et al. 1992). Soils account for an estimated 75% of the N_2O (Davidson 1991) and up to 40% of the NO produced globally (Conrad 1996a), but because of its short tropospheric residence time, soils are the dominant NO source over rural areas (Williams, Hutchinson et al. 1992).

In soils, microbial denitrification and autotrophic nitrification are the principal

sources of gaseous N-oxides, although dissimilatory and assimilatory nitrate reduction, heterotrophic nitrification, and chemical processes also contribute (Conrad 1995; Conrad 1996a). NO and N_2O are obligate intermediates in the denitrification pathway, but the production mechanisms during ammonium oxidation are more complex. These may include decomposition of an intermediate, but are primarily chemical and enzymatic reductions of the product nitrite (NO_2^-) (Ritchie and Nicholas 1972). Ammonia-oxidizing bacteria enzymatically denitrify NO_2^- to NO and N_2O (Poth and Focht 1985; Remde and Conrad 1990). Self-decomposition of nitrous acid is a chemical source of NO (Nelson and Bremner 1970), but cannot account for all abiotic soil NO production (Blackmer and Cerrato 1986). Abiotic NO_2^- reduction is also driven by constituents of soil organic matter (Nelson and Bremner 1970; Blackmer and Cerrato 1986; McKenney et al. 1990), ferrous iron (Conrad 1996a), and perhaps hydroxylamine (Stüven et al. 1992).

Nitrification inhibitors are frequently used to distinguish between nitrification and denitrification as NO and N_2O sources. Autotrophic nitrification is usually the implied target of inhibition, but heterotrophic nitrification can also be affected (Conrad 1996a). Klemedtsson et al. (1988b) described a procedure to characterize N_2O production by using 2-5 Pa acetylene (C_2H_2) to inhibit nitrification and 10 kPa C_2H_2 to inhibit N_2O reduction by denitrifiers. The low C_2H_2 level is also useful in studying NO, but at a partial pressure of 10 kPa, C_2H_2 rapidly reacts with NO and O_2 (Dunfield and Knowles 1997).

Many studies have related soil water content, temperature, and nitrogen status to NO and N_2O fluxes (Williams, Hutchinson et al. 1992). However, it is difficult to apply the relationships obtained across soil types, in part because surface fluxes are the net results of productive and consumptive processes occurring within the soil column. Especially in the case of NO, where various groups of organisms are involved (Conrad 1996a), production and consumption can be differently regulated by environmental variables.

In soil, NO may be reduced to N_2O or N_2 (Remde et al. 1989; Remde and Conrad 1991a), or oxidized to nitrate (NO_3^-) (Baumgärtner et al. 1996, Dunfield and

Knowles 1997). Through its affect on diffusion rates, soil moisture influences the NO residence time in soil, and thereby also influences net NO oxidation or reduction (Drury et al. 1992; Hutchinson et al. 1993). However, the role of NO-consuming processes in regulating NO fluxes has seldom been investigated systematically. A simple model describing NO fluxes from measured NO diffusion, NO uptake, and NO production rates (Galbally and Johannson 1989) applies well in some situations but fails when soil NO turnover rates are not homogeneous with depth (Rudolph and Conrad 1996).

We investigated NO and N_2O fluxes in an organic soil (Dunfield et al. 1995; Dunfield and Knowles 1997) to determine their sources, the relative importance of NO production and consumption processes, and effects of water and nitrogen.

8.3 Materials and methods

8.3.1 Sources of gaseous N-oxides. Soil cores 35 cm deep were taken in 8 cm diameter, 50 cm long polyvinyl chloride tubes from a humisol (pH 6.8-7.2; 60% loss on ignition) on the Central Experimental Farm of Agriculture and Agri-Food Canada in Ottawa, as previously described (Dunfield et al. 1995). For the first experiment, cores were taken from three areas (blocks) several meters apart in December 1994, and stored at 17°C until dried to >0.4 gas-filled porosity (ϕ_g). Cores were then wetted to either $\phi_g=0.27 \pm 0.01$ (standard error of the mean [SEM] of 18 cores) or $\phi_g=0.065 \pm 0.008$, and used in a blocked factorial experiment incorporating 2 water contents (as above) $\times$ 3 fertilizer treatments $\times$ 3 C_2H_2 levels. Cores were watered with 40 ml (for $\phi_g=0.27$ cores) or 20 ml (for $\phi_g=0.065$ cores) of distilled H_2O containing no fertilizer, or containing 100 kg N ha^{-1} as $NaNO_3$ or as NH_4Cl. Control and $+NaNO_3$ cores were first tested at $\phi_g=0.27$, then wetted and used again at $\phi_g=0.065$. C_2H_2 treatments were control (no addition), +3 Pa C_2H_2 to inhibit nitrification, or +5 kPa C_2H_2 to inhibit N_2O reduction (Klemedtsson et al. 1988b).

The effects of these C_2H_2 levels on NO and N_2O production during denitrification were first tested by placing 6.0 g soil samples (135% H_2O d.w.) in 60-ml serum vials, capping air tight with butyl rubber stoppers, and creating an

anaerobic atmosphere by thrice evacuating the atmosphere and refilling with He. The inhibitory effect of C_2H_2 on nitrification was verified in slurries (1 g soil d.w. : 7 ml distilled H_2O) supplemented with 1 m*M* NH_4Cl, incubated aerobically and shaken at 250 rpm on a rotary shaker. Slurry samples were taken at intervals for $NO_3^-+NO_2^-$ determination.

Core N_2O fluxes were estimated by capping gas tight and measuring the linear increase in headspace mixing ratio over 2 h. Where required, C_2H_2 was injected into the headspace at the beginning of the incubation. Cores were then uncapped for at least 1 h before testing NO fluxes during a <12 min enclosure period. Within this period headspace NO mixing ratios increased nearly linearly with time, indicating little flux underestimation due to increasing headspace NO mixing ratios and therefore increasing consumption. Although cores were stored at 20-22°C, flux estimations were performed at 24-28°C.

The effects of NO_3^- on NO and N_2O production during nitrification were examined using 10-ml amounts of an aerobic soil slurry (1 g d.w. : 10 ml H_2O) in 60-ml serum vials, shaken at 250 rpm. Slurries were supplemented with no N, 20 m*M* $NaNO_3$, 10 m*M* NH_4Cl, 10 m*M* $NaNO_3$ + 10 m*M* NH_4Cl, or 10 m*M* NaCl + 10 m*M* NH_4Cl, each treatment replicated $\pm$5-10 Pa C_2H_2. Slurry samples were periodically taken for NO_2^- analysis.

8.3.2 NO production and consumption. Cores were sampled in three blocks in September 1995. These were wetted to $\phi_g=0.11 \pm 0.01$ (SEM 21 cores) and fertilized as before, except that 3 sets of triplicate cores were included for the unfertilized and the $+NH_4Cl$ treatments. After 14 and 44 days, triplicate control and $+NH_4Cl$ cores were divided into 0-2, 2-4, 4-6, and 6-10 cm depth intervals. 5.0-g subsamples were placed in 60-ml serum vials and sealed. The NO mixing ratio after >30 h was assumed to be the NO compensation point. After a further >20 h incubation under 5 Pa C_2H_2 to halt nitrification, NO uptake rate constants (k_{NO}) were estimated from the disappearance of 0.1-0.2 Pa added NO (Dunfield and Knowles 1997).

In June 1996 another set of cores was sampled from the humisol ($\phi_g=0.44$)

and, for comparison, from a humic gleysol (ϕ_g=0.29, pH 6.1, loss on ignition 7%) (Topp et al. 1996), for estimation of gross NO production and consumption. These coring tubes were equipped with ports at 1-cm intervals through which soil matrix air could be sampled with a syringe. Half (3-4) of the cores were fertilized with 100 kg N ha^{-1} as NH_4Cl in 40 ml H_2O, after which NO mixing ratios with depth and surface fluxes were measured at 1- to 7-day intervals. The rest of the cores were watered with 40 ml of H_2O and subsamples from 2-cm (0-14 cm) or 4-cm (14-28 cm) depth intervals assayed for k_{NO}. Gross NO production was then estimated as the sum of surface flux and the integration of ([NO] $\times$ k_{NO}) with depth.

Gross NO production from NH_4^+ was also measured using 1-mm sieved soil air-dried to various H_2O contents. Soil samples in 150-ml Erlenmeyer flasks were fertilized with 0.3-0.6 μmol g^{-1} (d.w.) NH_4Cl in 0.5-1.5 ml H_2O, mixed well, and flasks capped with SubaSeals (William Freeman Co., Barnsley, England). Controls received an equal amount of H_2O. In half of six replicates, NO mixing ratios were followed over time until no extra NO was present in $+NH_4Cl$ samples compared to controls. The remaining samples were periodically injected with 0.5-1 Pa NO to determine k_{NO}. The rate constants were measured within 10 minutes, assuming a zero-order production rate (Remde et al. 1989) and an NO compensation point at the mixing ratio measured immediately before adding NO. The gross NO production from NH_4^+ was estimated by integrating ([NO] $\times$ k_{NO}) over the course of the experiment.

8.3.3 Analyses. NO was measured using a Sievers chemiluminescence analyzer and N_2O by gas chromatography using a ^{63}Ni Perkin-Elmer electron capture detector. NO_2^- and NO_3^- were measured based on the Greiss-Ilosvay reaction (Dunfield and Knowles 1997). Analyses of variance (ANOVAs) were performed using SYSTAT (SYSTAT Inc, Evanston, Illinois). Data were often log-transformed to satisfy parametric assumptions. Multiple comparisons are Bonferroni contrasts.

8.4 Results

8.4.1 Sources of gaseous N-oxides. Nitrification in soil slurries was effectively

inhibited by as little as 1 Pa C_2H_2 (data not shown). 1-4 Pa C_2H_2 had minimal effect on denitrification activity (Fig. 8.1). 10 kPa C_2H_2 increased net N_2O production as expected, but also increased net NO production after 48 h (Fig. 8.1). Acetylene may have become a carbon source for denitrifiers, thereby accelerating nitrogen use.

Inhibiting nitrification eliminated most NO efflux both in dry (Fig. 8.2) and wet (Fig. 8.3) cores. Bursts of NO production after fertilization with NH_4Cl and after watering were due to nitrification (Fig. 8.2). The NO flux stimulation by NH_4^+ addition lasted longer in the wet soil cores (Fig. 8.3), perhaps because NH_4^+ oxidation was O_2-limited. The residual, but non-zero, flux in cores +3 Pa C_2H_2 (Figs. 8.2-8.3) may indicate incomplete C_2H_2 diffusion throughout the soil, although cores were preincubated with C_2H_2 for 2 h before testing NO flux. Alternatively, there may be a small contribution to NO flux by denitrifiers or other bacteria, or ongoing chemical reduction of NO_2^- produced before nitrification was halted.

3 Pa C_2H_2 had no significant effect on N_2O flux in ϕ_g=0.065 cores (Fig. 8.3). Under these near-saturated conditions, N_2O production was obviously due to denitrification, and was strongly stimulated by 3 kPa C_2H_2. N_2O fluxes were measured starting just 11 d after wetting, before denitrification could become N-limited, and fertilization had no significant effect on flux (Fig. 8.3). In the drier cores, N_2O production resulted from nitrification of NH_4^+ fertilizer (Fig. 8.2). However, statistical comparison of other treatments in the dry cores shown in Fig. 8.2 was difficult because of a non-normal distribution. A few cores exhibited N_2O fluxes up to an order of magnitude higher than most cores, indicating isolated N_2O production hotspots.

Gas fluxes were measured at temperatures about 5°C higher than the storage temperature, so integrations of NO and N_2O fluxes over time for Figs. 8.2-8.3 are subject to some error. As a general rule, efflux doubles for every 10°C increase (Williams, Hutchinson et al. 1992), so the error should not be >50%. In Fig. 8.2, 0.054% of the added NH_4^+ was lost as N_2O and 0.25% as NO. 1.04% of the NH_4^+ was lost as NO in Fig. 8.3, and 0.56% in Fig. 8.4, although fertilizer N was still being converted to NO when these experiments were ceased.

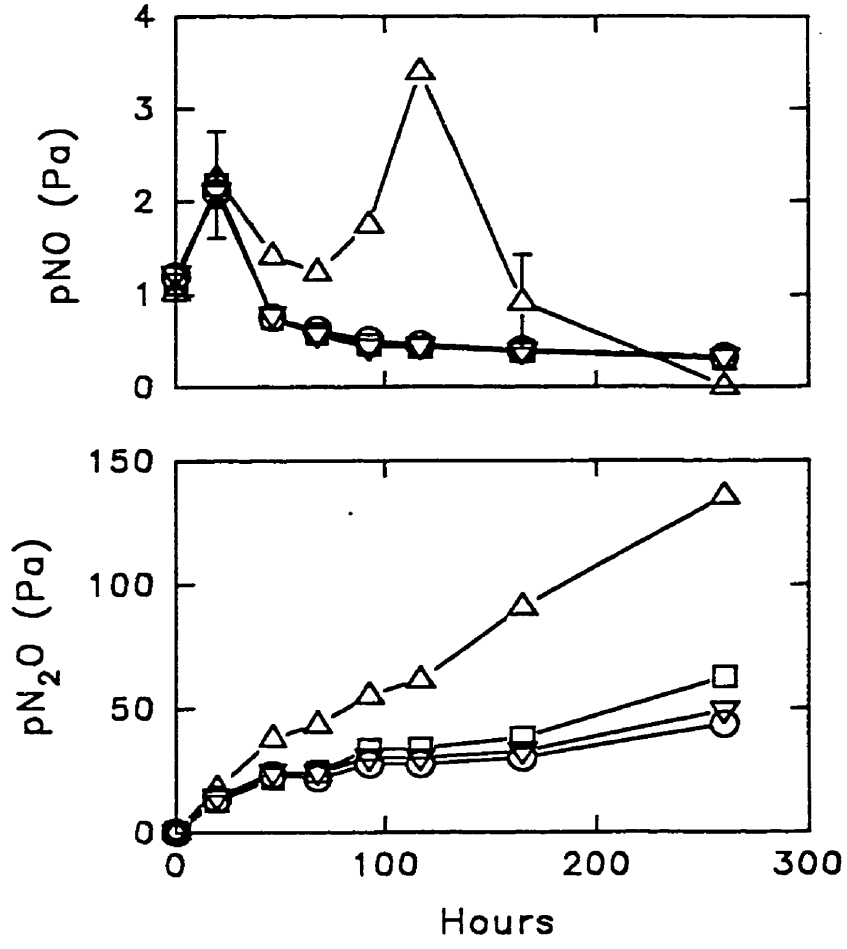

Fig. 8.1 Effects of C_2H_2 additions on anaerobic accumulations of NO and N_2O in closed flasks containing humisol samples. Flasks were supplemented with 10% (▵), 4 Pa (□), 1 Pa (▽), or no (○) C_2H_2. Data are means of triplicates ± 1 standard error of the mean (SEM). Where error bars are not visible they are contained within the symbol.

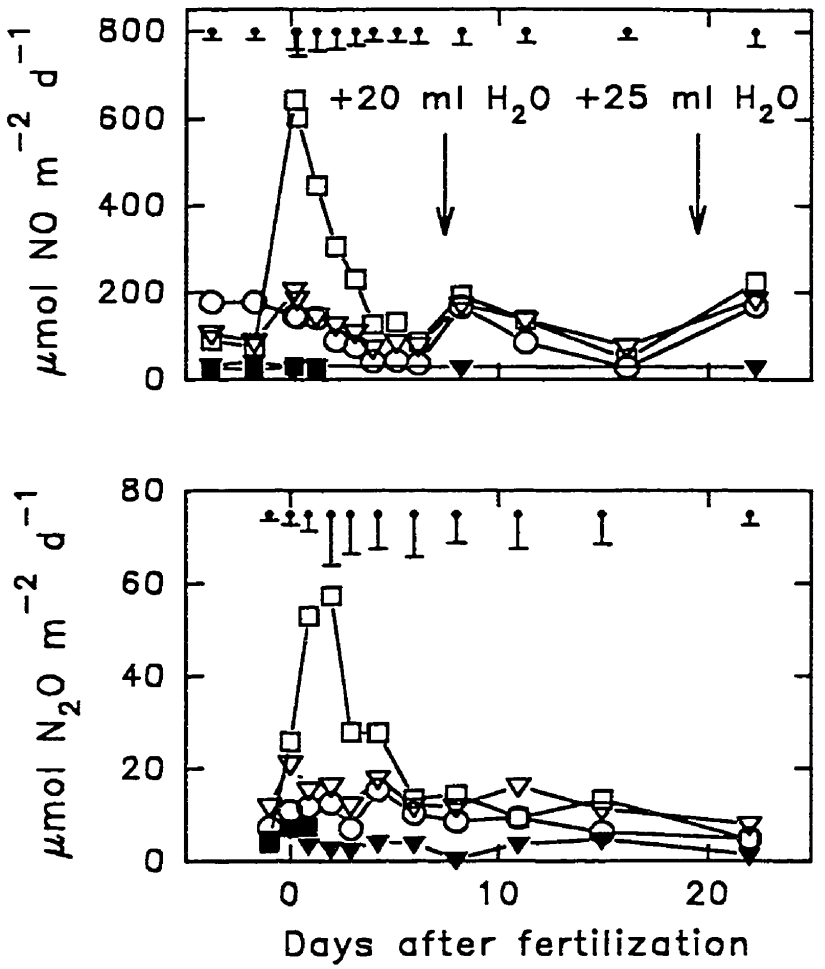

Fig. 8.2 NO and N_2O emissions from humisol cores at initial ϕ_g=0.27. Cores were watered with 40 ml H_2O (○), or fertilized with 100 kg N ha^{-1} as NH_4Cl (□,■), or $NaNO_3$ (▽,▼) in 40 ml H_2O on day 0. Filled symbols represent cores with 3 Pa C_2H_2 added. Fluxes are means of triplicate cores, with 1 SEM pooled for each sampling date shown at the top of each panel.

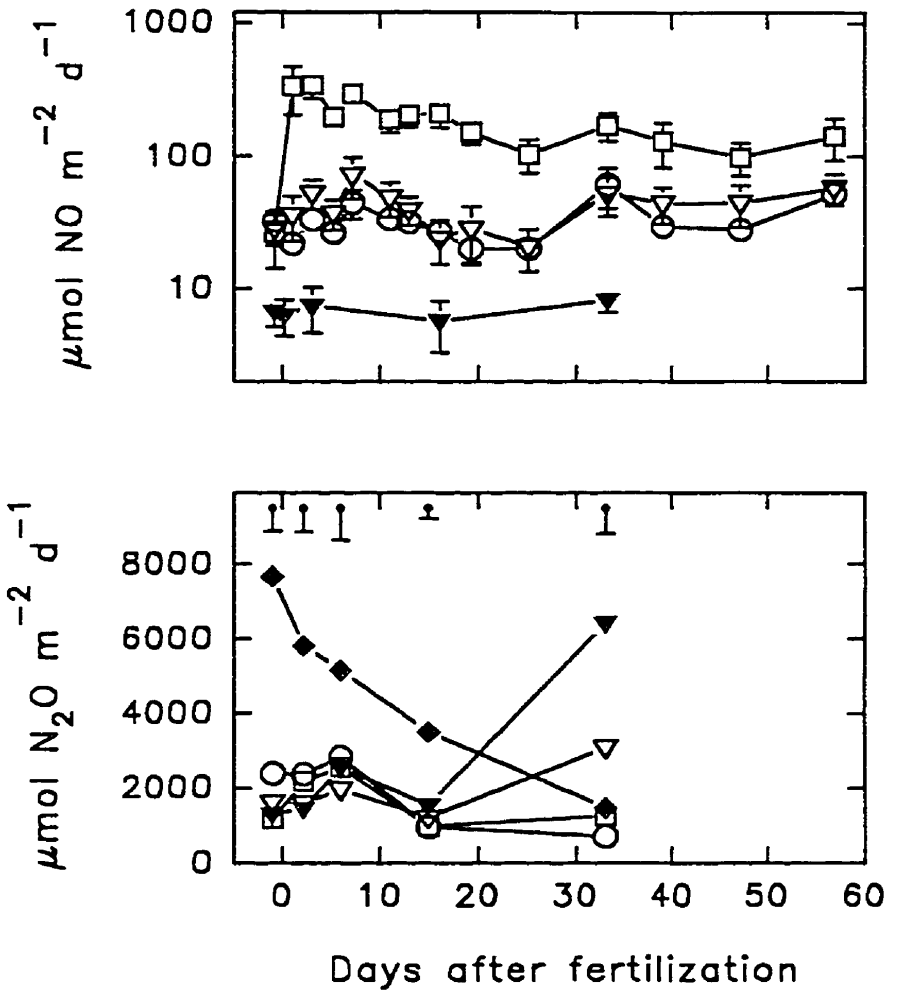

Fig. 8.3 NO and N_2O emissions from humisol cores at initial ϕ_g=0.065. Cores were watered with 20 ml H_2O (○), or fertilized with 100 kg N ha^{-1} as NH_4Cl (□), or $NaNO_3$ (▽, ▼, ◆) in 20 ml H_2O on day 0. The three +$NaNO_3$ treatments are: no C_2H_2 (▽), +3 Pa C_2H_2 (▼), and +3 kPa C_2H_2 (◆). NO fluxes are means of triplicates ±1 SEM. N_2O fluxes are means only with 1 SEM pooled for each sampling date shown at the top of the panel.

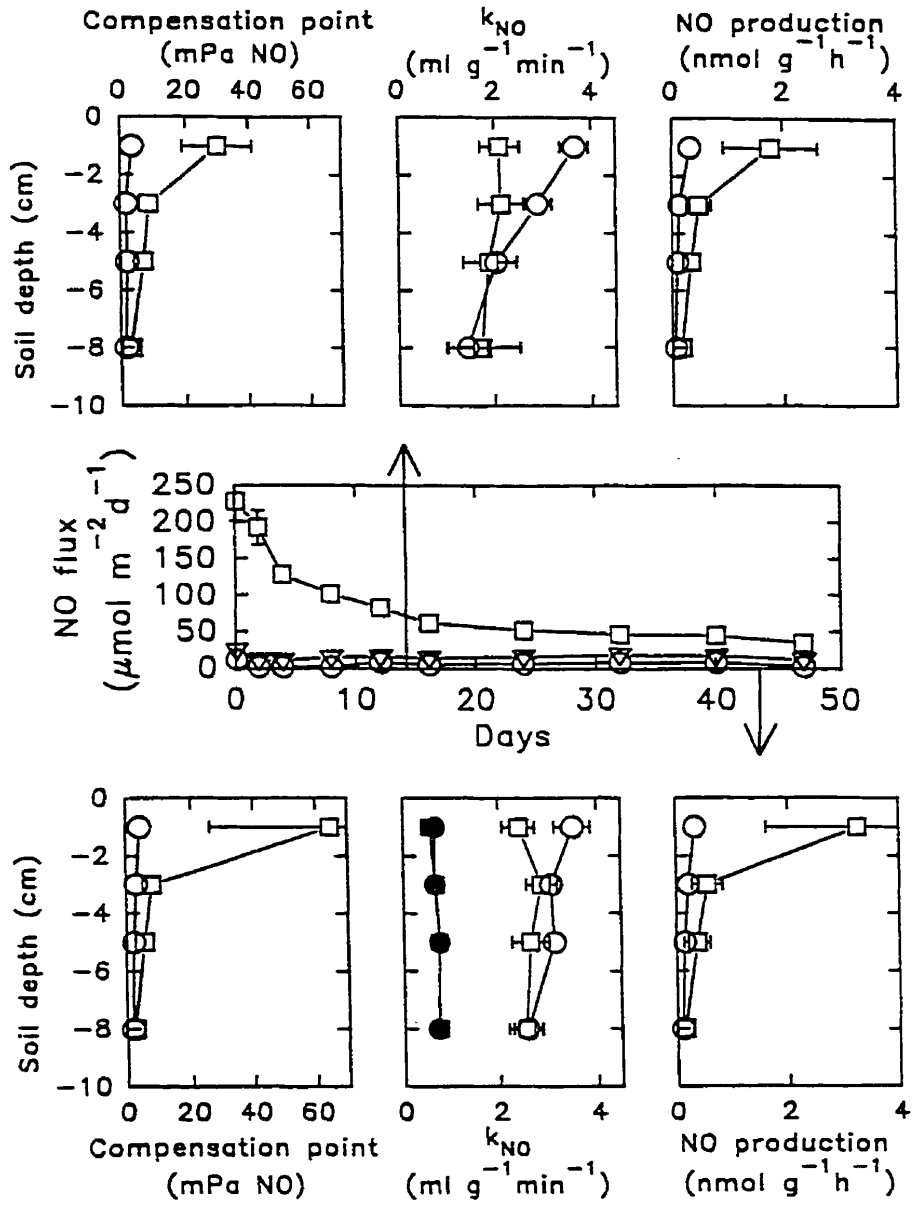

Fig. 8.4 NO emissions from humisol cores at initial ϕ_g=0.11 (centre panel). Cores were unfertilized (○), or fertilized with 100 kg N ha^{-1} as NH_4Cl (□) or $NaNO_3$ (▽) on day zero. At the times indicated by arrows, triplicate cores per treatment were sacrificed and divided into depth subsamples. The top three panels show the NO compensation points, NO uptake rate constants, and NO production rates of these subsamples on the first sampling time. The bottom three panels summarize the second sampling. Data are means of at least triplicates ±1 SEM. Where error bars are not seen they are contained within the symbol. The filled symbols represent samples autoclaved for 1 h on each of 3 consecutive days.

In the experiment shown in Fig. 8.4 (centre panel), $NaNO_3$ as well as NH_4Cl fertilizer significantly stimulated NO flux ($P<0.005$, repeated measures ANOVA). Control NO fluxes were lower than in the first experiment (Fig. 8.3), so denitrification may have been proportionally greater as an NO source, accounting for the effect of NO_3^-. However, a direct effect of NO_3^- on nitrification is also possible. In soil slurries, $NaNO_3$ addition increased N_2O production and NO compensation points during nitrification of NH_4^+ (Fig. 8.5). Although not shown in Fig. 8.5, the effect of $NaNO_3$ on NO was significant even without added NH_4^+. Gaseous N-oxide production was eliminated by 7 Pa C_2H_2, and so was solely the result of nitrification (Fig. 8.5).

The initial pH of the slurry with 10 m*M* added NH_4^+ (Fig. 8.5) was 6.4, and this decreased by <0.1 with $NaNO_3$ addition. However, NO_2^- concentrations were greatly increased by $NaNO_3$ addition. For example (in slurries with NH_4^+ added) addition of 10 m*M* $NaNO_3$ resulted in NO_2^- concentration increases from 36.8 $\pm$1.3 to 91.8 $\pm$4.6 μM after 2 d, and from 48.3 $\pm$1.6 to 88.7 $\pm$6.5 μM after 4 d. The stimulation of N_2O, NO, and NO_2^- concentrations by $NaNO_3$ (Fig. 8.5) was partially (40-50%) reproduced with 10 m*M* NaCl (data not shown).

8.4.2 NO production and consumption. The stimulation of NO flux in NH_4^+-fertilized soil was primarily due to altered N turnover in the upper 2 cm of soil (Fig. 8.4). While most of the increases in NO fluxes and NO compensation points resulted from stimulated production rates, there was also a significant inhibition of k_{NO} ($P<0.05$). This inhibition resulted from reduced microbial activity rather than reduced surface sorption, as there was no inhibition of k_{NO} in autoclaved samples (Fig. 8.4).

Both the humisol and the humic gleysol had relatively consistent k_{NO} values with soil depth, although the absolute rate was much higher in the humisol (Fig. 8.6). The shapes of the NO mixing ratio profiles with soil depth indicate that NO production was confined mainly to the upper 2-8 cm of soil, and that NO was consumed as it diffused downwards (Fig. 8.6). Because of downward diffusion, NO

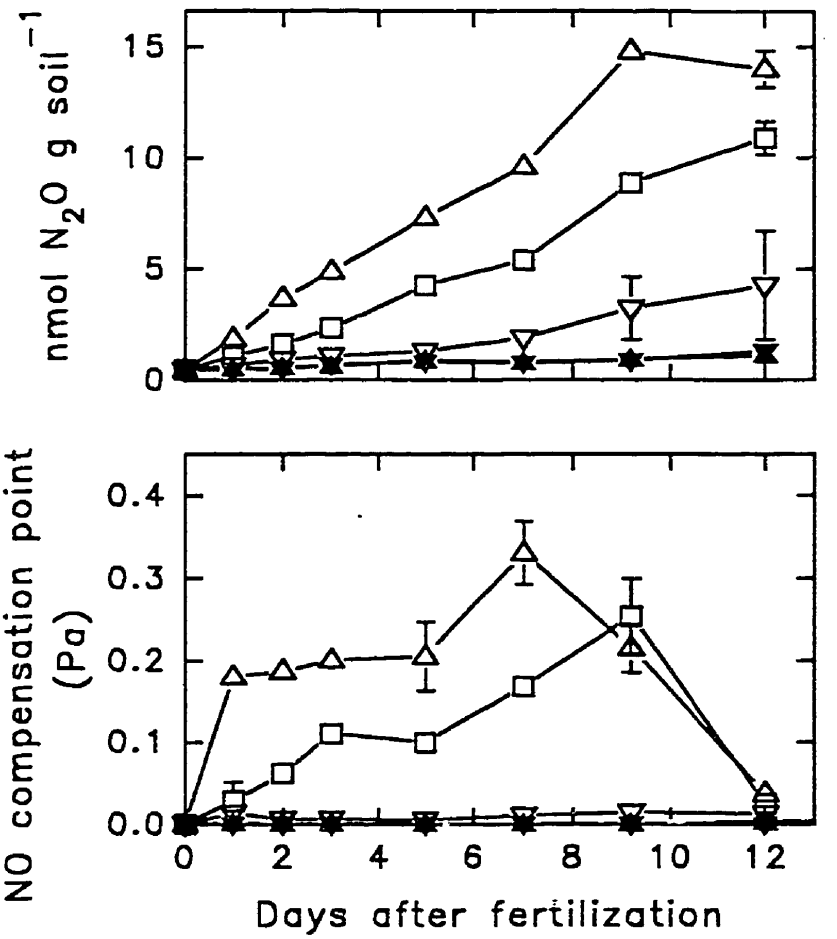

Fig. 8.5 N_2O accumulation and NO compensation points in closed aerobic vials containing humisol slurries supplemented with: 10 m*M* NH_4Cl (□), 20 m*M* $NaNO_3$ (▽), 20 m*M* $NaNO_3$ + 7 Pa C_2H_2 (▼), 10 m*M* NH_4Cl + 10 m*M* $NaNO_3$ (△), or 10 m*M* NH_4Cl + 10 m*M* $NaNO_3$ + 7 Pa C_2H_2 (▲). Data are means of duplicates or triplicates ±1 SEM.

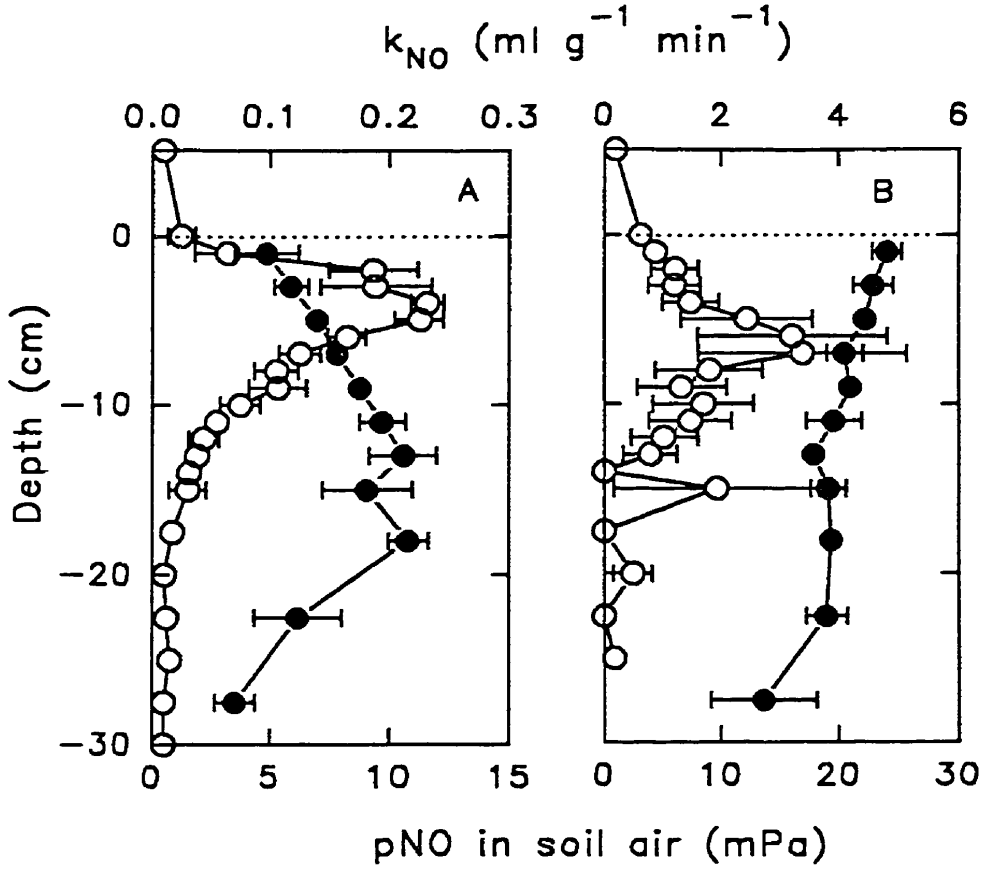

Fig. 8.6 Depth profiles of NO uptake rate constants (●) and soil gas NO partial pressures 4 days after fertilization with 100 kg N ha^{-1} as NH_4Cl (○), in soil cores from a humic gleysol (*panel A*) and a humisol (*panel B*). Data are means of 3 (A) or 4 (B) cores ±1 SEM.

mixing ratios in fertilized cores were well above ambient levels to 15-20 cm depth.

Integrated ([NO] × k_{NO}) over depth plus surface efflux represented the gross NO production rate of a soil core. Gross NO production rates were compared to surface fluxes in the humisol (Fig. 8.7) and humic gleysol (Fig. 8.8). In the humisol, the majority (on average 95%) of NO produced was consumed rather than emitted (Fig. 8.7). In contrast, with its lower k_{NO} value (Fig. 8.6), the humic gleysol consumed a lower percentage of the gross NO produced (on average 38%) (Fig. 8.8). These percentage estimates suffer from two potential errors. First, k_{NO}s were estimated from gaseous NO uptake rates and may be diffusion-limited. They would therefore underestimate NO uptake in soil where NO was being produced in solution. Second, the gross NO production estimate did not account for the inhibitory effect of NH_4^+ on k_{NO}. However, because most of the NH_4^+ is retained in the upper 2-8 cm of the soil, because k_{NO} is inhibited also only in the surface soil, and because these are counteracting errors, the estimates should be reasonable. Unfertilized cores were not included in this experiment, but the following estimates were made assuming pre-fertilization fluxes as background rates. Within the duration of the experiments, the gross NO production in the humisol and humic gleysol were nearly equal proportions of the added ammonium (3.03% versus 3.85%), but only a small portion of it (0.195%) was surface emission from the humisol while most (2.18%) was emitted from the humic gleysol.

More precise estimates of gross NO production from NH_4^+ were obtained with experiments using sieved soil in closed flasks. A typical experiment is shown in Fig. 8.9. The inhibition of NO uptake during nitrification of NH_4^+ is again evident. Up to 26% of the NH_4^+ added to soil was converted to NO, the ratio increasing with the dryness of the initial sample (Table 8.1). This may reflect underestimation of k_{NO} in wetter soil, or a chemical or microbial effect of drying.

8.5 Discussion

When soil air is rapidly stripped, denitrification is a major NO source (McKenney et al. 1982). However, denitrification generally only occurs in near-saturated soils

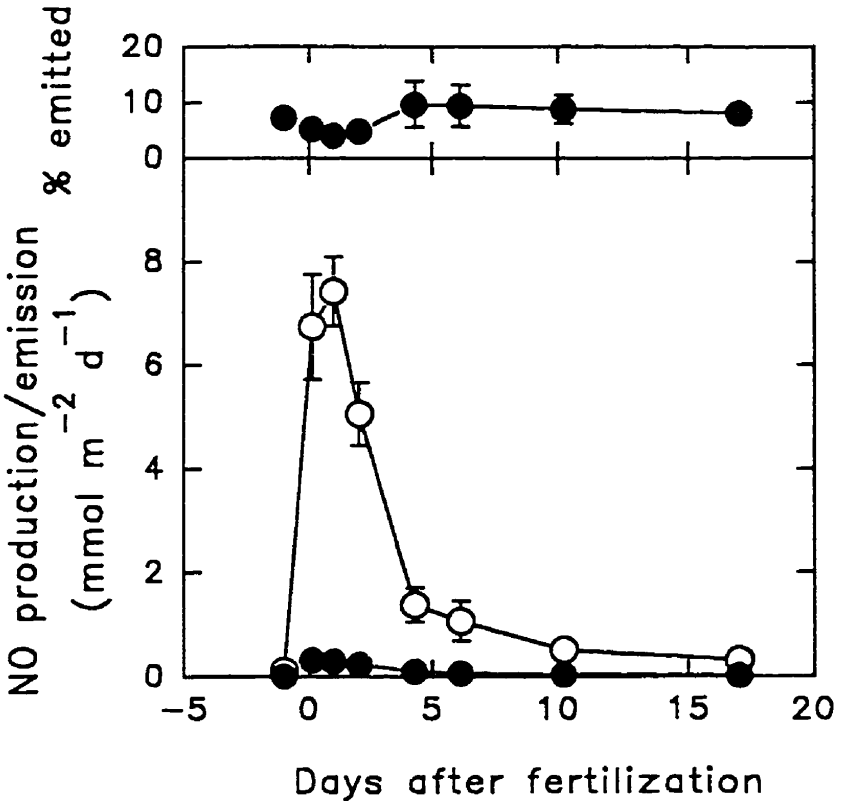

Fig. 8.7 Surface NO emission (●) and gross NO production (○) rates in humisol cores fertilized with 100 kg N ha^{-1} as NH_4Cl on day 0. Gross production is the emission plus total NO consumption estimated by integrating ([NO] × k_{NO}) to 30-cm depth. The top panel shows the emission rate as a percentage of the total production. Data are means of four cores ±1 SEM.

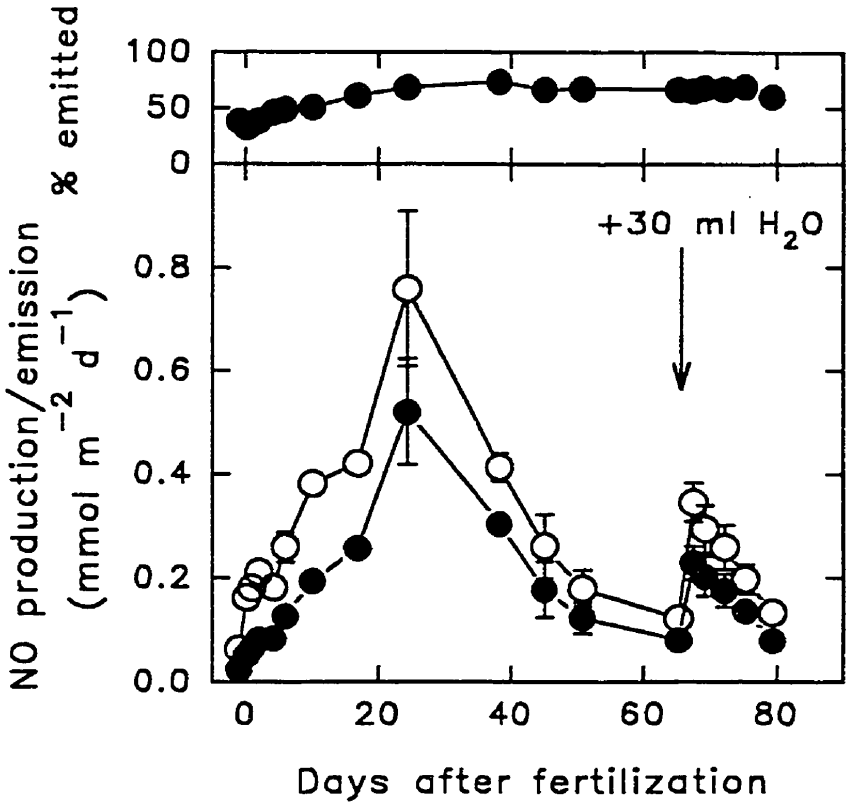

Fig. 8.8 Surface NO emission (●) and gross NO production (○) rates in cores of a humic gleysol fertilized with 100 kg N ha^{-1} as NH_4Cl on day 0. The top panel shows the emission rate as a percentage of the total production. Data are means of three cores ±1 SEM.

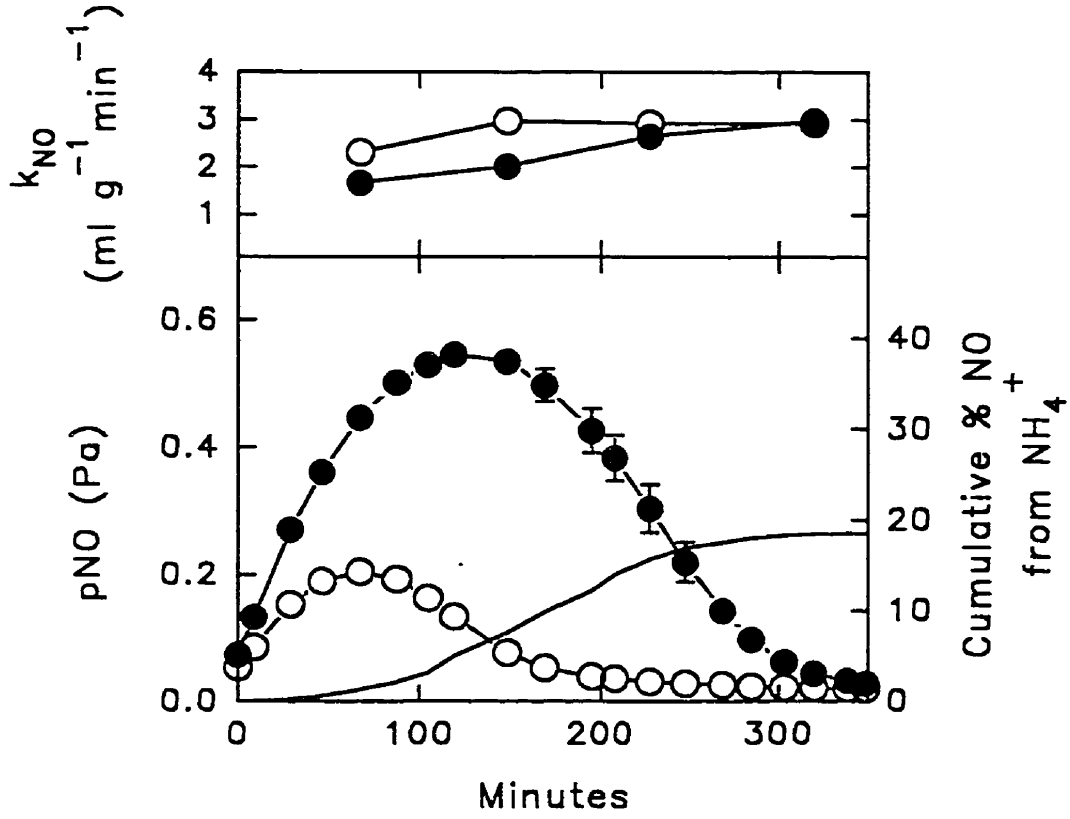

Fig. 8.9 NO partial pressures in closed 150-ml flasks containing 5.0 g humisol (91% H_2O d.w.) after adding 0.5 ml H_2O (○) or 0.5 ml of 2 m*M* NH_4Cl (●). The line indicates the cumulative gross %NO produced from NH_4^+, based on integration of ([NO] × k_{NO}) with time. Data are means of triplicates ±1 SEM.

Table 8.1 Gross NO production from added NH_4^+ in humisol samples air dried to various H_2O contents. Data are means of triplicates ± 1 SEM.

Trial	% H_2O d.w.		% NO from NH_4^+
	Air dried to	Rewetted to	
1	108	129	5.03 $\pm$0.44
2	88.5	107	9.04 $\pm$1.51
3	73.8	91.2	18.5 $\pm$1.18
4	20.0	56.1	26.1 $\pm$0.57

where diffusion is slow compared to microbial NO reduction, and little NO escapes the soil (Drury et al. 1992). Nitrate fertilizer addition often increases soil NO flux (Williams, Hutchinson et al. 1992; Cárdenas et al. 1993). This effect can be interpreted as resulting from denitrification, but direct evidence of a denitrification source is rare (Remde and Conrad 1991c). Nitrification has been shown to be the dominant NO source in a variety of soils through the use of the nitrification inhibitors C_2H_2 (Davidson 1992; Davidson et al. 1993), nitrapyrin (Tortoso and Hutchinson 1990; Remde and Conrad 1991c; Hutchinson et al. 1993), and dicyandiamide (Skiba et al. 1993). Even in near-saturated soil, or soil wetted to a degree where denitrification becomes a major N_2O source, nitrification can remain the principal NO source (Davidson 1992; Skiba et al. 1993). Our results agree with this trend. Even in near-saturated cores where active denitrification occurred and produced N_2O, nitrification provided most of the NO emitted from the soil surface. Denitrification might have dominated NO production in subsurface soil, but nitrification presumably dominated in the surface aerobic layer and thereby controlled atmospheric flux.

In contrast to NO, N_2O is frequently a combined product of soil denitrification and nitrification (Robertson and Tiedje 1987; Klemedtsson et al. 1988a; Davidson et al. 1993; Martikainen et al. 1993), the balance changing with soil conditions. Often nitrification is the major source below water saturation, and denitrification near or above saturation (Aulakh et al. 1984; Davidson et al. 1986; Davidson 1992; Davidson et al. 1993; Skiba et al. 1993; Mummey et al. 1994), although there are many exceptions. Nitrification can be the major source in wet conditions (Klemedtsson et al. 1988a; Hutchinson et al. 1993) while studies using $^{15}NO_3^-$ show that this can be the major precursor of N_2O even in aerobic conditions (Speir et al. 1995a). The effect of H_2O is complex because it influences nitrogen mineralization, diffusion of products out of soil, and diffusion of O_2. These effects are compounded by differences in structure, C and N status of various soils.

Our results correspond to the simple situation of high H_2O content favouring denitrification. Only nitrification of fertilizer NH_4^+, not denitrification of fertilizer NO_3^-, stimulated N_2O emissions from dry soil. However, because of high variability,

we could not significantly assign N_2O fluxes to a single source in unfertilized dry soil. These fluxes may have been partially due to nitrification, and partially to denitrification in anaerobic microsites. In near-saturated cores the N_2O emissions associated with denitrification drowned out any contribution from nitrification, although fluxes were measured shortly after the cores were wetted, before denitrification could become N-limited. In N-depleted samples nitrification in aerobic surface soil could become the major N_2O source, in a similar way as it dominated NO flux.

Only 0.05% of NH_4^+ fertilizer was emitted as N_2O through nitrification. This is at the low end of values measured in other neutral soils of 0.17-0.93% (Maag and Vinther 1996), 0.1-0.8% (Aulakh et al. 1984), 0.15-9% (Martikainen et al. 1993) 0.02% (Tortoso and Hutchinson 1990), and 0.02-0.04% (Davidson et al. 1993). Considering the large denitrification potential of organic soils, and the high N_2O emissions which are observed in very wet humisol (results of this paper and Dunfield et al. 1995), ammonium oxidation contributes little to the total annual N_2O flux.

However, larger amounts of NO were released by nitrification, 0.2-1.0% over the time periods studied. Typically 1-4% of the NH_4^+ oxidized in soil is emitted to the atmosphere as NO, but a wide range of 0.1-11% has been reported (Shepherd et al. 1991; Williams, Hutchinson et al. 1992). The humisol fluxes were relatively low in this range, but would have been much greater save for its high NO uptake potential. As much as 95% of the total NO produced was reconsumed within the soil column (at ϕ_g=0.44). This consumption compensated for a high gross NO production rate, which accounted for as much as 26% of the nitrified NH_4^+ in rewetted air-dried soil. This proportion is higher than previous reports with soil (Williams, Hutchinson et al. 1992) or nitrifying bacteria (Lipschultz et al. 1981; Stüven et al. 1992), and may result from reactions of NO_2^- with soil organic matter. Such a high proportion is potentially important in the field, as mineralization events are triggered by progressive drying and rewetting of surface soil. Bursts of NO production often follow wetting events (Davidson 1992; Davidson et al. 1993).

Nitrogen monoxide consumption in this humisol is primarily a microbial

oxidation, probably mediated by heterotrophs (Dunfield and Knowles 1997). *Nitrobacter* also oxidize NO to NO_2^- and then to NO_3^- (Freitag and Bock 1990), while NO_3^- is the major product of NO oxidation by soil heterotrophs (Koschorreck et al. 1996). Given that up to 26% of the NH_4^+ oxidized in this humisol passes through NO, the pathway $NO_2^- \rightarrow NO \rightarrow NO_3^-$ mediated by soil heterotrophs represents a significant alternate pathway of nitrification, bypassing NO_2^- oxidation by *Nitrobacter*.

We have shown for the first time that ammonium-based fertilizer can decrease soil NO consumption potential. Although increased NO flux after NH_4^+ fertilization was primarily the result of increased NO production, NO consumption rate constants were also reduced by about half. This possibility was previously tested but found insignificant in a cambisol and a luvisol (Remde and Conrad 1991c; Baumgärtner and Conrad 1992a). Our observations may be related to the high rates of NO production and consumption in this fertile organic soil. Possible explanations are: (i) extended exposure to elevated NO concentrations depletes the microbial factors responsible for NO uptake; (ii) NO_2^- toxicity during rapid nitrification inhibits the activity of the microbes responsible for NO oxidation; or (iii) the microbes responsible undergo regulatory responses during rapid soil NH_4^+ oxidation. The first two hypotheses seem unlikely considering that decreased NO uptake in depth subsamples was measured at least 20 h after nitrification was halted by C_2H_2 addition, while in loose soil samples the inhibition disappeared quickly after the added NH_4^+ was depleted.

We have also shown that stimulation of NO and N_2O production through NO_3^- addition (Williams, Hutchinson et al. 1992) should not always be accepted as proof of a denitrification source, since NO_3^- also stimulates nitrification-based NO and N_2O production. The effect of NO_3^- on gaseous N-oxide production is probably indirect, the direct cause being increased NO_2^- levels. High NO_3^- concentrations should decrease the energetic yield of, and slow down the $NO_2^- \rightarrow NO_3^-$ reaction. Nitrate is also a noncompetitive inhibitor of NO_2^- oxidation by *Nitrobacter* (Boon and Laudelout 1962). This inhibition increases with declining pH, and at pH 6.5, >70% inhibition by 100 m*M* NO_3^- (and to a lesser extent NaCl) has been noted (Hunik et al. 1993).

Although our NO_3^- concentrations were much lower (10 m*M*), such an inhibition would explain our observations.

Soil NO fluxes are often positively correlated to NO_3^- concentrations (Williams, Hutchinson et al. 1992). High soil NO_3^- concentrations may simply reflect rapid N turnover rates and high N/C ratios (Davidson 1992), but part of the correlation may result from the direct effect of NO_3^- on the products of nitrification, especially in fertilized soils.

In summary, we have shown that nitrification is the major source of NO but not N_2O from an organic soil. The net surface NO efflux is greatly attenuated by NO consumption. The dynamics of NO turnover in relation to soil nitrogen and soil diffusion are complex. NH_4^+ and NO_3^- can both affect nitrification, while NH_4^+ also affects NO uptake. Even while NO production is confined to the upper few cm of soil, oxidation of this NO occurs over a much greater depth. Such vertical zonation creates difficulties for simple mechanistic models (Rudolph and Conrad 1996), and more complex simulations might be necessary to create a useful predictive model of NO flux.

SECTION 9. SUMMARY

A humisol was examined as a model system and as a unique system of trace gas cycling. The homogeneity of the soil with depth made it ideal for studying atmospheric methane uptake with a one-dimensional Fickian diffusion model. The soil had a high water-holding capacity and diffusion could therefore be studied over a wide moisture range. The low variability of soil subsamples and the clear Michaelis-Menton pattern of methane oxidation also made it ideal for studying the kinetic mechanisms of inhibition of CH_4 oxidation by various forms of soil nitrogen. Organic soils also have unique properties. We attempted to assess this uniqueness, primarily with regard to gaseous N-oxide cycling.

The response of atmospheric CH_4 uptake to water content in intact soil cores was an exponential rise to a maximum. This was expected from the exponential dependence of gas diffusion rates in semi-porous media on gas-filled porosity (Campbell 1985), and suggests that atmospheric CH_4 uptake is to some extent constrained by gaseous diffusion through the soil matrix. The threshold of the relationship was attained at about ϕ_g=0.2 (130% H_2O). When soil was drier than this, diffusion was not the major factor limiting CH_4 uptake.

Kinetic experiments showed that $NaNO_3$ and other salts were noncompetitive inhibitors of methane oxidation. This inhibition was evident only at concentrations much higher than field application rates. There were specific, stronger NH_4^+ effects. NH_4^+ was a competitive inhibitor of CH_4 oxidation. The determined $K_{i(app)}$ value was low enough to suggest that even *in situ* soil NH_4^+ concentrations inhibit CH_4 oxidation, and that ammonium-based fertilizers can be potent suppressors of CH_4 oxidation. This strong competitive inhibition was worsened by the toxic effects of NO_2^-. This latter effect has been previously described (Schnell and King 1994). Our own results show more clearly that NO_2^- production is the result of enzyme competition and methanotroph NH_4^+ oxidation.

The net inhibitory effect of urea fertilizer in the field was ameliorated by several factors. The rapid nitrification rate of the humisol quickly depleted NH_4^+

from urea hydrolysis. The inhibition was reversible and therefore disappeared when NH_4^+ was depleted. NH_4^+ was also immobilized in the surface soil. Methane oxidation potential occurred throughout the organic layer, and at summertime soil moisture contents was not limited by the diffusion rate of atmospheric CH_4, so surface-immobilized NH_4^+ had little inhibitory effect on net CH_4 uptake.

Organic soils can support higher microbial activities and therefore potentially higher trace gas fluxes than mineral soils. Our humisol was a net source for methane in the spring. Reports on methanogenesis in non-wetland soils are scarce. Comparisons among soils are therefore difficult to make, but the methanogenic activity observed in the humisol was probably related to the high oxygen demand, carbon degradation, and water-holding capacity.

Organic soils are important point sources of N_2O (Sahrawat and Keeney 1986), which was also evident at our site. NO and N_2O are produced by the same microbial processes. It might therefore be expected that organic soils are also important point sources of NO. Studies were performed to examine the processes controlling NO cycling in the humisol, and comparisons made with other soils. Studies with soil organic amendments were also performed to more directly assess the role of soil organic matter in NO cycling.

The soil had both a high nitrification and a high denitrification potential. Denitrification was the main source of N_2O. NO was produced primarily through nitrification, even under wet, denitrifying conditions. Because large amounts of nitrogen are mineralized and nitrified from organic soils (Guthrie and Duxbury 1978), net yearly NO emissions may be high compared to other soils. We noted that high proportions of nitrified N were converted to NO under certain conditions, as much as 26% when air-dried soil was rewetted.

However, net NO emissions from soils are attenuated by NO consumption processes. The main consumption process in the humisol was microbial oxidation to nitrate. This process was apparently mediated by soil heterotrophs, and was not coupled to energy production. Probably because of the heterotrophic source, there were strong correlations of NO uptake rate constants with soil organic matter contents

and with soil CO_2 evolution rates across soil types. Therefore, organic soils had relatively high NO-uptake potentials. Amendment of a gleysol with composted dairy manure increased soil NO uptake rate constants, decreased NO compensation points, and decreased surface NO emissions during nitrification of fertilizer NH_4^+. NO consumption by soil heterotrophs is therefore a strong control of soil-surface NO fluxes.

When fertilized with NH_4^+, the humisol exhibited much higher NO-efflux rates than a gleysol because of its more rapid nitrification rate. However, when integrated over time the net % of fertilizer N emitted as NO across the soil surface was less in the humisol. In aerobic humisol cores, 95% of the NO produced was reoxidized rather than emitted to the atmosphere. Contrary to the hypothesis that fertilizer NO losses might be higher from organic soils than from mineral soils, the net surface emissions from humisol cores were less than from gleysol cores, due to their higher NO uptake rate constants.

These studies have shed some light on the mechanisms of trace gas cycling in organic soils, and the specific effects of organic matter on trace gas metabolism. They stress the need to study production and consumption processes simultaneously as controllers of net surface fluxes. They also stress the importance of applying mechanistic studies of microbial trace gas metabolism to intact soil systems, where spatial and temporal factors complicate the net effects of these mechanisms.

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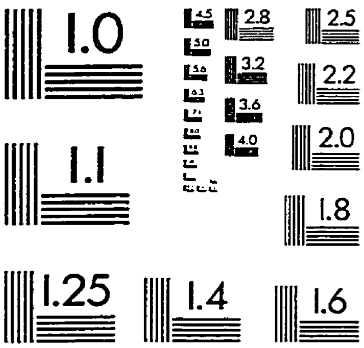
IMAGE EVALUATION TEST TARGET (QA-3)



150mm

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