Pitfalls in measuring nitrous oxide production in nitrifiers

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ABSTRACT

The microbial soil processes nitrification and denitrification are globally the most important sources of the greenhouse gas nitrous oxide (N_2O). Nitrifiers produce N_2O by nitrification and by nitrifier denitrification. In nitrification, N_2O develops during the oxidation of hydroxylamine (NH_2OH). In nitrifier denitrification, nitrifiers reduce nitrite (NO_2^-) via N_2O to N_2. Not much is known about this latter pathway yet. The differentiation between nitrification, nitrifier denitrification, denitrification and other soil sources of N_2O is usually based on incubations with combinations of 0.02 kPa acetylene (C_2H_2) and 100 kPa oxygen (O_2). C_2H_2 is supposed to inhibit nitrification and nitrifier denitrification, and O_2 is supposed to inhibit nitrifier denitrification and denitrification.

In this thesis, a survey of the sources of N_2O in four soils under a range of conditions showed that the incubation method with C_2H_2 and O_2 was not suitable for all soils. The addition of inhibitors led in several cases to increased N_2O production. Sometimes, negative fluxes were calculated for the different sources of N_2O, especially for nitrifier denitrification. Pure culture experiments with *Nitrosomonas europaea* and *Nitrosospira briensis* revealed that C_2H_2 did not inhibit the N_2O production by *N. briensis*. Large concentrations of O_2 (100 kPa) strongly reduced the N_2O production by both nitrifiers as expected, but also partly inhibited ammonia oxidation. A negative effect of 100 kPa O_2 on ammonia oxidation could be confirmed in a study with mutants of *N. europaea* that were deficient in nitrite reductase (NirK) or nitric oxide reductase (NORB), two enzymes needed for the nitrifier denitrification pathway. The similar amounts of N_2O produced by the NirK-deficient cells and the wild-type might indicate that nitrification rather than nitrifier denitrification is important for N_2O production. The NORB-deficient cells produced much larger amounts of N_2O than the wild-type and this mutant was furthermore not affected by C_2H_2. While side-effects of the mutation on pathways of N_2O production cannot be excluded, there are indication of a role of NORB in directing ammonia oxidation towards NO_2^- rather than N_2O and of an unknown pathway of N_2O production in nitrifiers. A sensitivity analysis revealed that an inhibition of the N_2O reductase of denitrifiers by C_2H_2 most likely caused some of the observed over- and underestimations of sources of N_2O in the soil survey. Furthermore, it is likely that C_2H_2 only inhibited part of nitrification and nitrifier denitrification and that O_2 also partly inhibited nitrification in the soil. This suggests that nitrifiers have probably been underestimated as producers of N_2O in studies using C_2H_2 and O_2 as inhibitors. Future studies should further investigate the pathways of N_2O production, including the indicated possible unknown pathway of nitrifiers. A combination of stable isotope studies of N and O and incubation studies with inhibitors other than C_2H_2 and O_2 might enable the differentiation between sources of N_2O in soils.

Keywords: nitrifier denitrification, nitrous oxide, nitrification, denitrification, ammonia oxidation, acetylene, oxygen, inhibitor.

CHAPTER ONE | GENERAL INTRODUCTION

not published previously

History of the Use of N_2O

The gas nitrous oxide (N_2O) was discovered in 1772 by Joseph Priestley (Figure 1), an English scientist and clergyman who was also the first to isolate oxygen (O_2), carbon monoxide (CO) and other gases (Mattson, 2001). Priestley was one of the last supporters of the phlogiston theory (Mattson, 2001), which postulates that all flammable materials contain phlogiston, a substance without color, odor, taste, or weight that is given off in burning. 'Phlogisticated' substances were thought to contain phlogiston and, on being burned, were 'dephlogisticated'. 'Dephlogisticated air' was O_2, and 'phlogisticated air' nitrogen (N_2) (The Columbia Electronic Encyclopedia, 2000). Priestley termed N_2O 'phlogisticated nitrous air', with 'nitrous air' being nitric oxide (NO).

Figure 1: Joseph Priestley (from Mattson, 2001).

In 1785, the surgical assistant Humphrey Davy inhaled N_2O in a self-study and discovered its intoxicating effect (History House, 2002). He started to offer his friends N_2O at parties. Due to the low cost and few side-effects, the so-called 'laughing gas' was considered a good alternative to alcohol. In 1844, the former medical student Gardner Quincy Colton toured America with a 'Grand Exhibition of the effects produced by inhaling Nitrous Oxid [sic]' (History House, 2002). During one of these shows, a volunteer who had inhaled N_2O stumbled and injured his leg. He said later that he had felt no pain while under the influence of N_2O. This was witnessed by the dentist Dr. Horace Wells. With the help of Colton and of another dentist, Wells tried out N_2O himself the next day. He did not experience pain during an extraction of one of his molars (Cameron and May, 1999). However, when Wells wanted to demonstrate the effects of N_2O in a tooth-pulling operation at Massachusetts Medical School a month later, he was not totally successful, since the patient still felt some discomfort (Higgins, 2002). The laughing and booing audience marked the end of Wells' carreer (Cameron and May, 1999). In 1846, one of Wells' former students, William Morton, was the first to successfully give a public demonstration of the effects of an anaesthetic during an operation. Morton used ether (Higgins, 2002). N_2O is being used as an anaesthetic since the

1890s (Thompson, 2002). An advantage of N_2O is that it works much quicker than ether. However, N_2O cannot be given in sufficient quantities to induce complete anaesthesia. The concentration of N_2O administered was at first difficult to control, so that problems arose since either too little N_2O was given to be effective, or too much, so that patients died from suffocation (Chris Thompson, personal communication).

Today, N_2O is still used in anaesthetic mixtures, especially in dental surgery. Besides, laughing gas remains popular as a drug in some circles. N_2O is also used for a variety of other purposes. Since it is a colourless gas that is inert at room temperature and not toxic in the needed concentrations, it is used as a propellant for whipped cream and other products (Socsil, 2001). It is also applied in the semi-conductor industry for etching microchips (Socsil, 2001). N_2O helps to speed engines, for example in racing cars, since it is not flammable but promotes combustion as it breaks down at high temperatures to one third O_2 and two thirds molecular nitrogen (N_2), thus providing more O_2 per unit than normal air (American Motorsports Promotions, 2000). It has even been proposed to use N_2O in engines of manned space flights (SpaceDev, 2001). Since the effects of inhaling N_2O are similar to the effects of nitrogen hypnosis, divers sometimes use N_2O to safely get to know the effects of deep dives (Bacharach, 2002, Hyperbaric Medicine Unit, 2002).

Role of N_2O in Global Warming

We have seen so far that N_2O is a very useful gas for humans. However, it is also a prominent greenhouse gas. The greenhouse effect is normally perceived as something dangerous. Nevertheless, it is not per definition negative. The so-called ‘natural greenhouse effect’ warms the earth to a comfortable mean temperature of $+16^{o}C$ instead of a mere $-18^{o}C$ (Upton, 1997). The warming works very similar to a greenhouse, where light, i.e. shortwave ultraviolet and visible radiation, comes in through the glass panels, and heat, i.e. longwave thermal infrared radiation, cannot escape. About 70% of the radiation that is absorbed from the sun reaches the earth’s surface (UNFCCC, 2002). This shortwave radiation then heats the surface, melts ice, evaporates water, etc. The warmed surfaces start to emit longwave infrared radiation. Greenhouse gases in the atmosphere prevent some of this infrared radiation from leaving the atmosphere (UNFCCC, 2002). Thus, some energy is trapped in the system. Important greenhouse gases besides N_2O are water (H_2O), carbon dioxide (CO_2) and methane (CH_4).

If a greenhouse is getting too hot in summer, two strategies can be taken: windows can be opened to enable the escape of heat, or the glass panels can be whitened to restrict the radiation from entering. Both possibilities also apply to the ‘Greenhouse Earth’.

The open windows of a hot greenhouse are comparable to gaps in absorption spectra of gases in the atmosphere. Every gas can only absorb radiation of certain wavelenghts (Figure 2). There are wavelengths were no absorption is taking place, so that this radiation can escape from the atmosphere like through an open window. However, due to the emission of more and different greenhouse gases, this window is getting smaller, so that more radiation is captured (Upton, 1997, Jansen, 2002). This leads to a change in the net radiative energy available to the earth, a so-called radiative forcing. Positive radiative forcings tend to warm the earth, negative radiative forcings cool it down (IPCC, 2001).

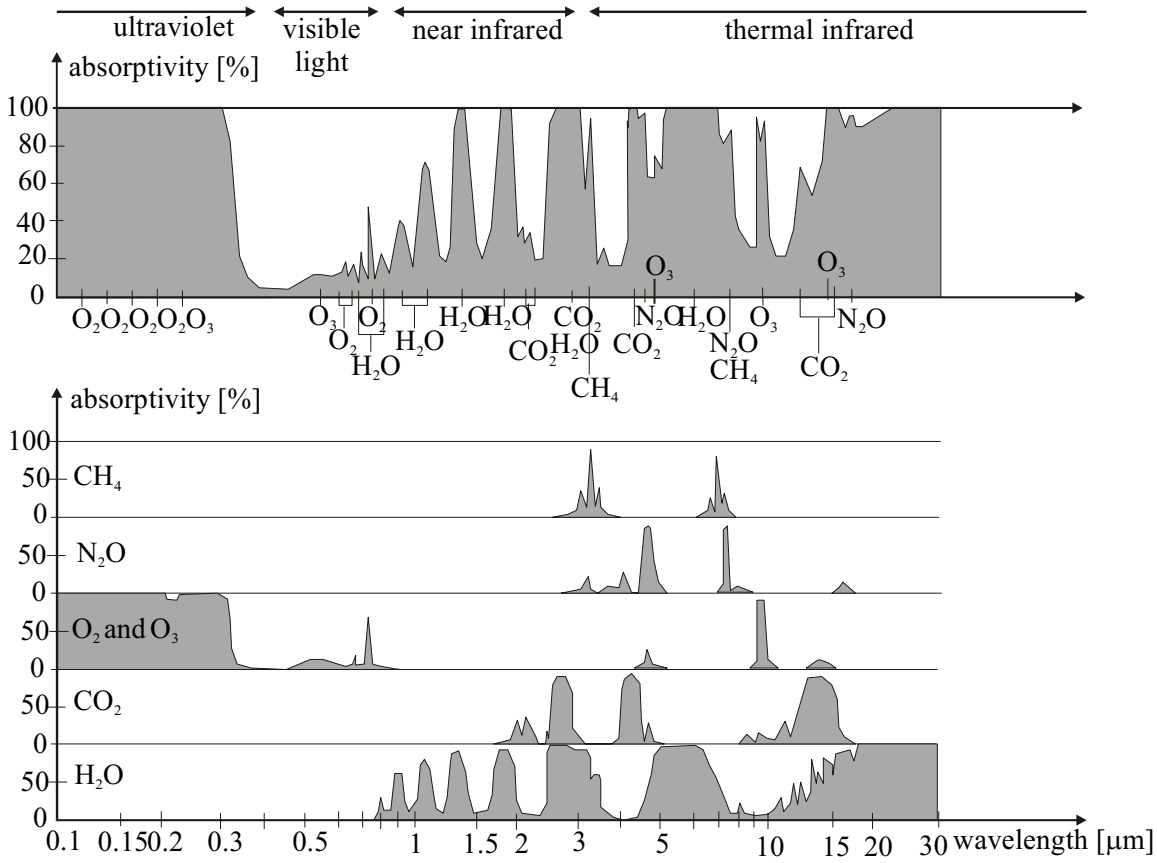

Figure 2: This figure shows the overall absorption spectrum of the atmosphere (above), which is below broken up in the spectra of the major greenhouse gases in their natural concentrations (from Jansen, 2002).

The whitening of the glass panels of a greenhouse to keep incoming shortwave radiation out can be compared to the working of aerosols in the atmosphere. Aerosols are very small airborne particles and droplets. Their actual impact on the climate is still not well understood, but it seems that tiny dust particles in the atmosphere can scatter incoming radiation (IPCC, 2001). They can furthermore initiate the building of clouds, which can reflect incoming radiation back to space. However, black aerosols, for example soot particles, can absorb thermal infrared radiation and might thus enhance a warming of the climate (IPCC, 2001).

Nowadays, many scientists all over the world agree that the global climate is changing due to human activities (IPCC, 2001). It is virtually certain (probability larger than 99%) that the sea surface temperature as well as the land air temperature have increased by 0.4 to 0.8^{o}C since the late 19th century. In the 20th century, there has been a massive retreat of mountain glaciers. It is likely (probability between 66 and 90%) that the 1990s was the warmest decade of the millenium with 1998 being the warmest year in the instrumental record since 1861. In the Northern Hemisphere, the mid-to-high latitude precipitation has very likely (probability between 90 and 99%) increased since 1900 by 5 to 10%. Much of this was due to heavy and extreme precipitation events. New models show that these changes are unlikely (10-33% chance) to be due to internal variability of the climate alone. When anthropogenic influences are included in these new models, the results fit the observations well (IPCC, 2001).

At present, N_2O causes 6% of the radiative forcing of all greenhouse gases (IPCC 2001). This percentage, as well as the N_2O concentration in the atmosphere, continues to increase. With a lifetime of 114 years (IPCC, 2001), N_2O produced at any given moment will continue to influence the global climate generations later. Thus, it is important that we increase the knowledge of the different possible sources of N_2O. We can only try to decrease the emissions with profound knowledge of the sources. If we want to stabilize N_2O in the atmosphere at today's concentrations, we have to decrease the emission of N_2O by about 50% (IPCC, 1996).

Role of N_2O in the Nitrogen Cycle

During the Industrial Era, the atmospheric concentration of N_2O has steadily increased. It is now 16% (46 ppb) larger than in 1750 (IPCC, 2001, Figure 3). In 1998, the concentration of N_2O amounted to 314 ppb. Between 1980 and 1998, it has increased at a rate of 0.8 ppb per year, which is equal to about 0.25% per year (IPCC, 2001).

The main global source of atmospheric N_2O is the soil (Bouwman, 1990). In the soil, different processes generate N_2O. The most important ones are microbial processes carried out by nitrifiers and denitrifiers (Granli and Bøckman, 1994). Nitrifiers are autotrophic microorganisms that obtain energy from the oxidation of ammonia (NH_3) or nitrite (NO_2^-). N_2O is a by-product of ammonia oxidation. Besides, nitrifiers produce N_2O as an intermediate when reducing NO_2^-. The reduction of NO_2^- by nitrifiers is called nitrifier denitrification (Poth and Focht, 1985) and is still largely unexplored. Denitrifiers are predominantly heterotrophic organisms. Most of them use nitrate (NO_3^-) or NO_2^- as alternatives to O_2 as an electron acceptor in low-oxygen conditions. In the reduction of NO_3^- via NO_2^- to N_2, N_2O is an intermediate and can be emitted (Granli and Bøckman, 1994). There are indications that soils

might not only be a source of N_2O, but can in some conditions also act as a sink. Especially nitrogen-limited soils might consume N_2O (Goossens et al., 2001, Glatzel and Stahr, 2001). However, this sink function of soils for N_2O is generally considered to be of only minor importance (Kerner, 1996, Regina et al., 1999).

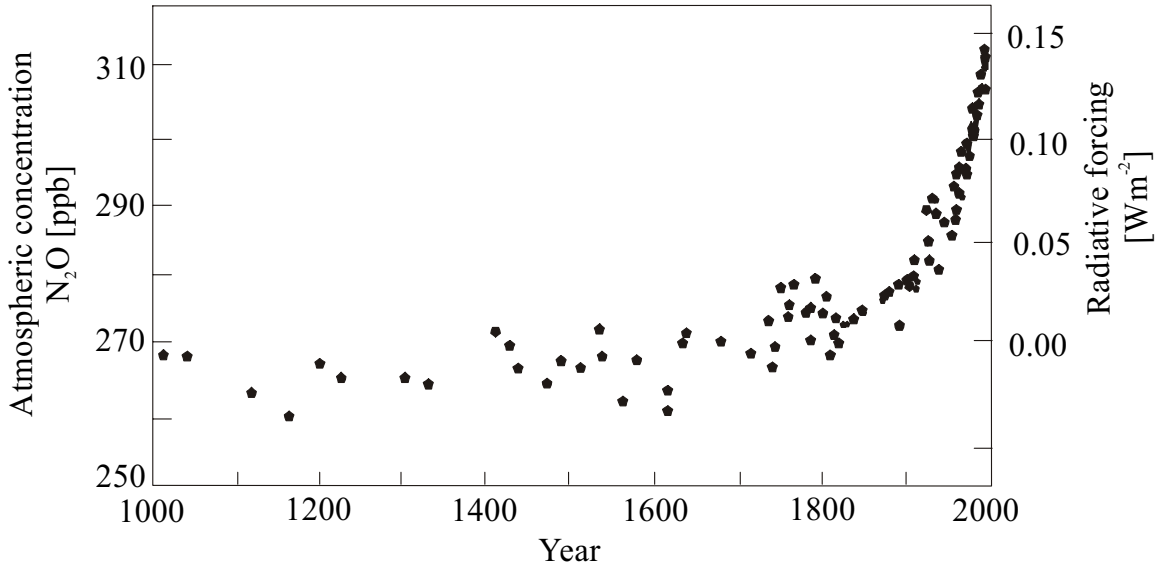

Figure 3: Atmospheric concentrations of N_2O over the past 1,000 years. The estimated radiative forcing is indicated on the right-hand side (from IPCC, 2001).

The N_2O emission of agricultural soils has increased due to the application of nitrogen fertilizers and increased use of leguminous crops (Granli and Bøckman, 1994, Mosier, 2001). Therefore, about 60% of the N_2O emission from soil is regarded as anthropogenic emission (Mosier et al., 1998a). Since the uncertainty in the estimated flux of N_2O from soil to the atmosphere is relatively large, the margins for this part of the anthropogenic emission of N_2O are relatively wide. Mosier et al. (1998a) estimated that between 1.25 and 5.75 Tg N_2O-N per year are emitted from agriculture due to the use of mineral fertilizers, animal wastes and N-fixation.

So far, we are still unable to predict the fate of a unit of nitrogen (N) applied to a specific agricultural field (Mosier et al., 1998a). Generally, plants only manage to take up 50% of the N applied. The other 50% is for the greater part lost via leaching, seepage, run-off, erosion and gaseous losses (FAO, 2001). It is still difficult to predict how much is lost as N_2O. Part of the N lost in other forms can be nitrified of denitrified at a later stage whereby again some N_2O may be released to the atmosphere (Mosier et al., 1998b). Despite intensive research efforts, known total inputs and outputs of nitrogen in agricultural systems are not balanced yet. This problem has already been pointed out by Allison in 1955. He reasoned that the imbalance was due to the impractibility of measuring all soil gains and losses of N in a single

field experiment. Augustin et al. (1997) could recover 53 to 69% of the applied ^{15}N labelled fertilizer in plants and soil of a fen grassland. They assumed that the rest had been lost in gaseous form. Clough et al. (1999) tried to measure all sinks of ^{15}N-labelled NO_3^- fertilizer in incubations of sealed soil columns. Immobilisation, entrapment in pore space, dissolution in soil water, and gas fluxes accounted for only 80% of the ^{15}N applied. The rest was not recovered. The authors discussed that the emission of N in gaseous form might have been underestimated in their study.

Figure 4: Biosphere 2 project (Biosphere 2 Center, 2002).

Thus, some of the imbalance in the N cycle might be accounted for by gaseous losses, partly as N_2O. Attempts to budget the global N_2O cycle have shown that on the one hand some sources are underestimated (e.g. Mosier et al., 1998b), but that on the other hand so far unidentified sinks might exist or known sinks might be underestimated (e.g. Rahn and Wahlen, 1997). How little the N_2O cycle is understood, can also be seen from the results of the Biosphere 2 project. In this massive glass-closed facility in southern Arizona (Figure 4), 8 people lived self-contained for two years. Then, the experiment had to be stopped prematurely, because N_2O concentrations had increased to 79 ppm, about 250 times larger than normal ambient concentrations (Broecker, 1996). Such large concentrations can affect vitamin B-12 synthesis and thus cause brain damage in humans (CGAnet, 2002).

As said before, N_2O can stay in the atmosphere for 114 years after its production (IPCC, 2001). The main known sink of N_2O is its destruction by UV radiation to nitric oxide (NO) taking place in the stratosphere (Bliefert, 1994). One of the reasons for the large N_2O concentrations in Biosphere 2 was the missing breakdown mechanism. The necessary UV radiation was blocked by the glass roof of Biosphere 2 (Allen and Nelson, 1999). Through the breakdown to NO, N_2O plays an indirect role in the increase of the ozone hole, since stratospheric NO contributes to the destruction of ozone (Bliefert, 1994).

Scope and Outline

The above sections show that N_2O has important impacts on human life, not only because of its use as a drug and anaesthetic, but also –more importantly– because it is a greenhouse gas. Since agricultural soils are the largest single source of N_2O (Mosier, 1998a), a range of studies has already dealt with the production of N_2O in soils. However, not all soil sources of N_2O are well understood. Especially the contribution of the two pathways in nitrifiers leading to the production of N_2O, namely the oxidation of NH_3 and the reduction of NO_2^-, has so far only been quantified under very few different conditions. The few studies carried out attribute up to 30% of the total N_2O production of a soil to nitrifier denitrification, the reduction of NO_2^- by nitrifiers (Webster and Hopkins, 1996a). Thus, nitrifier denitrification could be a large source of N_2O in some conditions. It is assumed to be favoured by suboxic conditions and large NO_2^- concentrations (Ritchie and Nicholas, 1972, Poth and Focht, 1985). This has so far not been demonstrated in soil experiments, however. There is a need to understand the different sources of N_2O, since adequate mitigation measures can only be taken with profound knowledge of the sources and influencing factors. Furthermore, good knowledge of the sources of N_2O might also help to get a better match between total input and output of N in agricultural systems.

The overall objective of my research was to increase the understanding of N_2O production by nitrifier denitrification, i.e. the reduction of NO_2^- by nitrifiers. Initially, the specific objectives were

i) to quantitatively assess the effects of a range of environmental conditions on N_2O production by nitrifier denitrification, so as to derive possible mitigation measures, and

ii) to come up with a best estimate of N_2O production by nitrifier denitrification in the Netherlands.

Soon after the start of my project it became clear that these specific objectives could not be satisfied. The mechanisms of nitrifier denitrification and of N_2O production by nitrifiers in general were far more complex than initially thought. Questions concerning the prominent measurement method arose. As a consequence, the specific objectives were adapted and became:

i) to test the prominent methodology for quantifying the N_2O production by nitrifier denitrification, and

ii) to assess the importance of nitrifier denitrification for N_2O production in pure cultures of *Nitrosomonas europaea* and *Nitrosospira briensis*.

This thesis presents the main results of my investigation. I began the study with a review of the role of nitrifier denitrification in the production of N_2O (Chapter 2). This clearly showed that quantitative information on the importance of this pathway for the production of N_2O was still lacking. I therefore carried out a survey of N_2O production by different soil sources, including nitrifier denitrification. This is described in Chapter 3. For that study, I used an incubation method described in the literature (Webster and Hopkins, 1996a). However, the results of the survey lead to questions concerning the reliability of this method. Therefore, I tested the method under controlled conditions in the laboratory with pure cultures of nitrifiers (Chapter 4). This cast further doubts on the reliability and specificness of the inhibitors. These doubts could finally be confirmed in a study with transformed nitrifiers lacking enzymes of the nitrifier denitrification pathway (Chapter 5). Besides results leading to a rejection of the inhibition method, Chapters 4 and 5 also give indications concerning the importance of nitrifier denitrification for N_2O production in pure cultures of nitrifiers. This is taken up and placed in a larger context in Chapter 6, the final discussion of the results.


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CHAPTER TWO

ROLE OF NITRIFIER DENITRIFICATION IN THE PRODUCTION OF NITROUS OXIDE

N. Wrage, G.L. Velthof, M.L. van Beusichem, O. Oenema

(published in slightly modified form as Wrage et al., 2001)

Nitrifier denitrification is the pathway of nitrification in which ammonia (NH_3) is oxidized to nitrite (NO_2^-), followed by the reduction of NO_2^- to nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2). The transformations are carried out by autotrophic nitrifiers. Thus, nitrifier denitrification differs from coupled nitrification and denitrification, where denitrifiers reduce NO_2^- or nitrate (NO_3^-) that was produced by nitrifiers. Nitrifier denitrification contributes to the development of the greenhouse gas N_2O and also causes losses of fertilizer nitrogen in agricultural soils.

In this review article, present knowledge about nitrifier denitrification is summarized in order to give an exact definition, to spread awareness of its pathway and controlling factors and to identify areas of research needed to improve global N_2O budgets. Due to experimental difficulties and a lack of awareness of nitrifier denitrification, not much is known about this mechanism of N_2O production yet. The few measurements carried out so far, attribute up to 30% of the total N_2O production to nitrifier denitrification. Low oxygen conditions coupled with low organic carbon contents of soils favour this pathway as might low pH. As nitrifier denitrification can lead to substantial N_2O emissions, there is a need to quantify this pathway in different soils under different conditions. New insights attained through quantification experiments should be used in the improvement of computer models to define sets of conditions that show where and when nitrifier denitrification is a significant source of N_2O. This may subsequently render the development of guidelines for low-emission farming practices necessary.

Introduction

Nitrous oxide (N_2O) is a greenhouse gas with important impacts on our environment. Its 100-year global warming potential is about 320 times as strong as that of carbon dioxide (CO_2). It has a lifetime of approximately 120 years (IPCC, 1996). The major sink for N_2O is the stratospheric reaction with atomic oxygen to nitric oxide (NO). The resultant NO induces the destruction of stratospheric ozone (Bliefert, 1994).

Considerable anthropogenic emissions of N_2O arise from agricultural soils. In 1997, the largest single source of N_2O globally was the use of nitrogen (N) fertilizers in agriculture. In the USA, N_2O from this source amounted to 405 kt of a total 1,011 kt N_2O produced (EIA, 1998). Apart from environmental problems, such losses lead to higher expenses for farmers due to loss of N fertilizer.

The production of N_2O results from microbial transformations (nitrification and denitrification) of nitrogenous compounds. The microbial processes are essentially the same whether they take place in soils, wastewater treatment plants, sediments or water bodies. More than 25 years ago, it was proposed that some nitrifiers could not only nitrify, but denitrify as well (Ritchie and Nicholas, 1972). It has been suggested that this pathway of nitrification, called nitrifier denitrification, might contribute to a major part of the loss of ammonium (NH_4^+) from soils in the form of NO or N_2O (Poth and Focht, 1985; Webster and Hopkins, 1996a). However, not much is known about nitrifier denitrification, partly because a simple method to measure nitrifier denitrification is still lacking.

The imbalance between total inputs and outputs of N in agricultural systems has puzzled scientists for more than 50 years (e.g. Allison, 1955). With the need to quantify all possible sources of N_2O, there is a renewed interest in N balance studies. It is still unclear where all the N is going (e.g. Clough et al., 1999). Does it escape via pathways that have not been taken into account so far? Does it accumulate in the system until an external trigger causes its release into the environment? To find answers to these questions, there is now renewed interest in nitrifier denitrification, as this might, under some conditions, contribute to the loss of N from agricultural systems and be a major source of N_2O and NO. Furthermore, interest in nitrifier denitrification is growing in wastewater treatment as new techniques like OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) are based on nitrifier denitrification (Verstraete and Philips, 1998).

Apart from the absence of a measurement method, another factor leading to the lack of understanding of nitrifier denitrification might be due to the different names attributed to this

pathway. Often the term 'nitrifier denitrification' is used synonymously with 'simultaneous' or 'coupled nitrification and denitrification' (e.g. Bock et al., 1995), although the latter terms usually describe a different set of pathways (e.g. dos Santos et al., 1996, see also below). Sometimes simply 'nitrification' is employed as a name for the production of N_2O via NO_2^- by nitrifiers (e.g. Goreau et al., 1980). A clear distinction between the different pathways is necessary as their N_2O production is affected by different environmental circumstances.

In this paper, present knowledge about nitrifier denitrification is summarized in order to spread awareness of the pathway and to identify areas of research needed to improve global N_2O budgets. It is essential to define exactly what is meant by nitrifier denitrification and to distinguish it from related pathways and processes. Before that is done, the related processes of importance concerning the terrestrial N cycle are briefly explained. After a following short description of the present knowledge about nitrifier denitrification, unanswered questions and hypotheses are discussed.

Nitrification, denitrification and related processes

A summary is presented below of the key issues of the terrestrial N cycle. For a more detailed review of the microbial processes nitrification and denitrification, the reader is referred to the papers by Delwiche (1981), Tiedje (1988), Kuenen and Robertson (1988), Firestone and Davidson (1989) and Williams et al. (1992).

Nitrification

Autotrophic nitrification

Nitrification is the oxidation of NH_4^+ or NH_3 to NO_3^- via NO_2^- (see Figure 5). These reactions are carried out by two groups of microorganisms: the first part up to NO_2^- is conducted by the so-called ammonia oxidizers or primary nitrifiers, whereas the second step is carried out by NO_2^--oxidizers or secondary nitrifiers (Bock et al., 1986). These two groups are together addressed as Nitrobacteriaceae (Buchanan, 1917). *Nitrosomonas europaea* is the best studied autotrophic ammonia oxidizer, but it is not the most common primary nitrifier in soils (Macdonald, 1986; Klemedtsson et al., 1999). *Nitrobacter winogradskyi* is a representative of the NO_2^--oxidizers.

Some intermediates are produced during nitrification (see Figure 5). The first one in ammonia oxidation is hydroxylamine (NH_2OH). The oxidation of NH_3 to NH_2OH is catalyzed by ammonia monooxygenase (Wood, 1986). Here, two electrons are needed for the reduction of one of the atoms of oxygen (O_2) to water. The electrons are derived from the next step, the

oxidation of NH_2OH to NO_2^- (Hollocher et al., 1981, see Figure 5). Ammonia monooxygenase has a broad range of substrates for catalytic oxidations. These 'substrates' can also inhibit the ammonia oxidation function of the enzyme, either competitively or by covalently binding to its active site. For instance, the well-known inhibitor acetylene (C_2H_2) is converted to a very reactive unsaturated epoxide that inhibits the ammonia monooxygenase through covalent binding (McCarty, 1999). C_2H_2 inhibits ammonia oxidation at concentrations between 0.1 and 10 Pa (Berg et al., 1982). Methyl fluoride (CH_3F, Hyman et al., 1994) is another inhibitor of ammonia monooxygenase. This inhibitor has the advantage that it does not, as C_2H_2 does at larger concentrations (10 kPa), affect denitrification (Oremland and Culbertson, 1992).

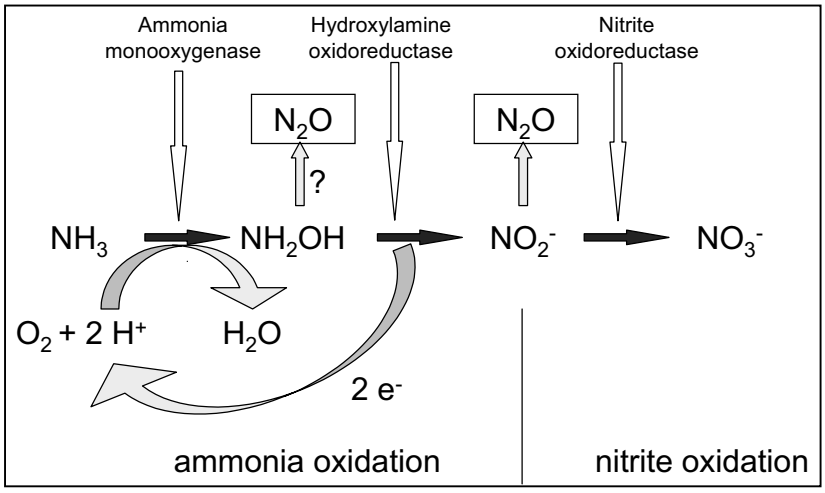

Figure 5: Nitrification: Outline of the pathway and enzymes involved (after Hynes and Knowles, 1984, Poth and Focht, 1985, and Wood, 1986).

The next step in ammonia oxidation is from NH_2OH to NO_2^-. This reaction is catalyzed by hydroxylamine oxidoreductase (McCarty, 1999). Hydrazine is an inhibitor of this enzyme (Nicholas and Jones, 1960).

The NO_2^- produced is further used by nitrite oxidizers in a one-step reaction to NO_3^-. The enzyme catalyzing this reaction is nitrite oxidoreductase (Bock et al., 1986). This reaction is inhibited by chlorate (Belser and Mays, 1980). Furthermore, NH_3 is in large amounts toxic to *Nitrobacter* (Chalk and Smith, 1983).

The Nitrobacteriaceae are aerobes and many are obligate autotrophs. The energy for the CO_2 fixation originates from nitrification. NH_3 and NO_2^- are not very effective energy sources, however. The electrons released during their oxidation can only be transferred to substances at the lower end of the respiration chain. Thus, the oxidation of NH_3 or NO_2^- cannot be directly coupled to the reduction of the first element of the respiration chain NAD (nicotine

amide dinucleotide). Energy has to be used to move electrons to this higher energy level, a process called reverse electron flow. This explains the slow growth of the nitrifying organisms and their relatively large substrate requirements make them difficult to cultivate. For the build-up of 1 g dry mass of *Nitrosomonas spec.*, 30 g NH_3 is needed (Schlegel, 1992). Thus, nitrifiers can be very important in terms of N transformations, even under circumstances where their population is not large. Not only is the end product, NO_3^-, produced in large quantities, but so are the described intermediates. The latter normally do not accumulate in soils, however.

N_2O is formed during ammonia oxidation through chemical decomposition of intermediates between NH_4^+ and NO_2^- such as NH_2OH or NO_2^- itself. This is usually regarded as a special form of chemodenitrification (Chalk and Smith, 1983), which is closely linked with ammonia oxidation as the latter is the source of the substrates for chemodenitrification. There is also evidence that incomplete oxidation of NH_2OH can lead to the development of N_2O (Hooper and Terry, 1979). The N_2O production via the nitrification pathway nitrifier denitrification is described later.

Thus, the ammonia oxidation to NO_2^- can be a source of N_2O. Furthermore, nitrification is via its products, NO_2^- and NO_3^-, coupled with denitrification, which is another significant process in terms of N_2O production, as described further below.

Heterotrophic nitrification

Apart from autotrophic nitrifiers using nitrification as an energy source for fixing carbon dioxide (CO_2), heterotrophic nitrifiers are also known. These nitrifiers use organic carbon (C) as a source of C and energy (Robertson and Kuenen, 1990; Castignetti, 1990). Heterotrophic nitrification is considered to be more common among fungi (Odu and Adeoye, 1970) than bacteria, and fungi may play an important role in heterotrophic nitrification in soils with a low pH. Some heterotrophic bacteria can also nitrify (Papen et al., 1989). Here, the classical distinction made between nitrifiers and denitrifiers begins to fade as these bacteria can often denitrify as well.

Although the substrate, intermediates and products of heterotrophic and autotrophic nitrification are the same, the enzymes of the two processes have been shown to differ from each other. Thus, the ammonia monooxygenase from heterotrophic nitrifiers as studied in *Pseudomonas denitrificans* is not inhibited by C_2H_2. Furthermore, the heterotrophs' hydroxylamine oxidoreductase is a non-haem iron enzyme, in contrast to the multi-haem enzyme found in autotrophs (Richardson et al., 1998). Heterotrophic nitrifiers can oxidize

organic forms of N such as urea as well as NH_3 (Papen et al., 1989). It has been suggested that heterotrophic nitrification might provide NO_3^- under aerobic conditions which could be used for denitrification when conditions turn favourable for this (Castignetti and Hollocher, 1984). In contrast to conventional denitrifiers (see below), these heterotrophic nitrifying bacteria are often able to denitrify under aerobic conditions (Robertson et al., 1989). N_2O is produced (as with denitrification) as an intermediate in the reduction of NO_2^- to N_2 (Anderson et al., 1993; Richardson et al., 1998). Under aerobic conditions, heterotrophic nitrifiers produce much more N_2O per cell than autotrophic nitrifiers (Papen et al., 1989; Anderson et al., 1993). Although heterotrophic nitrification is generally considered to be only a minor source of N_2O, it might produce significant amounts of N_2O under certain sets of circumstances such as low pH, large O_2 amounts and availability of organic material (Papen et al., 1989; Anderson et al., 1993).

Denitrification

Denitrification is the stepwise reduction of NO_3^- to N_2. Several intermediates are developed which can be set free (Figure 6).

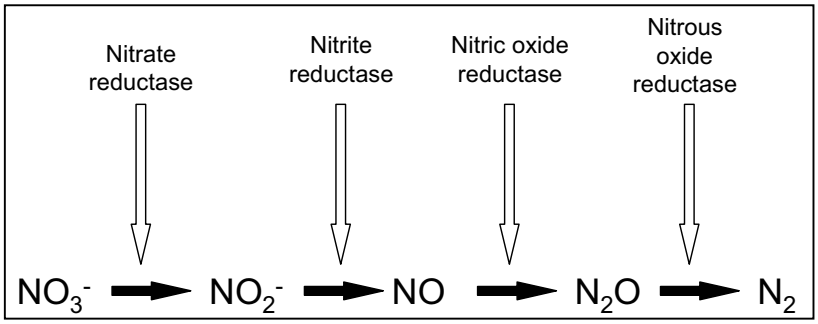

Figure 6: Denitrification: Outline of the pathway and enzymes involved (after Hochstein and Tomlinson, 1988).

The reactions are carried out by denitrifiers, which are widely distributed across the bacterial taxa, including *Pseudomonas, Bacillus, Thiobacillus, Propionibacterium* and others (Firestone, 1982). These predominantly heterotrophic microorganisms are facultative anaerobes that are able to use NO_3^- in place of O_2 as an electron acceptor in respiration to cope with low-oxygen or anaerobic conditions. Enzymes catalyzing the reactions are nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (Hochstein and Tomlinson, 1988, see Figure 6). In contrast to nitrification, N_2O is a regular intermediate of denitrification. The portion of the intermediate N_2O that is released is larger if the pH is low, because N_2O reductase is inhibited at low pH (Knowles, 1982). The ratio N_2O/N_2 also rises if NO_3^- is abundant in the soil because NO_3^- is preferred over N_2O as an electron acceptor

(Schlegel, 1992). If some O_2 is present, the ratio also increases as the inhibition of nitrous oxide reductase by O_2 is stronger than the inhibition of the other reductases of denitrification (Knowles, 1982). At large O_2 concentrations, the aerobic metabolism of denitrifiers is promoted so that the reduction of NO_3^- does not take place. Apart from such environmental factors, no specific inhibitors for denitrification are known yet, except for C_2H_2, which inhibits the reduction of N_2O. This leads to the accumulation of N_2O as the only end product of denitrification after addition of 10 kPa C_2H_2 (Yoshinari et al., 1977).

To summarize, N_2O is an intermediate of denitrification, which can be released in large quantities in low-oxygen environments with sufficient NO_3^- and metabolizable organic C.

Coupled nitrification-denitrification

Coupled nitrification-denitrification is mentioned here because it is often confused with nitrifier denitrification. Coupled nitrification-denitrification is not a separate process. The term is used to stress that NO_2^- or NO_3^- produced during nitrification can be utilised by denitrifiers. This coupling between nitrification and denitrification can take place in soils where favourable conditions for both nitrification and denitrification are present in neighboring microhabitats (e.g. Arah, 1997). In a study of these microhabitats, Khdyer and Cho (1983) investigated the degree of nitrification and denitrification after addition of urea uniformly mixed throughout soil columns under steady-state O_2 gradients. In the aerobic surface layer, nitrification took place whereas the anaerobic zone was dominated by denitrification. N_2O was mainly produced at the aerobic-anaerobic interface from where it could diffuse to the soil surface. This suggests that the production of N_2O is largest at conditions that are sub-optimal for both nitrifiers and denitrifiers. Comparable mechanisms are active in natural soils. Here, nitrification can take place in aerobic surface layers or cracks. Denitrification is mostly confined to anaerobic deeper layers, waterlogged areas or the interior of soil aggregates (Tiedje et al., 1984; Leffelaar, 1986). The interfaces between these areas are the places where the production of N_2O is largest. Coupled nitrification-denitrification is used in the treatment of wastewater, where high removal rates for N can be achieved through the provision of conditions that stimulate the linkage between the processes (dos Santos et al., 1996).

Chemodenitrification

Chemodenitrification is the chemical decomposition of intermediates from the oxidation of NH_4^+ to NO_2^- or of NO_2^- itself with organic (e.g. amines) or inorganic (e.g. Fe^{2+} or Cu^{2+}) compounds. It is a non-biological reaction usually taking place at low pH (van

Cleemput and Baert, 1984). The major product of these reactions under acid conditions is NO (van Cleemput and Baert, 1984), although N_2O also develops (Chalk and Smith, 1983). Chemodenitrification is closely linked with nitrification so that it is often difficult to determine whether NO and N_2O are developed through nitrification or chemodenitrification (Martikainen and De Boer, 1993).

What is nitrifier denitrification?

Nitrifier denitrification is a pathway of nitrification. In nitrifier denitrification, the oxidation of NH_3 to NO_2^- is followed by the reduction of NO_2^- to N_2O and N_2 (Figure 7). This sequence of reactions is carried out by only one group of microorganisms, namely autotrophic ammonia oxidizers. Thus nitrifier denitrification contrasts with coupled nitrification-denitrification, where different groups of coexisting microorganisms can together transform NH_3 to finally N_2.

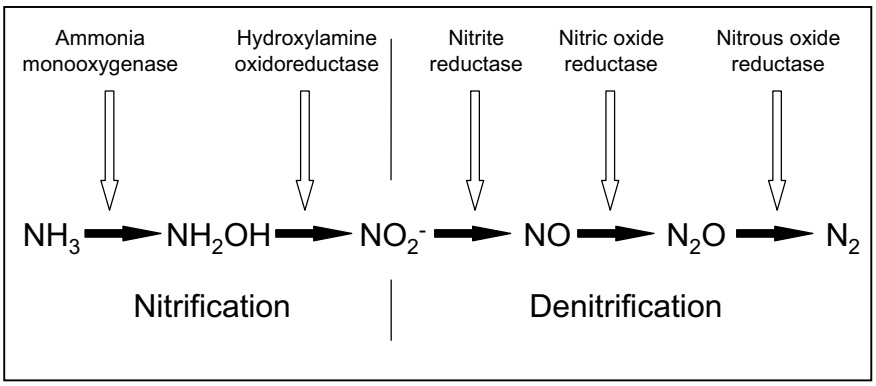

Figure 7: Nitrifier denitrification: Hypothetical pathway and probable enzymes (after Poth and Focht, 1985, Hooper, 1968).

There also exists a similar pathway to nitrifier denitrification in NO_2^--oxidizers: Several strains of the genus *Nitrobacter* have been reported to be able to produce N_2O via anaerobic reduction of NO_3^- with pyruvate as electron donor (Freitag et al., 1987). As even less information is available about this pathway than about nitrifier denitrification, this pathway is not covered in this article.

The first part of nitrifier denitrification (oxidation of NH_3 to NO_2^-) has been attributed to nitrification (ammonia oxidation), whereas the reduction of NO_2^- is regarded as denitrification (Poth and Focht, 1985). As with denitrification, NO_2^- is reduced via NO to N_2O (Poth and Focht, 1985) and further to N_2 (Poth, 1986). The organisms involved in nitrifier denitrification are probably mostly ammonia oxidizers (Kuai and Verstraete, 1998).

The enzymes required by ammonia oxidizers that carry out nitrifier denitrification are believed to be essentially the same as for ammonia oxidation and denitrification. A nitrite reductase first characterized by Hooper (1968) seems to be responsible for the reduction of NO_2^-.

According to the above definition, nitrifier denitrification differs from heterotrophic nitrification linked with aerobic denitrification (see heterotrophic nitrification). These latter processes are not considered as nitrifier denitrification here, as no autotrophic nitrifiers are involved and different sets of enzymes are used.

In Figure 8, an overview of reactions in which nitrification and denitrification in soils are involved is presented. To keep the scheme convenient, heterotrophic nitrification with aerobic denitrification has not been taken into account here. The overlapping boxes symbolize the possibility of a coupling between nitrification and denitrification. As nitrifier denitrification is a pathway of nitrification, the boxes for nitrification and nitrifier denitrification overlap, but separate into the different branches from NO_2^- onwards.

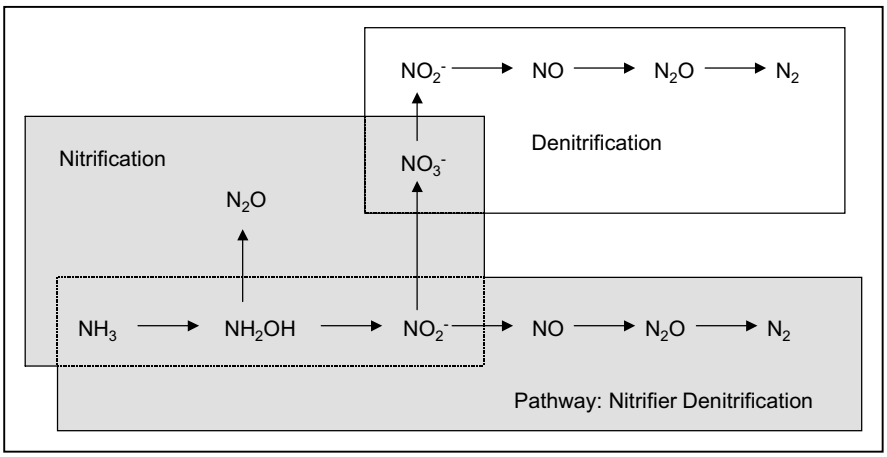

Figure 8: Transformations of mineral nitrogen in soil (for explanations see text).

Summarizing, only nitrifiers carry out nitrifier denitrification, whereas nitrifiers and denitrifiers are involved in coupled nitrification-denitrification. Furthermore, NO_3^- is not produced in nitrifier denitrification, but it may be formed as an intermediate in coupled nitrification-denitrification.

Present knowledge about nitrifier denitrification

A classical study on nitrifier denitrification was published by Ritchie and Nicholas (1972). Making experiments with *Nitrosomonas europaea* cells and cell free extracts adding ^{15}N labelled NH_4^+, NO_3^-, or NH_2OH, they found that *N. europaea* produces N_2O through the

reduction of NO_2^- with NH_2OH as an electron donor. This was shown to occur under aerobic as well as anaerobic conditions.

Poth and Focht (1985) found N_2O production in cultures of *N. europaea* through nitrifier denitrification only under conditions of oxygen stress. Washed cell suspensions transformed ^{15}N labelled NO_2^- to N_2O. The observed pattern of ^{15}N in N_2O was consistent exclusively with denitrification kinetics, showing that this part of nitrifier denitrification is indeed the same as in denitrification. NO_3^- could not be utilized by the cell suspensions. This is in agreement with the proposed pathway of nitrifier denitrification, where NO_2^- is reduced and NO_3^- is not formed (see Figures 7 and 8).

Muller et al. (1995) showed convincingly that nitrifier denitrification was the source of N_2 produced by sewage sludge cultured in recycling containers with a mixture of pure argon and low dissolved oxygen tensions. They argued that N_2 production by nitrifiers had not been observed before, because either the background levels of N_2 in air were too large for any newly formed N_2 to be detected, the concentration of nitrifiers was too small or the wrong strain of nitrifiers was used (according to Poth, 1986, *Nitrosomonas europaea* ATCC 19718 does not produce N_2).

Inhibition experiments have been carried out in attempts to quantify the contribution of nitrifier denitrification to total N losses. Robertson and Tiedje (1987) came to the conclusion that nitrifier denitrification was not an important pathway in the soils they studied. They added 10 Pa C_2H_2 to soil cores treated with 100 kPa O_2. They argued that nitrifier denitrification should already have been inhibited by O_2 as this suppresses denitrification (see also Table 1). Therefore supplementary C_2H_2 – inhibiting the first step of the ammonia oxidation of nitrification – should have no pronounced effect on the amount of N_2O released if nitrifier denitrification was its main source. However, the production of N_2O was further decreased upon addition of C_2H_2. This does not exclude nitrifier denitrification as an important source of N_2O. The results only showed that ammonia oxidation was an important process for the development of N_2O. How much of the N_2O production that was inhibited through addition of O_2 was caused by denitrification and nitrifier denitrification, respectively, was not evaluated by Robertson and Tiedje (1987).

Changing the inhibition experiments slightly, namely using one incubation with 10 Pa C_2H_2 to inhibit nitrification and nitrifier denitrification, one with 100 kPa O_2 to inhibit denitrification and nitrifier denitrification, and one with both 10 Pa C_2H_2 and 100 kPa O_2 to inhibit nitrification, denitrification and nitrifier denitrification (see Table 1), Webster and Hopkins (1996a) obtained the following results for a sandy-loam soil. Nitrifier denitrification was the

main source of N_2O from the drier soil (matric potential -1.0 kPa), whereas denitrifiers were identified as the dominant producers of N_2O from a wetter soil (matric potential -0.1 kPa). Of a total 192 pmol N_2O g^{-1} soil h^{-1} produced, 55 pmol N_2O g^{-1} soil h^{-1} (29%) were calculated to have been generated by nitrifier denitrification in the drier soil. In the wetter soil, the amount derived from nitrifier denitrification was estimated to be less than 7 pmol N_2O g^{-1} soil h^{-1} (3%) from a total of 282 pmol N_2O g^{-1} soil h^{-1}.

Table 1: Scheme of incubations carried out by Webster and Hopkins (1996a) and – slightly differently – by Robertson and Tiedje (1987) to determine the source of N_2O from soils (+: process can take place, -: process is inhibited)

Source of N_2O	Incubation without additions (Control)	Incubation with 10 Pa acetylene	Incubation with 100 kPa oxygen	Incubation with 10 Pa acetylene and 100 kPa oxygen
Nitrification	+	-	+	-
Nitrifier Denitrification	+	-	-	-
Denitrification	+	+	-	-
Other Sources	+	+	+	+

The pathway of nitrifier denitrification is receiving increasing attention lately in the field of wastewater treatment. Several wastewater treatment techniques have been developed that make use of either nitrifier denitrification or coupled nitrification-denitrification while omitting the intermediate production of NO_3^- in order to save energy (Verstraete and Philips, 1998). The so-called OLAND process (Oxygen-Limited Autotrophic Nitrification-Denitrification) utilises nitrifier denitrification through controlling the O_2 content in the reactor so that nitrification can only proceed up to NO_2^- which is subsequently used to oxidize another mole of NH_4^+ due to a shortage of electron acceptors (Verstraete and Philips, 1998). This shows that O_2 is an important factor controlling the occurrence of nitrifier denitrification. In the ANAMMOX process (anaerobic ammonium oxidation), NH_4^+ is oxidized to N_2 with NO_2^- as the electron acceptor by a newly identified planctomycete, *Brocadia anammoxidans* (Strous et al., 1999). This shows that the ability to perform reactions carried out during nitrifier denitrification is also present in other organisms.

Discussion

Although there are some techniques to assess the ecological significance of nitrifier denitrification, results are scarce. Therefore, the importance of this pathway in different soils under a range of conditions is still a matter of speculation. As has been mentioned above, the

amount of N_2O lost via nitrifier denitrification in soils measured so far varies between supposedly insignificant amounts (Robertson and Tiedje, 1987) and about 30% of the total N_2O production (Webster and Hopkins, 1996a). This may reflect slightly different methods or hint at the different importance of nitrifier denitrification for N_2O production in different soils under different conditions.

Apart from the quantification of nitrifier denitrification, other questions about this pathway still remain unsolved. One of these is its ecological and physiological importance for the microorganisms involved. The authors dealing with the subject suggest some potential answers. Thus, Ritchie and Nicholas (1972) mention the importance of a possible use of NO_2^- as an alternative for O_2 as an electron acceptor for microorganisms being temporarily subjected to anaerobic conditions. Poth and Focht (1985) propose several possible reasons for nitrifier denitrification: 1) the conservation of O_2 for the initial step of ammonia oxidation, 2) the removal of NO_2^- as a toxic product and 3) the decrease of competition for O_2 through the removal of the substrate for NO_2^--oxidizers.

Factors that are thought to influence nitrifier denitrification are summarized in Figure 9. The most important components are considered to be the amount of NH_3 and O_2 in the soil and the numbers of active nitrifiers with the ability to denitrify. The influence of rainfall and soil organic matter on mineralisation as well as other minor factors have not been taken into account for the sake of comprehensibility of the figure.

The circumstances favouring the different processes and pathways of the N-cycle lead to a distribution of nitrification, denitrification, heterotrophic nitrification with denitrification, coupled nitrification-denitrification and nitrifier denitrification in fertilized soils as shown in Figure 10. Nitrifier denitrification is shown separately from nitrification to stress that nitrifier denitrification only seems to take place in low-oxygen conditions. Nitrification as a whole does not take place in N poor soils, where heterotrophic nitrification with aerobic denitrification can take place if the system is rich in C. Denitrification takes place at large availabilities of organic C coupled with low O_2 contents of soils, as denitrifiers are heterotrophs that use NO_3^- anaerobically as an electron acceptor. Denitrifiers are able to compete at both low and high N contents. An area is shown in Figure 10 where nitrification and denitrification overlap. These are the conditions where coupled nitrification-denitrification can take place. Overall, it must be said that the borders are not as sharp as drawn, but that there can always be overlap between the different processes and pathways.

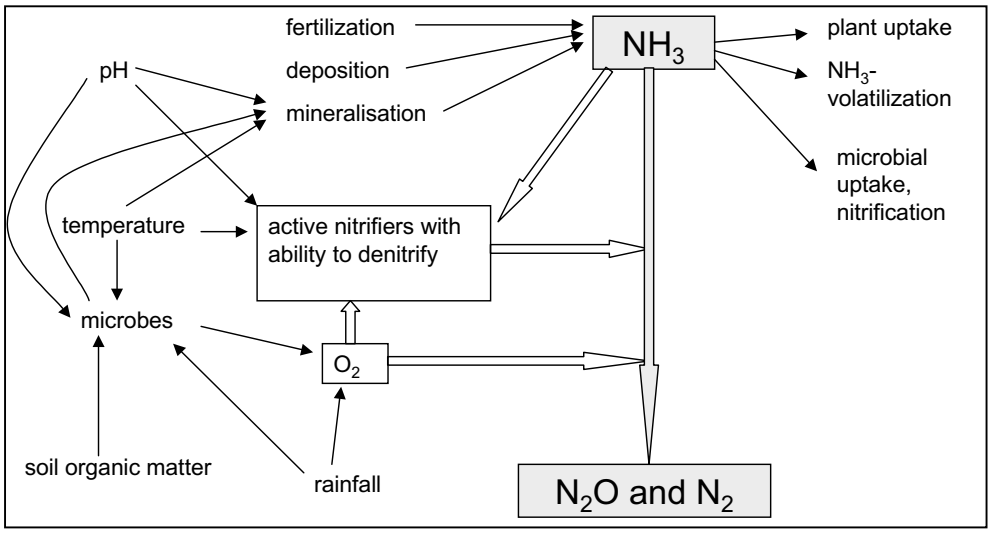

Figure 9: Environmental factors influencing nitrifier denitrification. Gray arrows indicate the pathway, the large white arrows show proximate factors and normal arrows other factors influencing nitrifier denitrification. The same scheme applies to the boxes.

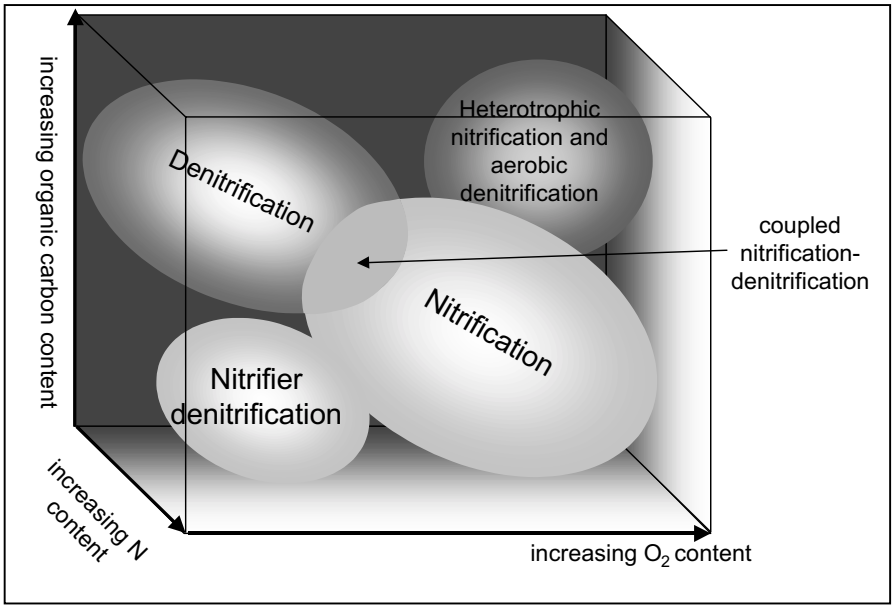

Figure 10: Possible ecological niche for the nitrification pathway nitrifier denitrification in fertilized soils.

The possibility of an ecological niche for nitrifier denitrification can also be assessed based on thermodynamic considerations. Table 2 shows the energy gain resulting from pure nitrification, nitrifier denitrification and denitrification at different conditions. The values indicate the different influence of changes in pH and temperature on the various processes and pathways.

Table 2: Changes in Gibbs free energy (ΔG) in nitrification, nitrifier denitrification and denitrification at different conditions, calculated from values published by Latimer (1953) and Thauer et al. (1977)

Process	$\Delta G^{0'}$ (25 °C, pH 7) [kJ/mol N]	ΔG (25 °C, pH 4) [kJ/mol N]	ΔG (10 °C, pH 7) [kJ/mol N]	ΔG (10 °C, pH 4) [kJ/mol N]
nitrification:				
$NH_3/NH_4^+ \rightarrow NO_2^-$	-274.7	-240.5	-278.1	-243.9
$NO_2^- \rightarrow NO_3^-$	-74.1	-74.1	-75.3	-75.3
nitrifier denitrification:				
$NH_3/NH_4^+ \rightarrow N_2O$	-264.2	-247.0	-265.8	-248.6
denitrification:				
$NO_3^- \rightarrow N_2O$	-389.6	-406.8	-392.6	-409.8

Ammonia oxidation is negatively affected by decreasing pH because of the development of 2 H^+-ions per oxidized NH_4^+. From a thermodynamic point of view, NO_2^- oxidation is not affected by pH as no H^+ is involved in this reaction. One H^+ mole is used for the development of water for each mole reduced NO_2^- in denitrification. That is the reason for higher energy gain in denitrification at lower pH. To calculate the change in free energy in nitrifier denitrification, it was assumed that after the oxidation of one mole NH_4^+ to NO_2^-, this NO_2^- reacts with another mole of NH_4^+ to form N_2 and water. Broda (1977) proposed this reaction. The conversion of equimolar amounts of NH_4^+ and NO_2^- to N_2 was shown by Bock et al. (1995) with *Nitrosomonas* cultures growing with molecular H as electron donor and NO_2^- as electron acceptor under anoxic conditions. Although the overall amount of energy gained is higher if a reaction pathway for nitrifier denitrification is chosen that combines ammonia oxidation with the steps of NO_2^- reduction to N_2 from denitrification (as in Figure 7, then $\Delta G^{0'}$ = -672.0 kJ/mol N), the amount of H^+-ions produced remains the same, at one H^+ per N. Thus, decreasing pH has a negative effect on nitrifier denitrification, but the influence is not as strong as in pure nitrification. Therefore, from a thermodynamic point of view, one might expect more nitrifiers to carry out nitrifier denitrification at decreasing pH. The influence of temperature changes is not so pronounced. Decreasing the temperature from 25 to 10°C has a slight positive effect on the energy gain from all the reactions. This effect is increasing in the order nitrite oxidation, nitrifier denitrification, denitrification, ammonium oxidation.

In the investigation of nitrifier denitrification, apart from inhibition studies with complete soil cores only experiments with *Nitrosomonas europaea* have been carried out. This is mainly

due to difficulties in the isolation and maintenance of nitrifiers from soils (Schmidt, 1982). Mostly enrichment is used as a technique to isolate nitrifiers. This may lead to the dominance of a single member of the soil population in the extract (Schmidt, 1982). Attempts to tackle this problem have been accomplished (e.g. Schmidt and Belser, 1982), but are even more tedious than the enrichments. Due to these problems, it is not yet known how widespread nitrifier denitrification is in the communities of nitrifiers in soils. We are not aware of any studies of nitrifier denitrification directly in the soil without the problems attached to inhibition or isolation of certain microorganisms.

In order to estimate the importance of the production of N_2O through nitrifiers in soils, more studies on nitrifier denitrification have to be carried out. Studies based on the scheme of incubations described above (see Table 1) might be useful in the attempt to quantify this pathway in different soils.

The use of stable isotopes both as tracers and in natural abundance monitoring might help to measure nitrifier denitrification without the restrictions resulting from the use of inhibitors. For nitrification, denitrification and nitrifier denitrification different N- and O-sources are used, which differ in their isotopic composition (Schmidt and Voerkelius, 1989). As furthermore the fractionation factors of at least nitrification and denitrification differ (Schmidt and Voerkelius, 1989), a difference in the $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios of N_2O from the different pathways in natural abundance measurements can be expected. This difference can be used to differentiate between the pathways. Webster and Hopkins (1996b) made an effort to use the natural abundances of ^{15}N and ^{18}O to differentiate between nitrification and denitrification as sources of N_2O. They concluded that denitrification was the main source of N_2O from a wetter soil while nitrification was its main source in a drier soil. As there are no data yet for the fractionation through hydroxylamine decomposition or nitrifier denitrification, no differentiation between these two possible pathways of N_2O production in nitrification could be made. In addition to natural abundance measurements, application of labelled NH_4^+, NO_3^- or NO_2^- in tracer studies should increase the knowledge of the reaction pathway of nitrifier denitrification.

Incubations of intact soil cores under different circumstances can give important insights into the conditions favouring nitrifier denitrification. The same should be done including incubations with pure or mixed cultures of nitrifiers to learn more about favourable conditions and the prevalence of nitrifier denitrification among different nitrifiers. The information gained in these experiments should subsequently be used in the improvement of terms for nitrifier denitrification in existing models of gaseous losses of N from farmland.

To sum up, we argue that nitrifier denitrification can be an important source of N_2O under certain circumstances, i.e. high N content, low organic C content, low O_2 pressure and maybe also low pH. In contrast to denitrification, this pathway of nitrification is not very well investigated yet, however. Thus, more effort should be put into the research of this pathway. The combination of the described methods (use of stable isotopes, inhibition experiments, pure culture experiments, modelling) would provide more information about the importance of nitrifier denitrification as a source of N_2O, both in terms of the amount of N_2O produced and the prevalence of nitrifier denitrification under different circumstances. Modelling should provide a means for the prediction of sets of conditions where nitrifier denitrification can indeed be significant as a source of N_2O. This knowledge could then be used for the development of guidelines for low-emission farming practices and maybe also in the improvement of techniques in wastewater treatment.

Acknowledgements

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CHAPTER THREE

CONTRIBUTION OF NITRIFIER DENITRIFICATION TO NITROUS OXIDE PRODUCTION IN GRASSLAND SOILS

N. Wrage, G.L. Velthof, H.J. Laanbroek, O. Oenema
(submitted to Soil Biology and Biochemistry)

Nitrifier denitrification is the reduction of nitrite (NO_2^-) by nitrifiers. It leads to the production of the greenhouse gas nitrous oxide (N_2O) as intermediate and possible end product. It is not known yet how important nitrifier denitrification is for the production of N_2O in soils. In this study, we explore N_2O production by nitrifier denitrification in relation to other N_2O producing processes like nitrification and denitrification in different soil conditions. The influence of the oxygen (O_2) content of the soil, different N sources, and pH was tested in four experiments. To differentiate between sources of N_2O, an incubation method with inhibitors was used (Webster and Hopkins, 1996a). Sets of four incubations included controls without addition of inhibitors, incubations with addition of small concentrations of acetylene (C_2H_2; 0.01-0.1 kPa), large concentrations of O_2 (100 kPa), or a combination of C_2H_2 and O_2. The results indicate that the availability of NO_2^- stimulated the apparent N_2O production by nitrifier denitrification. A decreasing O_2 content increased the total N_2O production, but decreased N_2O production by nitrifier denitrification. No significant effect of pH could be found. The study revealed problems concerning the use of the inhibitors C_2H_2 and O_2. Therefore, it was not possible to draw accurate conclusions about the amounts of N_2O produced by different sources. Almost one third of all incubations with inhibitors produced more N_2O than the controls. Possible reasons for the problems are discussed. We conclude that the effect of these inhibitors on sources of N_2O and the limitations to the inhibitors' effectiveness should be studied in more detail before accurate conclusions about the partitioning of N_2O sources in soils can be made based on these inhibitors.

Introduction

Nitrification and denitrification are the most important microbial sources of nitrous oxide (N_2O) in soils (Granli and Bøckmann, 1994). Nitrifiers produce N_2O in two ways, nitrification and nitrifier denitrification. In nitrification, N_2O is produced as a by-product of ammonia (NH_3) oxidation. In nitrifier denitrification, N_2O is an intermediate of the reduction of nitrite (NO_2^-) to molecular nitrogen (N_2) (Wrage et al., 2001). Denitrifiers produce N_2O as an intermediate and possible end product of the reduction of nitrate (NO_3^-) to N_2. Besides these main sources, other sources like chemodenitrification or heterotrophic nitrification contribute under some conditions substantially to the production of N_2O (Chalk and Smith, 1983, Papen et al., 1989, Anderson et al., 1993).

There is increasing interest in measures that decrease the production of N_2O, since N_2O is an important greenhouse gas (IPCC, 1996). To decrease the N_2O production effectively, good knowledge of all its sources and controlling factors is essential. Soils contribute substantially to N_2O emissions, especially when fertilised (Mosier, 1994). Many studies have differentiated between different soil sources of N_2O. The differentiation between N_2O produced by nitrifiers and denitrifiers has often been based on the inhibition of nitrification by small concentrations of acetylene (C_2H_2). However, C_2H_2 does not allow a distinction between nitrification and nitrifier denitrification since it inhibits both these sources of N_2O (Robertson and Tiedje, 1987). In 1996, Webster and Hopkins (1996a) further developed a method invented by Robertson and Tiedje (1987), which allowed to study the N_2O production by nitrification, nitrifier denitrification, denitrification, and other sources in soils separately. The method is based on a set of four incubations, including incubations with small concentrations of C_2H_2 (0.01-0.1 kPa), but also incubations with large concentrations of oxygen (O_2; 100 kPa) to suppress denitrification and nitrifier denitrification, incubations with both C_2H_2 and O_2, and control incubations without additions of inhibitors.

An application of this incubation method showed that nitrifier denitrification did not contribute to the N_2O production from a wet soil, but produced almost 30% of the N_2O in a drier soil (matric potentials of -0.1 and -1.0 kPa, respectively; Webster and Hopkins, 1996a). Very few other studies have investigated nitrifier denitrification in soils. Thus, the potential of different soils for N_2O production by nitrifier denitrification has never been studied systematically. Furthermore, the impact of factors like O_2 or NO_2^- content, which have been shown to be important in laboratory experiments with pure cultures (Poth and Focht, 1985, Kester et al., 1997, Dundee and Hopkins, 2001), are still not known for soils. Evidently, the

importance of nitrifier denitrification as a source of N_2O in different soil conditions is not well understood yet (Wrage et al., 2001).

In this study, we used the method of Webster and Hopkins (1996a) in a survey of sources of N_2O production in grassland soils. Managed grassland systems were chosen since they generally emit more N_2O per surface area than arable or forest soils (Oenema et al., 1998), especially when fertilised and grazed intensively. We studied the sources of N_2O under a wide range of conditions, using different soils and management practices. In total, four experiments were carried out.

In two experiments, we studied the effect of the aeration of a soil on sources of N_2O by manipulating the soil's water content and by investigating soils with different texture. The hypothesis was that a better aeration should promote aerobic processes like nitrification, while less aeration should promote more anaerobic processes like denitrification or possibly nitrifier denitrification. A third experiment was set up to see whether a larger NO_2^- content of a soil caused by urine additions promotes nitrifier denitrification as suggested by Koops et al. (1997). In a fourth experiment, we studied the effects of pH and of amount and form of N fertiliser on sources of N_2O production.

The different experiments carried out in this study should shed light on the significance of different N_2O producing processes in soils under a range of conditions. When the importance and regulation of these processes is better understood, we can estimate their contribution to the N_2O emission with more confidence. This will facilitate the choice between mitigation options for N_2O.

Material and Methods

Site and soil description

We carried out four experiments with grassland soil from four locations. Per experiment, one or two soils were used. Except for soil 1, soils were sampled in 2000-2001. Soil 1 is a clayey soil from a dairy farm in the Netherlands. It was sampled in 1986, dried, homogenised, cleared from stones and big roots and stored until use. This soil had a pH of 7.8. It had a total nitrogen content of 0.05%, a carbon content of 2.4% and an organic matter content of 6.4%. We used this soil for an experiment with manipulated water content and one with addition of artificial urine. To avoid effects from re-wetting (Davidson, 1992), the soil was preincubated as described below before experiments were carried out.

Soils 2 and 3 are from unfertilised grassland fields situated close to each other in the Hungarian puszta. Soil 2 is a sandy soil with a pH of 7.4, soil 3 a clayey soil with a pH of 6.3. Differences in soil texture, bulk densities, and water content influenced the diffusion of gases in these two soils. Soil 2 had a sand content of 24% in the A-horizon, compared to 4% in soil 3. The bulk density was 1.29 g cm^{-3} for the upper 20 cm of soil 2, and 1.36 g cm^{-3} for soil 3. The water content in the upper 10 cm was 0.33 g g^{-1} for soil 2 and 0.42 g g^{-1} for soil 3. These soils were used to investigate the influence of soil texture on sources of N_2O.

Soil 4 is a sandy soil from a long-term grassland experimental site in Poland where plots with different fertiliser application and pH had been established in 1981, 19 years before this experiment. The fertilisers, ammonium nitrate (AN) and calcium nitrate (CN), were applied in two amounts (N_1 = 120 kg N ha^{-1} $year^{-1}$ and N_2 = 240 kg N ha^{-1} $year^{-1}$). The total N application was split into three equal doses. At the start of the long-term experiment, in 1981, half of the plots had been limed with 4.6 t CaO ha^{-1} (Ca_2; Ca_0: not limed). As a result of the various treatments, the pH in the topsoil of the plots ranged from 3.5 to 6.3. Combining the factors fertiliser type, fertiliser amount, and liming resulted in 8 treatments. These treatments were set up in a randomised block design with four replicates each. Further details about the field can be found in Oenema and Sapek (2000).

Incubations

Within each experiment, four incubations according to the inhibition method developed by Webster and Hopkins (1996a; Table 3) were set up per treatment to distinguish between sources of N_2O. Throughout this paper, the term 'treatment' is used for changes in e.g. nutrient status or water content, not for application of inhibitors.

Table 3: Inhibitors used and effects on soil processes generating N_2O (+: process can take place; -: process is blocked). After Webster and Hopkins (1996a). The letters given in brackets in the first row are the abbreviations used in the text for this incubation.

Affected Process	**Control (C)**	**with small concentrations of C_2H_2 (A)**	**O_2 atmosphere (O)**	**with small concentrations of C_2H_2 in O_2 (AO)**
Nitrification	+	-	+	-
Nitrifier Denitrification	+	-	-	-
Denitrification	+	+	-	-
Other	+	+	+	+

Small concentrations of acetylene (C_2H_2) were used to inhibit ammonia oxidation (Klemedtsson et al., 1990), and consequently to inhibit both autotrophic nitrification and nitrifier denitrification. In experiments with homogenised soil, 0.01 kPa C_2H_2 was applied. To account for more difficult diffusion of gases under wet conditions, 0.02 kPa C_2H_2 was used in experiments with wet soil (Klemedtsson et al., 1988). In experiments with intact soil cores, 0.1 kPa C_2H_2 was used. This should not have influenced the reduction of N_2O to N_2 in the last step of denitrification, which is inhibited by C_2H_2 concentrations above 1 kPa (Klemedtsson et al., 1990).

To suppress denitrification, large concentrations of O_2 (approximately 100 kPa) were used. Nitrifier denitrification should also be inhibited by large concentrations of O_2 (Robertson and Tiedje, 1987). Large O_2 concentrations were established by flushing with pure O_2. In incubations with both C_2H_2 and O_2, neither nitrification pathways nor denitrification should take place. Thus, N_2O in these incubations had to be produced by other sources, such as chemodenitrification.

Experiment 1: Water content

In a first experiment, we manipulated the water content of a soil. A larger water content generally promotes denitrification (Bollmann and Conrad, 1998) and probably nitrifier denitrification (Dundee and Hopkins, 2001), but simultaneously increases consumption of N_2O by restricting gas diffusion (Hosen et al., 2000). For this experiment, 20 g of soil 1 was added to 250 ml serum bottles and nutrient solution as described by Verhagen and Laanbroek (1991) added to field capacity (4 ml per 20 g of soil). The bottles were preincubated with open lids at room temperature. After 4 days of preincubation, 4 ml of water was added to half of the bottles used for the experiment to increase the water content (0.4 g water g^{-1} dry soil). The next day, all bottles were closed and the inhibitors added (Table 3). The serum bottles were incubated horizontally at room temperature and shaken three times per day to increase the contact between soil and inhibitors. The amount of N_2O released was measured with a gas chromatograph with electron capture detector (PU 4400 Unicam Analytical Systems, Philips, The Netherlands) after 0 and 24 hours. The experiment was set up in triplicate.

Experiment 2: Soil texture

In a second experiment, we investigated the effect of soil texture on sources of N_2O. Soil texture influences the O_2 content of a soil. Thus, a light texture should promote aerobic

processes like nitrification and a heavy texture those more important under suboxic conditions, like nitrifier denitrification and denitrification (Bollmann and Conrad, 1998).

For this experiment, soils 2 and 3 were used. The upper 15 cm of both soils was sampled, homogenised and cleared from roots and big stones. 100 g of the prepared soil was filled into 500 ml serum bottles. Inhibitors were added according to the scheme in Table 3. Incubations were carried out at room temperature. The N_2O production was measured immediately after addition of the inhibitors, and again after 6 and 24 hours. The measurements were done using a photoacoustic infrared gas analyser (TGA, Brüel and Kjær 1302, Estavillo et al., 2002) equipped with traps for carbon dioxide (CO_2) and water. The experiment was done in triplicate.

Experiment 3: Artificial urine

Addition of artificial urine should increase the production of NO_2^- in soils. NO_2^- is a prerequisite for nitrifier denitrification. Koops et al. (1997) have shown that nitrifiers can contribute substantially to N_2O production from urine patches. The authors assumed that nitrifier denitrification was a major source of N_2O in their experiments.

For this experiment, 100 g of soil 1 (dried, homogenised and cleared from stones and big roots) was mixed with 20 ml of artificial urine in three concentrations. This increased the water content to field capacity. The artificial urine solution with the largest concentration ('Urine 1') contained per liter 17.7 g urea, 7.4 g hippuric acid, 0.2 g creatinine, 0.4 g allantoin, 0.1 g uric acid, 0.9 g ammonium chloride, 14.2 g $KHCO_3$ and 10.5 g KCl (after de Klein and van Logtestijn, 1994). For the solution 'Urine ½', half the concentrations were used for all chemicals except for $KHCO_3$ and KCl, where the same amounts as in 'Urine 1' were added. The 'Urine 0' solution contained only $KHCO_3$ and KCl in the same concentration as above.

The soil was preincubated in 500 ml serum bottles with open lids at room temperature for three days. Then, the bottles were closed and incubations with inhibitors started (according to Table 3). The N_2O production in these incubations was measured after 0, 5, and 24 hours with a TGA. The experiment was set up with four replicates. In this experiment, we also measured the concentrations of NH_4^+, NO_3^- and NO_2^- after 0 and 24 hours. To this end, KCl extractions were made (200 ml 1M KCl per 100 g soil) and measured by segmented flow analysis as described in Houba et al. (2000).

Experiment 4: Mineral fertilisers and pH

We studied the effects of different N fertiliser applications and of a range of soil pH in a unique field site in Poland. Application of ammonium nitrate potentially increases the N_2O production by nitrifiers by providing the substrate for nitrification. Calcium nitrate can only influence nitrification via shifts in pH. Therefore, it is expected to have a smaller impact on nitrifiers than ammonium nitrate. Increasing pH has been shown to decrease N_2O emissions from grassland (Yamulki et al., 1997). Nitrifiers have a rather high pH optimum of 8.5 (Hynes and Knowles, 1984). Thus, with rising pH, not only the amount of N_2O produced might change, but also the ratio between its sources.

For this experiment, soil 4 was collected from the eight different treatments (see above) one week after fertilisation. Six soil cores (25 mm diameter) of the top 5 cm of soil were pooled per plot and incubated in 1-liter Kilner jars with septum fittings. After addition of the inhibitors, the closed jars were incubated for 24 hours at ambient air temperature (about 8°C at night and 18°C at day; Sapek and Barszczewski, 2000). Then, the N_2O concentrations were measured using a TGA. The experiment was carried out with four replicates.

Calculations

Measured values for N_2O were corrected for N_2O present in the jars at the beginning of the experiments. The amount of N_2O produced by the different sources was calculated as follows.

$$N_2O_{Nitrification} = N_2O_O - N_2O_{AO} \quad \textbf{Equation 1}$$

$$N_2O_{Denitrification} = N_2O_A - N_2O_{AO} \quad \textbf{Equation 2}$$

$$N_2O_{Nitrifier\ Denitrification} = N_2O_C - N_2O_{Nitrification} - N_2O_{Denitrification} - N_2O_{AO} = N_2O_C - N_2O_O - N_2O_A + N_2O_{AO} \quad \textbf{Equation 3}$$

$$N_2O_{Other} = N_2O_{AO} \quad \textbf{Equation 4}$$

The subscripts C, A, O and AO refer to the incubation type that was used to differentiate between the processes as shown in Table 3.

All effects were evaluated with the statistics program SPSS for Windows 8.0 (Norusis, 1986). Data were tested for normality using the Kolmogorov-Smirnov test. Differences between treatments in normally distributed data were analysed using analysis of variance (ANOVA, $\alpha = 0.05$). In some cases, the data had to be transformed before analysis due to inhomogeneity of variances (Levene's test). The LSD test was used for multiple comparisons between means. When the data was not distributed normally, the Kruskal-Wallis test was used to evaluate

differences ($\alpha = 0.05$). Here, Schaich-Hamerle analysis was carried out as a post-hoc test using a macro procedure in Excel. All experiments were carried out with 3 or 4 replicates as stated in the description of the experiments.

Results

Total production of N_2O

All soils were producing N_2O in the same order of magnitude. However, the different treatments had strong impacts on the total amount of N_2O produced. Least N_2O was produced by soil 4 in a limed treatment receiving 120 kg calcium nitrate per year (4 nmol N_2O-N kg^{-1} h^{-1}, Table 7), most by soil 1 under wet conditions (184 nmol N_2O-N kg^{-1} h^{-1}, Table 4). Generally, more fertilisation and less aeration increased the N_2O production. A lot of variability in N_2O production could be observed both between and within experiments. In all experiments, negative values were calculated for some sources of N_2O, especially for nitrification and for nitrifier denitrification.

Table 4: N_2O-Production (nmol N_2O-N kg^{-1} h^{-1}) by different sources in two experiments with (1) manipulated water content and (2) different soil textures (means and standard deviations, n=3). Different superscript letters indicate significant differences (α=0.05) between the treatments of each experiment. For further explanation see text.

Treatment	Nitrification	Nitrifier Denitrification	Denitrification	Other Sources	Total Production
Water (1):					
soil moist	18 ± 13^a	-2 ± 5^a	22 ± 2^a	12 ± 1^a	34 ± 5^a
soil wet	-33 ± 216^a	-6003 ± 40^b	6301 ± 270^b	386 ± 72^b	184 ± 40^b
Texture (2):					
sand	-3 ± 4^a	3 ± 3^a	-1 ± 6^a	15 ± 8^a	14 ± 3^a
clay	4 ± 3^a	2 ± 6^a	22 ± 25^a	10 ± 5^a	38 ± 6^b

Experiment 1: Water content

The total production of N_2O was significantly larger in the wetter soil (Table 4). This was due to an increased N_2O production by denitrification and other sources. The N_2O production by nitrifier denitrification was significantly smaller under wetter conditions. The values for nitrifier denitrification were highly negative in the wet soil. These negative rates for nitrifier denitrification were matched by equally large, but positive, rates for

denitrification. No significant differences between the drier and wetter soil conditions were found for nitrification.

Experiment 2: Soil texture

The results from the soils differing in texture were consistent with those of the experiment with different water contents (Table 4). The total production of N_2O was significantly larger in the more heavily textured soil. The effects of different texture on the sources of N_2O were not significant (α=0.05). Denitrification was somewhat larger in the clay soil than in the sandy soil. Nitrification, nitrifier denitrification and other sources were about equal in both soils.

Experiment 3: Artificial urine

The total N_2O production in experiment 3 was similar to that in experiment 1, where the same soil had been used (Table 4 and 5). The total N_2O production increased with larger concentration of the applied urine solution (Table 5), but the differences were not statistically significant. The N_2O production from nitrifier denitrification and from other sources was significantly larger in the treatment with the largest urine concentration compared to the 'Urine 0' treatment that had received only $KHCO_3$ and KCl. The treatment with largest urine concentration produced significantly less N_2O by nitrification. Denitrification tended to increase with larger urine concentration, but this trend was not significant.

Table 5: N_2O production (nmol N_2O-N kg^{-1} h^{-1}) by different sources in a fertilisation experiment with addition of artificial urine (means and standard deviations, n=4). Different superscript letters indicate significant differences (α=0.05) between the treatments.

Treatment	Nitrification	Nitrifier Denitrification	Denitrification	Other Sources	Total Production
Urine 0	3 ± 1^{a}	33 ± 38^{a}	17 ± 13^{a}	13 ± 0^{a}	39 ± 38^{a}
Urine ½	4 ± 1^{a}	55 ± 15^{ab}	16 ± 10^{a}	23 ± 1^{ab}	52 ± 15^{a}
Urine 1	-5 ± 2^{b}	108 ± 44^{b}	47 ± 37^{a}	51 ± 20^{b}	99 ± 44^{a}

On the days of measurement, i.e. the third and fourth day after addition of fertiliser, both the NH_4^+ and the NO_2^- content were larger in treatments that had received urine solution with larger concentrations (Table 6). Initially, the NO_3^- concentration was approximately the same in all treatments. Changes in mineral N concentrations in 24 hours and differences between incubations with different inhibitors were too small to be measured accurately against the background concentrations (results not shown).

Table 6: Mineral N concentrations in a fertilisation experiment with artificial urine (means and standard deviations, n = 4). The concentrations were measured on the third day after addition of artificial urine, i.e. the day when N_2O measurements started.

Treatment	NH_4^+ [mg N $kg_{dry\ soil}^{-1}$]	NO_2^-[mg N $kg_{dry\ soil}^{-1}$]	NO_3^-[mg N $kg_{dry\ soil}^{-1}$]
Urine 0	11 ± 2	1 ± 0	29 ± 1
Urine ½	857 ± 12	8 ± 0	27 ± 1
Urine 1	1604 ± 2	16 ± 2	24 ± 1

Experiment 4: Mineral fertilisers and pH

The N_2O production in experiment 4 did not differ significantly between the eight treatments (α=0.05) (Table 7). However, when comparing all treatments receiving 120 kg N ha^{-1} $year^{-1}$ to those receiving 240 kg N ha^{-1} $year^{-1}$, i.e. N_1 against N_2, the total N_2O production was found to be significantly larger with larger N fertilisation. This was mainly due to slight (insignificant) increases in N_2O production from nitrifier denitrification and other sources. Use of CN fertiliser instead of AN did not have significant effects on the N_2O production from any source. Liming significantly increased the N_2O production only from sources other than nitrification, denitrification or nitrifier denitrification. No correlation between pH and N_2O production could be found.

Discussion

General trends of N_2O production

With 4 to 184 nmol N_2O-N kg^{-1} soil h^{-1}, the total N_2O production was at the lower end of that measured in comparable incubation studies. Robertson and Tiedje (1987) found a total production of 7 to 607 nmol N_2O-N kg^{-1} soil h^{-1} in two oxic forest soils that were known to produce significant amounts of N_2O. Webster and Hopkins (1996a) measured a production of 384 to 564 nmol N_2O-N kg^{-1} soil h^{-1} in grassland soil from an iron-podzol with sandy-loam texture.

The experiments investigating the effect of water content and texture on N_2O production showed a larger total N_2O production with increasing water content and with heavier texture of the soil. This coincided with larger N_2O production by denitrification. It is likely that these effects were caused by a lower O_2 availability in the soils. Other studies also showed the importance of denitrification for N_2O production under wet, suboxic conditions (Bollmann and Conrad, 1998). The N_2O production by nitrifier denitrification tended to decrease with larger water content and heavier texture of the soil. This is in line with the results of Webster

and Hopkins (1996a). In pure culture studies, nitrifier denitrification has been shown to become more important for N_2O production with declining O_2 concentrations (Whittaker et al., 2000, Dundee and Webster, 2001). Studies of the enzymes of nitrifiers have revealed that nitrite reductase, the enzyme carrying out the first step of the denitrifying pathway of nitrifiers, is always present in *Nitrosomonas europaea*, but is activated by low O_2 pressure (Whittaker et al., 2000). Besides O_2, NO_2^- is important for nitrifier denitrification (Bock et al., 1995). In pure culture studies with nitrifiers, NO_2^- can accumulate. In soils, NO_2^- is usually oxidised to NO_3^- or reduced by denitrifiers. It rarely accumulates. This limited NO_2^- availability might restrict nitrifier denitrification in soils compared to pure culture studies and could explain observed differences.

In the fertilisation experiments, the total N_2O production increased with increasing N addition. This coincided with a larger contribution of nitrifier denitrification to the total N_2O production, especially after application of artificial urine. This is in line with the results from Koops et al. (1997). The clear reaction of nitrifier denitrification to application of artificial urine could hint at the importance of NO_2^-. Larger concentrations of artificial urine led to larger NO_2^- concentrations in the soil. NO_2^- concentrations in soil are usually small, but can

Table 7: N_2O production (nmol N_2O-N kg^{-1} h^{-1}) by different sources in a fertilisation experiments with mineral N fertilisers (means and standard deviations, n=4). There were no significant differences (α=0.05) between treatments. The second part of the table shows the results (p-values) of the Kruskal-Wallis test for data summerized per factor. Significant effects are marked with an asterisk. Ca_0: no liming, Ca_2: 4.6 t CaO ha^{-1} in 1981; N_1, N_2: 120 or 240 kg N ha^{-1} $year^{-1}$, respectively; AN: ammonium nitrate as fertiliser, CN: calcium nitrate as fertiliser. For further explanations see text.

Treatment	Nitrification	Nitrifier Denitrification	Denitrification	Other Sources	Total Production
Ca_0 N_1AN	9 ± 19	24 ± 78	-2 ± 10	7 ± 5	38 ± 75
Ca_0 N_1CN	-4 ± 6	0 ± 20	1 ± 16	8 ± 5	8 ± 11
Ca_0 N_2AN	3 ± 8	23 ± 78	8 ± 29	9 ± 3	44 ± 72
Ca_0 N_2CN	64 ± 125	-104 ± 189	95 ± 86	7 ± 4	62 ± 112
Ca_2 N_1AN	8 ± 18	-4 ± 55	21 ± 13	11 ± 6	36 ± 50
Ca_2 N_1CN	45 ± 92	-193 ± 292	141 ± 278	11 ± 2	4 ± 3
Ca_2 N_2AN	-14 ± 63	-93 ± 142	79 ± 141	39 ± 62	11 ± 3
Ca_2 N_2CN	7 ± 15	53 ± 136	2 ± 27	16 ± 5	78 ± 132
Ca_0 against Ca_2	0.678	0.851	0.546	0.018*	0.407
N_1 against N_2	0.451	0.386	0.792	0.522	0.032*
AN against CN	0.821	0.851	0.851	0.678	0.792

increase after urine or manure addition. Thus, nitrifier denitrification could be an important source of N_2O on grazed or manure-fertilised grasslands. The total N_2O production is known to be larger from urine patches than from the surrounding soil (Oenema et al., 1997).

Negative fluxes

Many negative values were calculated for sources of N_2O (Tables 4, 5, and 7). One would suggest that these negative values indicate consumption of atmospheric N_2O by the soil as for example described by Freney et al. (1978). However, other possibilities for the negative values cannot be excluded. We believe that small fluxes, large spatial heterogeneity and the inhibitors themselves cause possible artifacts.

Small fluxes can cause problems when measurements have to be carried out close to the instruments' detection limits. Resulting measurement errors propagate when data are used in calculations. This might explain the higher frequency of negative data in the two-step calculation of nitrifier denitrification (Equation 3). When fluxes were small, N_2O from ambient air left after flushing with O_2 might have had an influence. However, in the experiments where measurements were done more than twice during the incubation time, the N_2O production was generally found to proceed linearly, also in incubations with O_2. Thus, incomplete removal of N_2O can be excluded as an important source of negative data in treatments with small N_2O production.

In studies of N_2O emissions from grasslands, problems due to large spatial variability are often encountered (see for example Dendooven et al., 1999, Velthof et al., 2000). In experiments 1, 2 and 3, where homogenised soil samples were used to account for this problem, the spatial heterogeneity should be negligible. However, the frequency with which negative values were calculated in these experiments was similar to that in experiment 4.

Incomplete inhibition

In most cases, the inhibitors decreased the N_2O production (Table 8). However, sometimes the N_2O production was larger in incubations with C_2H_2 and in incubations with O_2 than in the control incubations. In 47% of all cases, the application of a single inhibitor decreased the N_2O production more than the application of a combination of C_2H_2 and O_2. Conditions promoting diffusion of gases (and therefore of inhibitors) in soil, like drier soil or lighter soil texture did not lead to a better performance of the inhibitors. Possible scenarios of problems caused by the inhibitors are summarised in Table 9 and are now discussed in more detail.

Nitrification is an aerobic process and might have been stimulated by the addition of O_2. This would lead to an overestimation of nitrification as a source of N_2O and consequently to an underestimation of nitrifier denitrification (see Equation 3 and Table 9a). Other studies have reported that O_2 can stimulate ammonia oxidation, but at the same time suppresses the N_2O production by nitrifiers (Jørgensen et al., 1984). Thus, a stimulation of nitrification by O_2 might be compensated for by smaller N_2O production per unit of NH_3 nitrified. However, the effect of 100 kPa O_2 on nitrification has not been studied yet. Such large concentrations of O_2 might also suppress nitrification and other processes due to the large oxidative stress.

Table 8: Average N_2O production with inhibitors in 24 h of incubation, expressed in percent of the N_2O production in the control. For an explanation of the experiments and treatments see text. A: with acetylene, O: with oxygen, AO: with acetylene and oxygen.

Treatment	A [% C]	O [% C]	AO [% C]
water content experiment:			
soil moist	74	53	34
soil wet	3512	65	210
soil texture experiment:			
sand	101	86	111
clay	87	36	25
artificial urine experiment:			
Urine 0	75	40	79
Urine ½	75	53	44
Urine 1	99	47	51
mineral fertiliser experiment:			
Ca_0 N_1AN	13	43	19
Ca_0 N_1CN	104	85	91
Ca_0 N_2AN	40	29	21
Ca_0 N_2CN	165	114	12
Ca_2 N_1AN	90	53	31
Ca_2 N_1CN	3501	1295	261
Ca_2 N_2AN	1092	233	359
Ca_2 N_2CN	23	29	20

Incomplete suppression of nitrifier denitrification by O_2 might be another possible cause for the observed values (Table 9b). So far, total inhibition of nitrifier denitrification by large concentrations of O_2 has not been verified. Dundee and Hopkins (2001) still found production of N_2O with 100 kPa O_2 in pure cultures of *Nitrosomonas europaea* and *Nitrosolubus*

multiformis, but did not check whether this was only due to nitrification. Incompletely blocked nitrifier denitrification could have been a source of N_2O in incubations with O_2. N_2O production by nitrification would then have been overestimated. This would lead to an underestimation of N_2O production from nitrifier denitrification (Table 9b).

Table 9: Possible problems caused by the inhibitors C_2H_2 and O_2 and effects on the N_2O production rates. +: overestimation; -: underestimation; 0: no effect.

Possible problems caused by the inhibitors O_2 and C_2H_2	**Effect on calculated N_2O production rates**			
	Nitrification	**Nitrifier denitrification**	**Denitrification**	**Other Sources**
a) Stimulation of nitrification by O_2	+	-	0	0
b) Incomplete suppression of nitrifier denitrification by O_2	+	-	0	0
c) Incomplete suppression of denitrification by O_2	0	0	-	+
d) Incomplete inhibition of ammonia oxidation by C_2H_2	-	-	+	+
e) Inhibition of N_2O reductase in denitrifiers by C_2H_2	0	-	+	0

Incomplete suppression of denitrification by O_2 (Table 9c) could significantly influence the results due to the often observed importance of denitrification for N_2O production (Bollmann and Conrad, 1998). Since denitrification is an anaerobic process, it is supposed to be inhibited by large O_2 concentrations. However, such an inhibition might only stop the production of new enzymes, without affecting the reactions carried out by enzyme that is already present (Zumft, 1997). Furthermore, it is known that some heterotrophic bacteria are capable of aerobic denitrification (Robertson and Kuenen, 1984). Since denitrification potentially produces large amounts of N_2O, incomplete suppression of denitrification could result in large underestimations of denitrification and large overestimations of other sources (Table 9c).

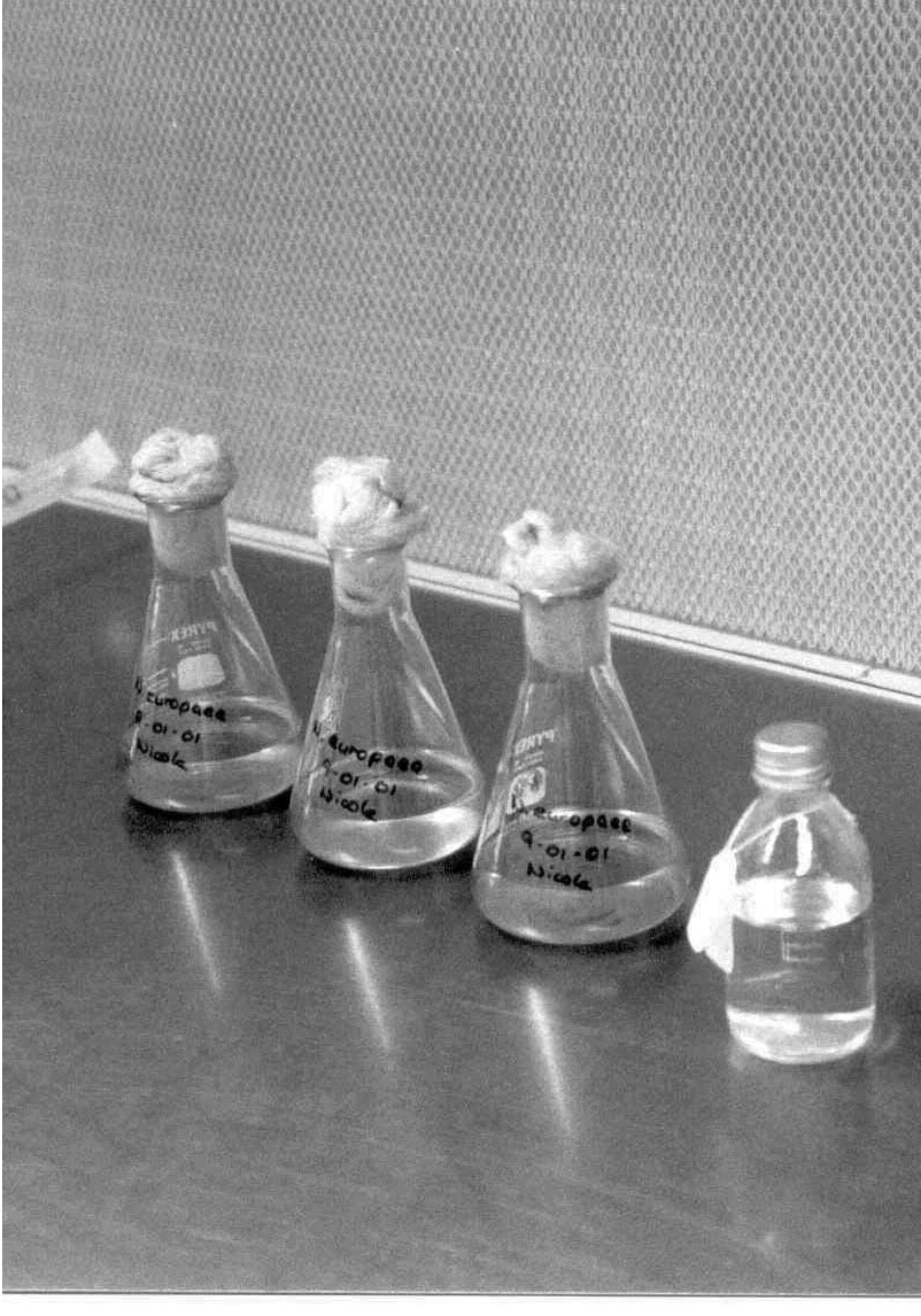
Surprisingly, the N_2O production sometimes seemed to be stimulated by addition of C_2H_2 or O_2 (Table 8). For C_2H_2, two artifacts might contribute to this stimulation: Incomplete inhibition of ammonia oxidation by C_2H_2 or a suppression of the N_2O reductase in denitrifiers. Incomplete inhibition of ammonia oxidation would lead to an underestimation of nitrification and nitrifier denitrification and an overestimation of denitrification and other sources (Table 9d). However, this artifact should not lead to increased values of N_2O production in

incubations with C_2H_2 compared to the controls. Inhibition of N_2O reductase would lead to a potentially large underestimation of nitrifier denitrification and overestimation of denitrification (Table 9e). The N_2O production in incubations with C_2H_2 could be larger than that in the controls. The enzyme N_2O reductase is normally not affected by small concentrations of C_2H_2. However, Ryden et al. (1979) found a beginning inhibition of this enzyme at C_2H_2 concentrations as small as 0.01 kPa. Inhibition of N_2O reductase can lead to much larger values of N_2O production in incubations with C_2H_2 (Ryden et al., 1979, Ryden and Dawson, 1982). For O_2, reasons for stimulated N_2O production are less clear. Robertson and Tiedje (1987) also found a stimulation of N_2O production by O_2 in one of the soils they studied. They suggested that this was due to an inhibition of N_2O reduction to N_2 by denitrifiers. N_2O might in that soil have been produced by other sources than nitrification and denitrification, since a significant portion of N_2O production was not inhibited by either C_2H_2 or O_2. In our study, stimulation of N_2O production by O_2 did not always coincide with a large N_2O production by other sources, i.e. sources not derived from nitrifiers or denitrifiers.

Evidently, the effects of the inhibitors may be more complex than initially thought. We believe that most of the calculated negative values do not indicate sinks for N_2O, but are a result of inhibition and analytical artifacts. Other studies that have used this inhibition method also discuss possible problems with the inhibition (Robertson and Tiedje, 1987, Webster and Hopkins, 1996a). Webster and Hopkins (1996a) discuss the possibility of an overestimation of nitrification and an underestimation of nitrifier denitrification and denitrification. However, since the method has never been applied as extensively as in this study, the limits of the method have never been seen so clearly.

Conclusions

This investigation revealed possible problems and uncertainties concerning the use of O_2 and C_2H_2 as inhibitors in soils. As a consequence, no clear statement can be made about the importance of different N_2O producing processes in soil based on the experiments carried out. However, some trends could be found. Generally, the availability of NO_2^- stimulated the N_2O production by nitrifier denitrification. More anoxic conditions generally decreased N_2O production by nitrifier denitrification in this study. Together with O_2, the availability of NO_2^- might be an important regulator of nitrifier denitrification in soils. More studies are needed to understand the controlling factors of nitrifier denitrification in soils. However, the inhibitors C_2H_2 and O_2 should first be tested thoroughly on their effects on different N_2O producing processes. Tests could for example be done in pure cultures of microorganisms to enable a differentiation of the inhibitors' effects on different groups of microorganisms.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CHAPTER FOUR

EFFECTIVENESS OF ACETYLENE AND OXYGEN AS INHIBITORS OF NITROUS OXIDE PRODUCTION IN *NITROSOMONAS EUROPAEA* AND *NITROSOSPIRA BRIENSIS*

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Autotrophic ammonia-oxidizing bacteria produce nitrous oxide (N_2O) as a by-product of nitrification or as an intermediate in nitrifier denitrification. In soil incubations, acetylene (C_2H_2) and oxygen (O_2) are used to distinguish between these sources. C_2H_2 inhibits ammonia oxidation and should therefore inhibit both N_2O production by nitrification and by nitrifier denitrification. O_2 suppresses the reduction pathway nitrifier denitrification. However, doubts concerning the reliability of C_2H_2 and O_2 as inhibitors have arisen recently. Therefore, we tested in this study the influence of C_2H_2 and O_2 alone and in combination on the N_2O production in pure cultures of the ammonia oxidizers *Nitrosomonas europaea* and *Nitrosospira briensis*.

C_2H_2 inhibited the nitrite production in both ammonia oxidizers and the N_2O production in *N. europaea*. Surprisingly, it did not affect the N_2O production in *N. briensis*. The variable response of ammonia oxidizers to C_2H_2 might have consequences for the use of C_2H_2 as an inhibitor of nitrification in soils.

Different partial pressures of O_2 (suboxic to 100 kPa O_2) were tested for their effectiveness in inhibiting N_2O production via nitrifier denitrification. A partial pressure of 100 kPa O_2 yielded minimal N_2O production by both ammonia-oxidizing species and seemed to effectively inhibit N_2O emission from nitrifier denitrification. However, a negative effect of 100 kPa O_2 on ammonia oxidation itself could not be excluded. Nitrifier denitrification was the main source of N_2O in both ammonia oxidizers under the conditions studied.

Introduction

Nitrification is one of the microbial processes leading to the production of nitrous oxide (N_2O) in soils. N_2O is an important greenhouse gas with a global warming potential of 320 relative to CO_2 (IPCC, 1996). In the stratosphere, it is converted to nitric oxide (NO), which plays a role in the destruction of the ozone layer (Crutzen, 1981).

Autotrophic ammonia-oxidizing bacteria produce N_2O as a by-product of nitrification or as an intermediate of nitrifier denitrification. As a by-product of nitrification, N_2O is formed during the spontaneous decomposition of intermediates of ammonia oxidation, e.g. hydroxylamine (NH_2OH) and nitric acid, under acid conditions (Chalk and Smith, 1983). Furthermore, incomplete oxidation of NH_2OH might lead to the development of N_2O during nitrification (Hooper and Terry, 1979). In nitrifier denitrification or autotrophic denitrification, ammonia oxidizers first oxidize ammonia (NH_3) to nitrite (NO_2^-) and subsequently reduce this NO_2^- to molecular nitrogen (N_2) (Poth and Focht, 1985, Poth, 1986, Wrage et al., 2001). N_2O can be released here as an intermediate in the reduction pathway from NO_2^- to N_2 (Poth et al., 1986, Muller et al., 1995).

Soils are the main site of N_2O production, and in the literature, various measures are discussed to minimize the emission of N_2O from soils (Granli and Bøckman, 1994, Mosier, 1994, Oenema et al., 1998). However, mitigation options for N_2O can only be evaluated with profound knowledge of all production pathways and their mutual interactions. Nitrification and denitrification have both been suggested to be dominant sources of N_2O in soils in different environmental conditions (Granli and Bøckman, 1994). Good understanding of the different sources of N_2O in nitrifiers is still lacking, but essential for choosing the right mitigation options.

Soil incubations with combinations of small concentrations of acetylene (C_2H_2) and large concentrations of molecular oxygen (O_2; 100 kPa) have been used to differentiate between nitrification, denitrification, nitrifier denitrification and other sources of N_2O production in soils in order to estimate their importance under various conditions (Robertson and Tiedje, 1987, Webster and Hopkins, 1996a; Table 10). This inhibition method has been used repeatedly in soil incubation studies (Robertson and Tiedje, 1987, Webster and Hopkins, 1996a, Wrage et al., 2000). However, the inhibitors did not always cause the expected reduction in the production of N_2O compared to the controls. Sometimes, the production of N_2O was even larger after addition of the inhibitors (Wrage et al., 2000).

Table 10: Incubation method used (modified after Webster and Hopkins, 1996a). C_2H_2: acetylene, O_2: oxygen, +: process can take place, -: process is inhibited.

Process	**C** (control)	**A** (+ 0.02 kPa C_2H_2)*	**O** (+ 100 kPa O_2)*	**AO** (+ 0.02 kPa C_2H_2 + 100 kPa O_2)*
nitrification	+	-	+	-
nitrifier denitrification	+	-	-	-
denitrification	+	+	-	-
other sources	+	+	+	+

* 1 kPa ≈ 1% (v/v)

C_2H_2 is often used as an inhibitor in soils (e.g. de Boer et al., 1992, Lång et al., 1993, Zhu and Carreiro, 1999). It has been shown to inhibit nitrification already at small concentrations (0.1-10 Pa) (Berg et al., 1982). However, problems with the use of small concentrations of C_2H_2 have repeatedly been encountered. Thus, small concentrations of C_2H_2 did not always inhibit nitrification in soils (Lång et al., 1993, Garrido et al., 2000). In some cases, this might have been due to the presence of heterotrophic nitrification, which is not sensitive to C_2H_2 (Lång et al., 1993, Richardson et al., 1998). However, an only 50% inhibition of ammonia mono-oxygenase with 25 Pa C_2H_2 was also found in a laboratory experiment with cell-free extracts of the autotrophic nitrifier *Nitrosomonas eutropha* (Schmidt and Bock, 1998). Complete inhibition required addition of 80 Pa C_2H_2, which is four to eight times more than normally used in soil incubations. So far, very few studies have systematically checked how reliable C_2H_2 is as an inhibitor of ammonia oxidation.

O_2 is an important factor influencing microbial reactions. Nitrification depends on sufficient amounts of O_2, while denitrification mainly takes place when O_2 is limiting (Granli and Bøckman, 1994). Artificially applied large partial pressures of O_2 (100 kPa, i.e. approximately 100% v/v) are used to inhibit nitrifier denitrification and heterotrophic denitrification in soils (Robertson and Tiedje, 1987, Webster and Hopkins, 1996a). Concerns have been raised that O_2 might not be reliable and specific enough as an inhibitor (Dundee and Hopkins, 2001). Thus, aerobic denitrification, or incomplete penetration of O_2 into the soil could influence the results (Dundee and Hopkins, 2001). Furthermore, studies of cell-free extracts of *Nitrosomonas eutropha* suggest that ammonia monooxygenase is also negatively affected by O_2; a partial inhibition occurred already at ambient concentrations of O_2 (Schmidt and Bock, 1998).

In this study, we investigated the effects of C_2H_2 and O_2 on NO_2^- and N_2O production by ammonia oxidizers. To simplify the system, pure cultures of ammonia oxidizers were used. Combinations of O_2 and C_2H_2 according to the method of Webster and Hopkins (1996a) (Table 10) were tested. This should show whether the concentrations of C_2H_2 normally applied in soils can inhibit ammonia oxidation and whether C_2H_2 and O_2 interfere when used in combination. In a second experiment, different partial pressures of O_2 (suboxic, 20, 40, 60, 80, and 100 kPa O_2) were tested for their effectiveness in inhibiting N_2O production by nitrifier denitrification. NO_2^- was measured as an indicator for influences of large partial pressures of O_2 on ammonia oxidation itself. The ammonia oxidizers *Nitrosomonas europaea* and *Nitrosospira briensis* were used in both experiments to account for differences between species of ammonia oxidizers. *N. europaea* is able to grow fast under optimal conditions. It is often used as a model organism in laboratory studies and has frequently been found in environments high in N like water treatment plants (Kowalchuk and Stephen, 2001). *N. briensis* is better adapted to environments less abundant in N and is common in a number of arable soils of neutral pH receiving fertilizers (Kowalchuk and Stephen, 2001).

Material and Methods

Microorganisms

The ammonia oxidizers used were *Nitrosomonas europaea* (ATCC 19178) and *Nitrosospira briensis* (ATCC 25971). The cultures were grown on a mineral medium containing (per liter) 660 mg of $(NH_4)_2SO_4$, 585 mg of NaCl, 49 mg of $MgSO_4 \times 7\ H_2O$, 147 mg of $CaCl_2 \times 2\ H_2O$, 75 mg of KCl, 54 mg of KH_2PO_4, 10 g of Hepes, and 1 ml trace element solution (after Verhagen and Laanbroek, 1991). As a pH indicator, 5 ml/l of a 0.4 % (w/v) bromothymol blue stock solution was added. The pH was adjusted to 7.8 with 5 M NaOH before autoclaving (121°C, 30 minutes). *N. europaea* was grown on this medium for 7 days, *N. briensis* for 14 days, due to a lower specific growth rate. The growing cultures were kept at 20°C in the dark. Shaking of the cultures was not considered necessary since the surface-volume ratio in the incubation bottles was high (100 ml culture in 250 ml Erlenmeyer flasks).

The ammonia oxidizers were harvested by centrifugation (15 000 RPM, 15 minutes, 4°C) and washed once. They were quantified by measuring the optical density at 436 nm (spectrophotometer model 100-20 by Hitachi Scientific Instruments, Tiel, The Netherlands). The results of this quantification method were consistent with counting after DAPI (4,6-diamidino-2-phenylindol) staining. This was tested with dilutions of 1- and 3-week old

cultures of both *N. europaea* and *N. briensis* ($r^2 = 0.99$ for 0 to 3×10^7 *N. europaea* cells per ml, $r^2 = 0.96$ for 0 to 1.5×10^7 *N. briensis* cells per ml).

General set-up of incubation experiments

The general set-up of the incubation experiments was as follows. Serum bottles (250 ml) with 10 ml mineral medium (see above) without KH_2PO_4 were autoclaved (121°C, 30 minutes). While still hot, they were closed to create negative pressure in the bottles during cooling. Then, the headspace was filled up with the gas that formed the main constituent of the respective incubation atmosphere (see below). When the headspace was filled (recognizable by the movement of the septa and the decrease of gas flow to the bottles), flushing was continued for another 10 seconds (flux: 11 min^{-1}) by piercing a second needle through the septa. This flushing method was chosen as a compromise between methods used in soil incubation studies (e.g. Wrage et al., 2000, Estavillo et al., 2002) and those normally used in pure culture studies (e.g. Kester et al., 1997, Dundee and Hopkins, 2001, Schmidt et al., 2001b). The incubation atmospheres were completed by adding other gases as appropriate (see below). To avoid contamination with other microorganisms, gases (including flushing) were added via 0.2 µm filters (disposable filter unit red rim, Schleicher & Schuell GmbH, Dassel, Germany). After addition of all gases, the septa of the serum bottles were briefly pierced to make sure that no pressure had built up in the bottles. The harvested and washed microorganisms were added to these prepared bottles by syringe. The bottles were then incubated at 20°C in the dark. In both experiments, all treatments were carried out with four replicates. On top of that, both experiments were repeated three times to make sure that the observed trends were reproducible.

Gas measurements were carried out after the addition of microorganisms to the bottles and again after 24 hours. The same time periods are normally used in soil incubation studies (Wrage et al., 2000, Estavillo et al., 2002). Preliminary experiments had shown the gas production in the cultures to proceed linearly in this time period (r^2 between 0.97 and 0.99). N_2O was analyzed in 5 ml samples with a gas chromatograph with electron capture detector (PU 4400 Unicam Analytical Systems, Philips, The Netherlands). The system was calibrated with 4.5 µl l^{-1} N_2O in N_2 (Hoek Loos, Schiedam, The Netherlands). The detection limit was 8 nl l^{-1}. Preliminary tests had shown that multiple gas samples up to a total of 40 ml could be taken from 250 ml serum bottles without influencing the linearity of gas measurements.

The culture media were analyzed colorimetrically for nitrite (NO_2^-), nitrate (NO_3^-) and ammonium (NH_4^+) (Houba et al., 2000). To this end, samples of 1.5 ml of the culture medium were taken from each bottle at the beginning and end of the inhibition experiment. To inhibit

microbial activity in the time before these samples could be analyzed, they were centrifuged (15 000 RPM, 15 minutes) and the supernatants frozen (-13°C).

Inhibition experiments

Influence of combinations of O_2 and C_2H_2

To test for the influence of O_2 and C_2H_2 alone and in combination, pure cultures of *N. europaea* and *N. briensis* were incubated with 100 kPa O_2 and 0.02 kPa C_2H_2 as shown in Table 10. For the control treatment (C) and the treatment with 0.02 kPa C_2H_2 (A), the incubation bottles were flushed with N_2. O_2 and CO_2 were added to reestablish ambient concentrations. The bottles for the treatments with 100 kPa O_2 (O) and with 100 kPa O_2 plus 0.02 kPa C_2H_2 (AO) were flushed with O_2, and CO_2 was added to reestablish ambient concentrations. C_2H_2 was added to treatments A and AO.

Influence of elevated partial pressures of O_2

To test for the influence of elevated partial pressures of O_2 on ammonia oxidizers, pure cultures of *N. europaea* and *N. briensis* were incubated under 6 different incubation atmospheres. The incubation atmospheres contained 20, 40, 60, 80 or 100 kPa O_2 in N_2. Furthermore, flushing with N_2 created a suboxic treatment. All treatments were supplied with CO_2 to reestablish ambient air concentrations. The partial pressures of O_2 given are only approximate to the conditions in the bottles, due to the flushing method, use of O_2 by ammonia oxidizers during the incubation, and diffusion constraints of gases in liquids.

Statistics

All experiments were set up with four replicates per treatment. Changes in gas concentration and mineral N concentrations were calculated per bottle by subtracting the amount measured at time 0 from that measured after 24 hours. Results are presented as means with standard deviations (n = 4). Since the standard deviations appeared to be very large, every experiment was repeated three times. These repetitions were also analyzed statistically. Treatment effects within every repetition and between repetitions were analyzed statistically with SPSS for Windows (Norusis, 1986) and with Excel. Gaussian distribution of the data and homogeneity of variance were tested with Kolmogorov-Smirnov's and Levene's tests, respectively. When the data was not distributed normally, the Kruskal-Wallis test was used to find differences between treatments. In case of significant effects (α = 0.05), Schaich-Hamerle analysis was used for multiple comparison of means. When the data followed a normal distribution, comparisons between treatments were done using ANOVA (α = 0.05)

Table 11: Amounts of N_2O and NO_2^- produced by *Nitrosomonas europaea* and *Nitrosospira briensis* in incubations with C_2H_2 and O_2. The experiment was repeated three times with each ammonia oxidizer. Shown are means and standard deviations (n = 4) of every repetition of the experiment. Results of the statistical analyses are shown as superscript letters for each repetition ($\alpha = 0.05$). Different letters in columns 'r.e.' (= repetition effects) show differences between repetitions, those in rows 'treatment effect' show differences between treatments for pooled data from the three repetitions. C: control; A: with 0.02 kPa C_2H_2; O: with 100 kPa O_2; and AO: with both 0.02 kPa C_2H_2 and 100 kPa O_2.

	N_2O [fmol cell^{-1} 24h^{-1}]					**NO_2^- [pmol cell^{-1} 24h^{-1}]**				
	Nitrosomonas europaea					***Nitrosomonas europaea***				
repetition	**C**	**A**	**O**	**AO**	**r.e.**	**C**	**A**	**O**	**AO**	**r.e.**
1	128±69 ^{a}	39±2 ^{ab}	30±10 ^{ab}	30±2 ^{b}	a	18.5±13.8 ^{a}	1.1±0.2*^{ab}	7.7±2.7 ^{a}	1.1±4.5*^{b}	a
2	60±38 ^{a}	30±6 ^{a}	23±23*^{ab}	8±4*^{b}	ab	1.7±10.2 ^{a}	0.5±0.3*^{a}	6.4±4.2 ^{a}	-0.1±0.2*^{a}	a
3	188±123 ^{a}	16±15*^{ab}	-5±32*^{ab}	-16±4*^{b}	b	32.0±25.5 ^{a}	0.6±0.1*^{a}	13.3±9.0 ^{a}	0.4±0.2*^{a}	a
treatment effect	a	b	b	b		a	ab	a	b	
	Nitrosospira briensis					***Nitrosospira briensis***				
1	89±29 ^{a}	89±20 ^{a}	20±36*^{b}	22±25*^{b}	a	2.8±2.6 ^{a}	0.2±1.7*^{a}	2.3±1.8 ^{a}	1.4±0.3*^{a}	a
2	33±10 ^{a}	32±7 ^{a}	5±3*^{b}	9±5*^{b}	a	10.4±4.4 ^{a}	0.2±0.2*^{b}	7.7±1.4 ^{a}	0.5±0.1*^{b}	a
3	68±6 ^{a}	53±5 ^{a}	24±18*^{b}	21±9*^{b}	a	5.7±5.8 ^{a}	0.8±0.2*^{a}	5.7±5.8 ^{a}	0.9±0.1*^{a}	a
treatment effect	a	a	b	b		a	b	a	ab	

* Asterisks indicate values that were below or not significantly different from the detection limit. Differences in the detection limit between repetitions are due to variable numbers of cells in the experiment and the expression of the values per cell here.

with subsequent LSD test as post-hoc test. When variances were not homogeneous, the data was transformed before ANOVA. In most cases, ANOVA was used to compare means between replicates within each repetition of the experiments, while the Kruskal-Wallis test was used to compare means between repetitions.

Results

Influence of combinations of O_2 and C_2H_2

The ammonia oxidizers *N. europaea* and *N. briensis* produced comparable amounts of N_2O in the control treatments (Table 11). The production tended to be somewhat larger for *N. europaea* than for *N. briensis* (not significant). The two ammonia oxidizers reacted differently to the inhibitors.

N. europaea was clearly influenced by all added inhibitors (Table 11). In the pooled data of the three repetitions, the N_2O production was significantly lower in the treatments with presence of inhibitors (A, O, and AO) than in the control treatments ($p \leq 0.001$, Kruskal-Wallis test). In treatments A, O, and AO, the amount of N_2O produced was close to -or below- the detection limit. The production did not differ significantly between these three treatments.

In contrast to *N. europaea*, *N. briensis* did not react to C_2H_2 addition with a decrease in N_2O production (Table 11). The production in treatment A was not significantly different from that in the controls. In treatments O and AO, the N_2O production by *N. briensis* was significantly decreased compared with the controls ($p \leq 0.001$, Kruskal-Wallis test). The production in these treatments was again below or not significantly different from the detection limit.

The pattern of NO_2^- production was comparable for *N. europaea* and *N. briensis* (Table 11), but the amounts of NO_2^- produced by *N. briensis* were at the lower end of the range observed for *N. europaea*. Both ammonia-oxidizing species produced NO_2^- in treatments C and O and did not produce NO_2^- in the presence of C_2H_2. For both ammonia oxidizers, the NO_2^- production in treatment O was not significantly different from that in the controls. However, *N. europaea* showed a slight trend to lower NO_2^- production in treatment O. NO_3^- could not be detected. Only very little NH_4^+ was consumed during the incubation period, so that changes in NH_4^+ concentrations could not be measured against the large background concentrations (around 10 mM). The amount of N_2O produced in the control treatments was for both ammonia oxidizers between 0.3 and 3.5% of the amount of NO_2^- produced.

Influence of elevated partial pressures of O_2

The amounts of N_2O produced at different partial pressures of O_2 were comparable to those in the experiment with different inhibitors described above. Larger levels of O_2 significantly decreased the N_2O production by both *N. europaea* and *N. briensis* ($p \leq 0.001$, Kruskal-Wallis test; Table 12). While 80 kPa O_2 totally inhibited N_2O production in *N. europaea*, *N. briensis* still produced some N_2O in incubations with 100 kPa O_2.

Significant changes in NH_4^+ concentrations could not be measured. Neither was NO_3^- detected. For *N. europaea*, the NO_2^- production was largest in the treatment with 20 kPa O_2 and decreased with larger O_2 concentrations (Table 12). However, this trend was not significant. No trend could either be found for *N. briensis*, where the production of NO_2^- was low (Table 12). Generally, *N. briensis* and *N. europaea* produced similar amounts of N_2O per NO_2^- produced (1.33±0.72% for *N. briensis* as opposed to 0.98±1.06% for *N. europaea*). No significant trend in this percentage could be found with increasing O_2 concentrations.

Discussion

This study was carried out to investigate the influence of O_2 and C_2H_2 on ammonia oxidizers in pure culture. The experimental set-up was close to the set-up commonly used in soil incubations to allow comparison. Addition of C_2H_2 inhibited N_2O production to a large extent in *N. europaea*, while having only a minor effect on *N. briensis*.

Since C_2H_2 inhibits ammonia oxidation, it should stop N_2O production by nitrification as well as by nitrifier denitrification (Webster and Hopkins, 1996a, Table 10). Yet, significant amounts of N_2O were produced in incubations of *N. briensis* with C_2H_2. We can exclude chemical sources for the production of N_2O in experiments with *N. briensis*, as bottles incubated without ammonia oxidizers did not show N_2O production. Small amounts of NH_2OH may have been left in the incubated cells after washing. While this NH_2OH could have been a source of N_2O during the incubation, it is unlikely that it would yield amounts of N_2O similar to those in the control incubations, where NH_3 could be oxidized without constraints. We therefore have to conclude that *N. briensis* seems to be insensitive to C_2H_2 as far as N_2O production is concerned. To see whether larger concentrations of C_2H_2 could inhibit N_2O production by *N. briensis*, we tested C_2H_2 concentrations of up to 1 kPa. To date, a consistent inhibition of N_2O production by *N. briensis* at larger C_2H_2 concentrations could not be measured (unpublished results).

Table 12: Amounts of N_2O and NO_2^- produced by *Nitrosomonas europaea* and *Nitrosospira briensis* in incubations with different partial pressures of O_2 (suboxic, 20, 40, 60, 80, and 100 kPa O_2). The experiment was repeated three times with each ammonia oxidizer. Shown are means and standard deviations (n = 4) of every repetition. Results of the statistical analyses are shown as superscript letters for each repetition ($\alpha = 0.05$). Different letters in columns 'r.e.' (= repetition effects) show differences between repetitions, those in rows 't.e.' (= treatment effects) show differences between treatments for pooled data from the three repetitions.

	N_2O [fmol/(cell x 24h)]							**NO_2^- [pmol cell^{-1} 24h^{-1}]**						
	Nitrosomonas europaea							***Nitrosomonas europaea***						
repetition	**sub[1]**	**20**	**40**	**60**	**80**	**100**	**r.e.**	**sub[1]**	**20**	**40**	**60**	**80**	**100**	**r.e.**
1	123±64 ^{a}	88±12 ^{a}	53±47 ^{ab}	23±18* ^{b}	6±6* ^{c}	0±0* ^{c}	a	19±30 ^{a}	50±9 ^{a}	31±33 ^{a}	18±9 ^{a}	17±5 ^{a}	8±9 ^{a}	a
2	36±12 ^{a}	42±42 ^{a}	30±6 ^{ab}	18±6 ^{bc}	6±0* ^{c}	0±0* ^{d}	a	1±2 ^{a}	6±10 ^{a}	1±8 ^{a}	3±1 ^{a}	1±1* ^{a}	2±1 ^{a}	b
3	96±40 ^{a}	116±52 ^{a}	66±35 ^{ab}	28±11* ^{bc}	17±9* ^{cd}	8±11* ^{d}	a	14±17 ^{a}	28±14 ^{a}	13±8 ^{a}	3±10 ^{a}	9±4 ^{a}	7±5 ^{a}	a
t.e.	a	a	ab	abc	bc	c		a	a	a	a	a	a	
	Nitrosospira briensis							***Nitrosospira briensis***						
1	123±14 ^{a}	96±31 ^{a}	103±26 ^{a}	47±6 ^{b}	53±20 ^{b}	36±6 ^{b}	a	5±3 ^{a}	3±3 ^{a}	7±4 ^{a}	4±3 ^{a}	3±2 ^{a}	4±1 ^{a}	a
2	92±10 ^{a}	57±7 ^{bc}	70±14 ^{b}	59±3 ^{bc}	56±7 ^{c}	54±5 ^{c}	a	7±7 ^{a}	5±3 ^{a}	9±8 ^{a}	4±14 ^{a}	12±5 ^{a}	1±7* ^{a}	a
3	74±32 ^{abc}	50±8 ^{ab}	44±2 ^{a}	31±3* ^{bc}	30±2 ^{bc}	24±5* ^{c}	b	3±3 ^{a}	7±2 ^{a}	5±3 ^{a}	6±1 ^{a}	8±2 ^{a}	4±2 ^{a}	a
t.e.	a	ab	ab	b	b	b		a	a	a	a	a	a	

[1] sub = suboxic; the bottles were flushed with nitrogen for the incubation, so that only traces of oxygen were left.

* Asterisks indicate values that were below or not significantly different from the detection limit. Differences in the detection limit between repetitions are due to variable numbers of cells in the experiment and the expression of the values per cell here.

This study seems to be the first one where the effect of C_2H_2 on N_2O production by *N. briensis* is tested. In an isolate of *N. briensis* from a Finnish ombrotrophic peat soil, 1 kPa C_2H_2 successfully inhibited ammonia oxidation measured as NO_2^- production (Jiang and Bakken, 1999). Unfortunately, N_2O production was not measured in that study. In contrast to N_2O production, the production of NO_2^- by *N. briensis* in our experiment was completely inhibited by C_2H_2 (detection limit: 3 μmol l^{-1}). We do not know yet how the N_2O production could proceed while NO_2^- production ceased. The current thinking of C_2H_2 inhibition of ammonia oxidation suggests that C_2H_2 interferes with the oxidation of NO to NO_2, the direct oxygen source for ammonia oxidation (Schmidt et al., 2001a). Then, both N_2O and NO_2^- production should be inhibited by C_2H_2. More investigations into the nature and operation of the enzymes involved are needed.

Insensitivity by *N. briensis* to C_2H_2 in terms of N_2O production would have severe implications for the use of C_2H_2 as an inhibitor of N_2O production by ammonia oxidizers in soils. In most soils, *Nitrosospira* species are the most common ammonia oxidizers (Kowalchuk and Stephen, 2001). Insensitivity to C_2H_2 might be more widespread in this group of ammonia oxidizers. Thus, in another study (Schmidt and Bock, 1998), problems with the inhibition of ammonia oxidation in *N. eutropha* were encountered. The lack of response of some ammonia oxidizers to C_2H_2 might be responsible for the observed problems with C_2H_2 inhibition of N_2O production by nitrification in soils (e.g. Wrage et al., 2000). So far, continued nitrification in incubations with C_2H_2 has been assigned to heterotrophic nitrification (e.g. Lång et al., 1993). Following the results from this study, N_2O production after application of C_2H_2 might also be due to autotrophic ammonia oxidizers.

Our results indicate that the measurement of only N_2O production in soil incubation studies with small concentrations of C_2H_2 might be misleading when some autotrophic ammonia oxidizers produce N_2O irrespective of C_2H_2. If the inhibition of NO_2^- production by C_2H_2 as observed in *N. briensis* is universal for ammonia oxidizers, measurements of the changes in the substrate and products of nitrification, NH_3, NO_2^- and NO_3^-, in incubations with C_2H_2 might help to differentiate between the sources of N_2O production. While a need for more research on effects of C_2H_2 on different ammonia oxidizers remains, we can conclude that for the time being it is better to measure changes in NH_3, NO_2^- and NO_3^- as well as N_2O production if statements about nitrification are to be made based on incubations with C_2H_2.

Large partial pressures of O_2 inhibited the N_2O production in both ammonia oxidizers studied. The inhibition of N_2O production was complete in *N. europaea* at 80 kPa O_2, while *N. briensis* still produced some N_2O with 100 kPa O_2. We conclude that partial pressures of less

than 100 kPa O_2 are probably not sufficient to inhibit nitrifier denitrification in soils. It is very difficult –if not impossible– to establish such large concentrations of O_2 in soil incubations. Even after extensive flushing, there might still be microhabitats, especially inside aggregates, with smaller O_2 concentrations. These problems might be more serious in wet soils and undisturbed soil columns than in dry and crumbled soil. Furthermore, such large partial pressures of O_2 might have negative effects on ammonia oxidation itself and on other soil processes. When 100 kPa O_2 is to be used in soils to differentiate between sources of N_2O, it is a prerequisite that only autotrophic and heterotrophic denitrification are inhibited, and not nitrification itself. In cell-free extracts, a sensitivity of ammonia monooxygenase to ambient concentrations of O_2 was found (Schmidt and Bock, 1998). Our results with pure cultures of nitrifiers do not support this finding. The NO_2^- production did not differ significantly between treatments with different O_2 concentrations. However, at O_2 concentrations larger than ambient, *N. europaea* tended to produce less NO_2^-, suggesting that ammonia oxidation in this organism was negatively affected by large O_2 concentrations. The NO_2^- production in *N. briensis* was not influenced by different partial pressures of O_2. Thus, the ammonium oxidation in this organism does not seem to have been negatively affected by large concentrations of O_2.

The variation between replicates and between repetitions was large, although all experiments were carried out in a similar way and under semi-controlled conditions. The microorganisms were always grown under constant conditions and for the same time before starting an experiment. However, the numbers of microorganisms used for the experiments varied between 5 and 17 x 10^6 cells per liter. Furthermore, the cultures might have been in different growth phases. This may have influenced the speed with which they could start to grow and produce gases at the beginning of the experiment. Due to methodological constraints, gas measurements could only be carried out twice during the incubation time. If the N_2O production did not in all cases proceed linearly between these measurements, this will have contributed to the large variability. However, the trends observed were the same in the three repetitions of the experiments, and the amounts of N_2O and NO_2^- produced did not differ significantly between the repetitions of the experiments in most cases.

To differentiate between the sources of N_2O in the two ammonia oxidizers studied, the following equations have been proposed (after Webster and Hopkins, 1996a):

$$N_2O_{Nitrification} = N_2O_O - N_2O_{AO} \qquad \textbf{Equation 5}$$

$$N_2O_{\text{Nitrifier Denitrification}} = N_2O_C - N_2O_O \qquad \textbf{Equation 6}$$

'N_2O' is the amount of N_2O produced, and the subscripts on the right-hand side of the equations refer to the treatments as shown in Table 10. Thus, the importance of nitrifier denitrification can be derived from the difference in gas production between treatments C and O. The gas produced by nitrification can at the most be as large as the amount produced in treatment O. If no production could be measured in treatment O, we assumed a production equal to the detection limit for the calculation. However, we should keep in mind that ammonia oxidation might have been negatively affected by large O_2 concentrations, as discussed before. This means that the amount of N_2O produced by nitrification directly could be underestimated and the amount produced by nitrifier denitrification overestimated. Since the NO_2^- production did not differ significantly between the control treatments and those with 100 kPa O_2, the possible underestimation of nitrification (Equation 5) and overestimation of nitrifier denitrification (Equation 6) should be limited.

For both ammonia oxidizers, nitrifier denitrification was the most important source of N_2O. About 80% of the N_2O production in *N. europaea* was caused by nitrifier denitrification. *N. briensis* produced approximately 65% of the N_2O emitted via nitrifier denitrification. *N. europaea* did not only produce a larger part of its N_2O by nitrifier denitrification than *N. briensis*, but the absolute amount of N_2O produced by nitrifier denitrification was also larger for *N. europaea* (48-180 fmol N_2O $cell^{-1}$ $24h^{-1}$ by *N. europaea* as opposed to 21-61 fmol N_2O $cell^{-1}$ $24h^{-1}$ by *N. briensis*).

We can conclude that O_2 worked as an inhibitor of nitrifier denitrification in both ammonia oxidizers studied. Since concentrations of 100 kPa O_2 seem necessary to inhibit production of N_2O via nitrifier denitrification, the applicability of this method to soils might be limited. Furthermore, side effects of large concentrations of O_2 on ammonia oxidation and on other soil processes cannot be excluded. C_2H_2 effectively inhibited N_2O production in *N. europaea*, but not in *N. briensis*. The NO_2^- production was inhibited by C_2H_2 in both *N. europaea* and *N. briensis*. The lack of inhibition of N_2O production by C_2H_2 addition in *N. briensis* cannot be explained yet. Screening of different ammonia oxidizers is needed to clarify whether insensitivity to C_2H_2 is a more common phenomenon in ammonia oxidizers. Measurements of changes in the substrate and products of nitrification, NH_3, NO_2^- and NO_3^-, as well as of N_2O production might allow better estimates of nitrification than measurements of only one of the variables when soil incubations with C_2H_2 have to be carried out. Under the conditions studied, nitrifier denitrification was the main source of N_2O in both ammonia oxidizers.


PHILIPS

CHAPTER FIVE

NITROUS OXIDE PRODUCTION BY MUTANTS OF *NITROSOMONAS EUROPAEA* THAT ARE DEFICIENT IN NITRITE REDUCTASE OR NITRIC OXIDE REDUCTASE

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Ammonia-oxidizing bacteria produce nitrous oxide (N_2O) in nitrification during the oxidation of hydroxylamine (NH_2OH) and in nitrifier denitrification during the reduction of nitrite (NO_2^-). Inhibitions with small concentrations of acetylene (C_2H_2) and large concentrations of oxygen (O_2) have been used to differentiate between these two sources with varying success. C_2H_2 inhibits ammonia oxidation and therefore N_2O production from both nitrification and nitrifier denitrification, while 100 kPa O_2 should inhibit only nitrifier denitrification. We tested that method in incubations of the wild-type of *Nitrosomonas europaea*, one mutant of the same strain that was deficient in nitrite reductase (NirK) and one that was deficient in nitric oxide reductase (NORB). This should at the same time reveal possible problems with the incubation method and show which of the two pathways is more important for N_2O production by *N. europaea* under the studied conditions.

The results indicated that 100 kPa O_2 was not suitable as an inhibitor of nitrifier denitrification, since it also adversely affected ammonia oxidation. The NirK-deficient cells produced as much N_2O as the wild-type. This might indicate that the known pathway of nitrifier denitrification, where the first step is catalysed by NirK, is not so important in terms of N_2O production by ammonia oxidizers. Surprisingly, the NORB-deficient cells produced approximately 60 times more N_2O than the wild-type in control incubations with ambient O_2 concentrations. At the same time, a net consumption of NO_2^- took place. We suggest that the enzyme nitric oxide reductase, that is missing in this mutant, might direct ammonia oxidation towards NO_2^- rather than N_2O as an end product. Large concentrations of O_2 seem to fulfil the same role, since the N_2O production by NORB-deficient cells was in incubations with 100 kPa O_2 not different from that by the wild-type. The NORB-deficient cells were insensitive to C_2H_2. This might hint at a so far unidentified way of N_2O production in ammonia oxidizers. The unexpected results show that a lot of unknowns remain in the metabolism of ammonia oxidizers.

Introduction

Nitrous oxide (N_2O) is an important greenhouse gas (IPCC, 2001). Its main source is the soil (Bouwman, 1990), where ammonia-oxidizing and denitrifying bacteria are the most important producers (Granli and Bøckmann, 1994). Ammonia-oxidizing bacteria produce N_2O during nitrification and nitrifier denitrification (Figure 11A). In nitrification, N_2O is a by-product of ammonia oxidation. It can be formed by the enzyme hydroxylamine oxidoreductase during the oxidation of hydroxylamine (NH_2OH, Hooper and Terry, 1979). Furthermore, chemical production of N_2O from NH_2OH or NO_2^- produced in nitrification can occur in acid conditions (Chalk and Smith, 1983, van Cleemput and Baert, 1984). In nitrifier denitrification, ammonia oxidizers produce N_2O as an intermediate of the reduction of nitrite (NO_2^-) to molecular nitrogen (N_2) (Poth and Focht, 1985, Poth, 1986) (Figure 11A).

The importance of nitrification and nitrifier denitrification as respective sources of N_2O in ammonia oxidizers has recently been addressed in several studies. In an iron-podzol with sandy-loam structure, nitrifier denitrification was found to be responsible for up to 30% of the total N_2O production, while nitrification only accounted for up to 2% (Webster and Hopkins, 1996a). Generally, nitrifier denitrification is considered to be more important in suboxic conditions in the presence of NO_2^- (Poth and Focht, 1985, Kester et al., 1997). Beaumont et al. (2002) studied a mutant of *Nitrosomonas europaea* where the gene that encodes a copper-type nitrite reductase (NirK) had been disrupted (Figure 11B). Nitrite reductase is the enzyme that reduces NO_2^- to NO. If this reduction is the first step in the denitrification pathway of nitrifying bacteria, N_2O production by nitrifier denitrification should not be possible in this mutant. Nevertheless, the NirK-deficient cells produced more N_2O than wild-type cells. The authors therefore concluded that the nitrifier denitrification pathway via NirK is not essential for N_2O production in *N. europaea* (Beaumont et al., 2002). Wrage et al. (Chapter 4) approached the question of the N_2O-producing pathways in *N. europaea* using an incubation method developed by Webster and Hopkins (1996a). In sets of four incubations, combinations of small concentrations of acetylene (C_2H_2, 0.02 kPa) and large concentrations of oxygen (O_2, 100 kPa) were added to pure cultures of the ammonia oxidizers *N. europaea* and *Nitrosospira briensis*. C_2H_2 is supposed to inhibit both nitrifier denitrification and nitrification, O_2 only nitrifier denitrification. Since between 60 and 80% of the N_2O production was inhibited by O_2, it was concluded that nitrifier denitrification was more important for N_2O production than nitrification.

The contradictory findings of the studies of Beaumont et al. (2002) and Wrage et al. (Chapter 4) concerning the importance of nitrification and nitrifier denitrification for N_2O production

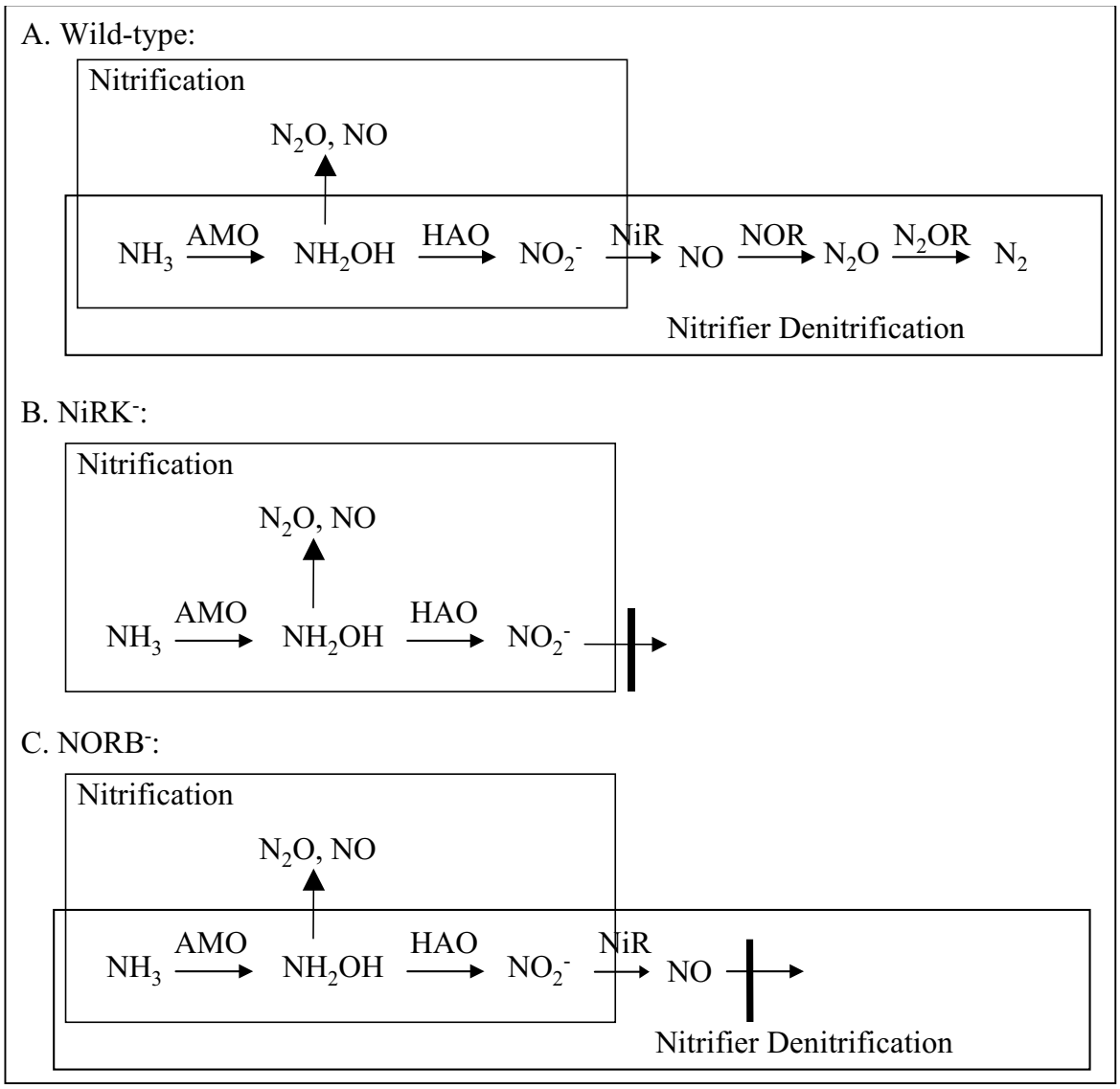

Figure 11: Postulated pathways of N_2O production in *Nitrosomonas europaea*. A. wild-type. B. NirK-deficient cells. C. NORB-deficient cells. For further explanations about the transformants see text. AMO: ammonia monooxygenase; HAO: hydroxylamine oxidoreductase; NiR: nitrite reductase; NOR: nitric oxide reductase; N_2OR: nitrous oxide reductase.

by nitrifiers might be intrinsic to the used methods. Wrage et al. (Chapter 4) discuss the possibility of an underestimation of nitrification in their study. They argue that the large concentrations of O_2 might not only inhibit nitrifier denitrification, but also negatively affect ammonia oxidation. Beaumont et al. (2002) studied the effect of a mutation in a gene of the nitrifier denitrification pathway on N_2O production. Due to the mutation, the NirK-deficient cells might produce more N_2O via a pathway that is not that important in the wild-type.

In this study, we combined the approaches used by Beaumont et al. (2002) and Wrage et al. (Chapter 4). With the incubation method used by Wrage et al. (Chapter 4), we investigated the

production of N_2O by the wild-type and NirK-deficient cells from Beaumont et al. (2002). Furthermore, the N_2O production by another mutant of *N. europaea* which is not expressing nitric oxide reductase (NORB) was studied. Similar to the NirK-deficient cells, NORB-deficient cells cannot produce N_2O by the known pathway of nitrifier denitrification (Figure 11C). However, NORB-deficient cells can reduce NO_2^- to nitric oxide (NO), so that less NO_2^- than in the NirK-deficient cells should accumulate. Since O_2 consumption is linked with ammonia oxidation and should therefore be inhibited by C_2H_2, the influence of C_2H_2 on the consumption of O_2 by the three strains was also studied.

The aims of this study were to test the effectiveness of C_2H_2 and O_2 as inhibitors of the pathways of N_2O production in ammonia oxidizing bacteria and to investigate whether nitrification or nitrifier denitrification is more important for N_2O production in *N. europaea* as a model organism of ammonia oxidizers.

Material and Methods

Microorganisms

The following microorganisms were used for this study: *N. europaea* strain ATCC 19178 (WT), a NirK-deficient mutant (NirK$^-$; ATCC 19178 derivative; *nirK*::pNIRsu, pBK11 derivative (Beaumont et al., 2002)), and a NORB-deficient mutant (NORB$^-$; ATCC 19178 derivative; *norB*::pNORsu, pBK11derivative). They were grown in liquid mineral medium as described by Hyman and Arp (1992). Since the NirK- and NORB-deficient cells were resistant to kanamycine, the medium for these mutants additionally contained 25 μg ml^{-1} kanamycine to prevent reversion of the mutation. The cultures were grown at 20^{o}C in the dark for one week.

The ammonia oxidizers were harvested by centrifugation (15 000 RPM, 15 minutes, 4^{o}C) and washed once. As an indication of the bacterial numbers, the optical density was measured at 600 nm (OD_{600}, spectrophotometer model Aquamate, Thermo Spectronic).

Incubation experiment

The harvested microorganisms were incubated according to the method developed by Webster and Hopkins (1996a) in its application for pure culture experiments as described by Wrage et al. (Chapter 4). In brief, sets of four incubations were set up, with either 0.02 kPa C_2H_2, 100 kPa O_2, 0.02 kPa C_2H_2 plus 100 kPa O_2 or no addition (control). Small concentrations of C_2H_2 should inhibit ammonia oxidation and thus N_2O production by both nitrification and nitrifier denitrification. An incubation atmosphere with 100 kPa O_2 is

supposed to inhibit only nitrifier denitrification (Webster and Hopkins, 1996a). Serum bottles (250 ml) containing 10 ml mineral medium were prepared as described elsewhere (Wrage et al., Chapter 4). When the appropriate incubation atmospheres had been created, the prepared microorganisms were added.

After addition of the microorganisms, the bottles were incubated at 20°C in the dark. After 24 and 48 hours, gas samples for N_2O measurements and liquid samples for NO_2^- measurements were taken. N_2O production during this period was linear. N_2O was measured in 5 ml samples with a gas chromatograph with electron capture detector (PU 4400 Unicam Analytical Systems, Philips, The Netherlands). The system was calibrated with 4.5 µl l^{-1} N_2O in N_2 (Hoek Loos, Schiedam, The Netherlands). The detection limit for N_2O was 8 nl l^{-1}. NO_2^- was measured with segmented-flow analysis (Houba et al., 2000). The detection limit for NO_2^- was approximately 0.04 mg N l^{-1}. The experiment was set up with 4 replicates per treatment and was repeated three times to see whether observed trends were reproducible.

Table 13: Incubations carried out to measure O_2 consumption. WT: wild-type, NirK: nitrite reductase, NORB: nitric oxide reductase. For further explanations, see text.

O_2 consumption experiment	**Incubation**	**Treatment**	**Replicates**
1	2 ml concentrated suspension of WT, NirK- or NORB-deficient cells directly in reaction vessel	with or without direct addition of 50 µl C_2H_2 to the suspension in the reaction vessel	2
2	10 ml suspension of WT, NirK- or NORB-deficient cells in 250 ml serum bottles	with 0.02 kPa C_2H_2 in headspace	1
3	2.5 ml suspension of NirK- or NORB-deficient cells in 100 ml serum bottles	with or without 0.02 kPa C_2H_2 in headspace	3

Oxygen consumption

An overview of the O_2 consumption experiments is shown in Table 13. Initially, the O_2 consumption was measured in concentrated suspensions of the wild-type, NirK-deficient cells and NORB-deficient cells (OD_{600} of 0.26, 0.26 and 0.27, respectively. Oxygraph with Clark electrode). To study the influence of C_2H_2, the O_2 consumption was also measured after adding 50 µl C_2H_2 directly to 2 ml of these concentrated suspensions in the reaction vessel of the oxygraph. This resulted in a concentration of C_2H_2 of about 2.5 kPa. These measurements were done in duplicate. To be able to better compare the results to those of the normally done

incubations, 10 ml of the suspensions were incubated in 250-ml serum bottles with 0.02 kPa C_2H_2 in the headspace for 1½ hours before O_2 consumption was measured. These incubations were first carried out without replication. Later, they were repeated with replication (n=3) in slightly modified form. To this end, 2.5 ml of suspensions of NirK-deficient cells and NORB-deficient cells (OD_{600} of 0.16 and 0.19, respectively) were incubated in 100 ml serum bottles with or without 0.02 kPa C_2H_2. These bottles were incubated at 20°C in the dark for 3 hours. Then, the O_2 consumption in the suspensions was measured (biological oxygen monitor, Yellow Springs Instruments Co., Inc., Model 5300, Yellow Springs, Ohio, USA).

Statistics

Except for the O_2 consumption experiments, all experiments were set up with four replicates. Because of the large standard deviations, the incubation experiment was repeated three times. These repetitions were also analysed statistically. Statistical analyses were carried out with SPSS for Windows 8.0 (Norusis, 1986). The data was first checked for normality with Kolmogorov-Smirnov's test. In case of normality, Levene's test was carried out to see whether the variances were homogenous. If they were not, the data was transformed before an ANOVA was carried out. Significant effects between treatments ($\alpha = 0.05$) were analysed with the LSD test. When the data was not normally distributed, the non-parametrical Kruskal-Wallis test was used for finding differences between treatments. In these cases, significant effects ($\alpha = 0.05$) were analysed with the Schaich-Hamerle test with a macro procedure in Excel. Generally, ANOVA was used to compare means between replicates within one repetition of experiments, while the Kruskal-Wallis test was used to compare means between repetitions and in pooled data.

Results

N_2O production

Generally, the NirK-deficient cells reacted to addition of inhibitors in a similar way as the wild-type, while the NORB-deficient cells behaved differently. The average N_2O production was with 44.08 nmol h^{-1} $(OD_{600}$ x ml$)^{-1}$ about 60 times larger in the NORB-deficient cells than in the wild-type (0.76 nmol h^{-1} $(OD_{600}$ x ml$)^{-1}$) or in the NirK-deficient cells (0.73 nmol h^{-1} $(OD_{600}$ x ml$)^{-1}$).

All inhibitors had effects on the N_2O production in the wild-type and NirK-deficient cells (Table 14). These effects were not in each repetition of the experiment significant due to the large variability of the data. However, analysis of pooled data of the three repetitions of the

experiment clearly showed that addition of C_2H_2 and C_2H_2 plus O_2 significantly ($\alpha = 0.05$) reduced the N_2O production compared with the controls. The N_2O production was then not significantly different from the detection limit. Addition of only O_2 also reduced the N_2O production in the wild-type and NirK-deficient cells, but the results using pooled data were not significantly different from those of the other three treatments, including the controls. In incubations with NORB-deficient cells, C_2H_2 did not reduce the N_2O production. In this strain, O_2 significantly decreased the N_2O production in both treatments with 100 kPa O_2, both in the presence and absence of C_2H_2.

Table 14: Production of N_2O by the wild-type and two mutants of *Nitrosomonas europaea* in incubations with C_2H_2 and O_2. Presented are means and standard deviations (n=4) for the three repetitions of the experiment. Results of the statistical analyses are given as superscript letters for each repetition (α=0.05). Column 'r.e.' (repetition effect) gives differences between repetitions, row 'trtmt. effect' (treatment effect) differences between treatments for pooled data from the three repetitions. C: control, A: with 0.02 kPa C_2H_2, O: with 100 kPa O_2, AO: with 0.02 kPa C_2H_2 plus 100 kPa O_2. OD_{600}: optical density of the cell suspensions at 600nm.

	N_2O [nmol h^{-1} (OD_{600} x ml)$^{-1}$]				
	***Nitrosomonas europaea* wild-type**				
repetition	**C**	**A**	**O**	**AO**	**r.e.**
1	0.63 ± 0.29^{a}	0.29 ± 0.17^{b}	0.29 ± 0.23^{b}	0.11 ± 0.11^{b}	ab
2	0.67 ± 0.29^{a}	0.19 ± 0.17^{a}	0.38 ± 0.58^{a}	-0.04 ± 0.32^{a}	a
3	0.97 ± 0.28^{a}	0.21 ± 0.08^{b}	0.76 ± 0.34^{a}	0.37 ± 0.12^{b}	b
trtmt. effect	a	b	ab	b	
	***Nitrosomonas europaea* NirK-deficient cells**				
1	0.43 ± 0.77^{a}	-0.06 ± 0.11^{b}	-0.03 ± 0.14^{b}	-0.17 ± 0.05^{b}	a
2	0.79 ± 0.77^{a}	-0.03 ± 0.09^{a}	0.13 ± 0.35^{a}	-0.05 ± 0.04^{a}	a
3	0.98 ± 0.96^{a}	-0.04 ± 0.11^{a}	0.16 ± 0.43^{a}	-0.07 ± 0.05^{a}	a
trtmt. effect	a	b	ab	b	
	***Nitrosomonas europaea* NORB-deficient cells**				
1	57.31 ± 73.72^{a}	54.55 ± 11.24^{a}	0.41 ± 0.41^{b}	0.07 ± 0.14^{b}	a
2	40.95 ± 33.71^{a}	57.76 ± 33.97^{a}	-0.24 ± 0.52^{b}	-0.16 ± 0.11^{b}	a
3	33.98 ± 24.22^{a}	43.45 ± 10.57^{a}	0.25 ± 0.37^{b}	0.46 ± 0.12^{b}	a
trtmt. effect	a	a	b	b	

NO_2^- production

The measurements of NO_2^- production again showed similar responses of the wild-type and NirK-deficient cells and a different behaviour of NORB-deficient cells (Table 15). The wild-type produced on average 223±161 nmol NO_2^- h^{-1} (OD_{600} x ml)$^{-1}$, while NirK-deficient cells produced on average in the controls 35±124 nmol NO_2^- h^{-1} (OD_{600} x ml)$^{-1}$. This difference was not significant due to the large variability of the data. In contrast to incubations with the wild-type or NirK-deficient cells, the NO_2^- concentration in incubations with NORB-deficient cells increased only in the 24 hours before the first measurement and decreased in

the 24 hours thereafter in all but one experiment (on average -16±141 nmol NO_2^- h^{-1} (OD_{600} x ml)$^{-1}$ in the controls). The difference in NO_2^- production between NORB-deficient cells and the other strains was significant.

Table 15: Production of NO_2^- by the wild-type and two mutants of *Nitrosomonas europaea* in incubations with C_2H_2 and O_2. Presented are means and standard deviations (n=4) for the three repetitions of the experiment. Results of the statistical analyses are given as superscript letters for each repetition (α=0.05). Column 'r.e.' (repetition effect) gives differences between repetitions, row 'trtmt. effect' (treatment effect) differences between treatments for pooled data from the three repetitions. C: control, A: with 0.02 kPa C_2H_2, O: with 100 kPa O_2, AO: with 0.02 kPa C_2H_2 plus 100 kPa O_2. OD_{600}: optical density of the cell suspensions at 600nm.

	NO_2^- [nmol h^{-1} (OD_{600} x ml)$^{-1}$]				
	***Nitrosomonas europaea* wild-type**				
repetition	**C**	**A**	**O**	**AO**	**r.e.**
1	151±186^a	8±3^a	-145±657^a	2±1^a	a
2	171±143^a	-18±37^a	96±171^a	0±6^a	a
3	346±98^a	1±1^b	87±92^b	14±31^b	a
trtmt. effect	a	b	ab	b	
	***Nitrosomonas europaea* NirK-deficient cells**				
1	-46±149^a	1±6^a	5±69^a	2±7^a	a
2	57±42^a	7±4^a	60±58^a	7±4^a	a
3	95±136^a	5±6^a	28±21^a	0±3^a	a
trtmt. effect	ab	ab	a	b	
	***Nitrosomonas europaea* NORB-deficient cells**				
1	-104±152^a	-63±15^a	-15±15^a	-12±1^a	a
2	99±142^a	-86±55^a	90±67^a	39±86^a	b
3	-42±32^a	-62±20^a	1±10^b	-5±6^b	ab
trtmt. effect	a	b	a	a	

The effects of the inhibitors on NO_2^- production in the wild-type were similar to those observed for N_2O production. C_2H_2 and a combination of C_2H_2 and O_2 decreased the NO_2^- production significantly (α = 0.05) in the wild-type. O_2 alone also decreased the NO_2^- production, but not significantly. In NirK-deficient cells, the trend was not so clear. Due to large standard deviations in the controls, no inhibitor significantly decreased the NO_2^- production. However, there was again a trend to lower production in treatments with C_2H_2, while the treatment with only O_2 produced approximately the same amount of NO_2^- as the controls. In incubations with NORB-deficient cells, the NO_2^- concentrations decreased in the controls and in the treatment with C_2H_2, while they increased (or decreased less) in both treatments with O_2. The decrease in NO_2^- concentrations in incubations with NORB-deficient cells was of the same magnitude as the increase in N_2O concentrations.

O_2 consumption

The O_2 consumption was in all strains of the same magnitude. In general, an inhibiting effect of C_2H_2 on O_2 consumption was observed (Table 16). This effect was larger when C_2H_2 had been added directly to the suspensions in the reaction vessel of the oxygraph than when the suspensions had been incubated in serum bottles with C_2H_2 only in the headspace. C_2H_2 inhibited O_2 consumption to a larger extent in the wild-type than in the transformed cells.

The comparison of NirK-deficient cells and NORB-deficient cells incubated with or without C_2H_2 again showed that C_2H_2 significantly reduced the O_2 consumption (Table 16). C_2H_2 reduced the O_2 consumption relative to that in the controls without C_2H_2 to a larger part in NirK-deficient cells than in NORB-deficient cells. The difference was not statistically significant, though.

Table 16: Consumption of O_2 in the wild-type and in the NirK- and NORB-deficient cells of *Nitrosomonas europaea*. Shown are means and standard deviations (n=3). Different superscript letters indicate significant differences (α=0.05) between treatments. C: direct measurement with concentrated microbial suspension; C+C_2H_2: direct measurement with concentrated microbial suspension with addition of 50 µl C_2H_2 per 2 ml suspension; I: pre-incubation of microbial suspension in serum bottles before oxygen consumption measurement; I+C_2H_2: pre-incubation of microbial suspension in serum bottles with 0.02 kPa C_2H_2 bottles before oxygen consumption measurement. OD_{600}: optical density of the cell suspensions at 600nm. For further explanations, see Table 13 and text.

	O_2 consumption [µmol h^{-1} (OD_{600} x ml)$^{-1}$]	
Experiment 1	**C**	**C+C_2H_2**
Wild-type	4.1	0.2
NirK-deficient cells	3.2	0.3
NORB-deficient cells	4.2	0.7
Experiment 2	**I**	**I+C_2H_2**
Wild-type	n.d.[1]	0.6
NirK-deficient cells	n.d.[1]	2.1
NORB-deficient cells	n.d.[1]	2.8
Experiment 3	**I**	**I+C_2H_2**
NirK-deficient cells	3.6±0.7[a]	0.6±0.3[b]
NORB-deficient cells	2.1±0.9[a]	0.6±0.1[b]

[1]not determined.

Discussion

This study provides important new insights into the effects of C_2H_2 and O_2 on N_2O production by the ammonia oxidizer *N. europaea*. It furthermore shows how incomplete our understanding of the metabolism of ammonia oxidizers still is.

The N_2O production by the wild-type in response to the inhibitors confirmed our expectations. C_2H_2, which inhibits ammonia oxidation (Berg et al., 1982, McCarty, 1999), decreased the N_2O production significantly. In incubations with 100 kPa O_2, where only nitrifier denitrification should be inhibited, the N_2O production was in between that found in the controls and that with C_2H_2 as inhibitor. The results for NO_2^- production by the wild-type are not in line with the expectations. If O_2 inhibits only nitrifier denitrification, the NO_2^- production in incubations with O_2 should be equal to or even larger than that in the controls, since no NO_2^- should be reduced to N_2O. However, the NO_2^- production was smaller in incubations with O_2 than in the controls. This could have two reasons: either nitrifier denitrification was not totally inhibited by 100 kPa O_2, or ammonia oxidation was negatively affected. A negative effect of large concentrations of O_2 on ammonia oxidation has also been suggested previously (Wrage et al., Chapter 4). It might be due to the formation of oxygen radicals, which increases with increasing O_2 concentrations (Schlegel, 1992).

The experiments with the NirK-deficient cells give further insight into the effect of O_2. Since these mutants cannot produce N_2O by nitrifier denitrification via nitrite reductase, 100 kPa O_2 is not expected to have an effect on their N_2O or NO_2^- production. However, the N_2O production in incubations with 100 kPa O_2 was decreased compared to that found in the controls. The NO_2^- production in incubations with 100 kPa O_2 was also slightly decreased compared to that in the controls. These results suggest that not only nitrifier denitrification, but also nitrification was influenced by such large O_2 concentrations. They might have decreased the incomplete oxidation of NH_2OH, one of the sources of N_2O in nitrification (Hooper and Terry, 1979). This could explain the larger effect of O_2 on N_2O production than on NO_2^- production. Furthermore, adverse effects of O_2 in such large concentrations on ammonia oxidation cannot be excluded as already pointed out above. Whatever the exact mechanism, 100 kPa O_2 seems to be unsuitable as an inhibitor of nitrifier denitrification, since it seems to also negatively affect other sources of N_2O in nitrifiers.

The NirK-deficient cells produced similar amounts of N_2O as the wild-type. This is different from the results of Beaumont et al. (2002), who found a much larger N_2O production in NirK-deficient cells than in the wild-type. The different results are probably due to differences in incubation methods and timing of the measurements. Beaumont et al. (2002) measured the

N_2O production in batch cultures in the early stationary phase of growth and in exponentially growing cultures. They incubated the cultures on a rotary shaker at 30°C. For our experiments, we incubated cultures that were in the early exponential phase without shaking at 20°C. The cell density in the suspensions was about 10 times lower in our experiments than in those of Beaumont et al. (2002). However, despite the experimental differences between both studies, the NirK-deficient cells did not produce less N_2O than the wild-type in either study. This could hint at the importance of nitrification itself for N_2O production compared to nitrifier denitrification.

Most remarkable was the observation that at normal O_2 concentrations, N_2O was the major end product of ammonia oxidation in the NORB-deficient cells (Table 14). In the absence of C_2H_2, but with normal O_2 concentrations, ammonia oxidation proceeded normally as can be concluded from the measured O_2 consumption rates (Table 16). Simultaneously with ammonia oxidation, a net consumption of NO_2^- from the medium was observed. Chemodenitrification, the chemical decomposition of NO_2^- to NO and N_2O at low pH (Chalk and Smith, 1983), was probably not an important reason for the observed NO_2^- consumption and large N_2O production. First of all, the mineral solution had a pH of 8. Secondly, the NO_2^- concentration in control incubations of the wild-type and of NORB-deficient cells was similar after 24 hours (0.08±0.09 mmol l^{-1} and 0.13±0.11 mmol l^{-1}, for NORB-deficient cells and wild-type, respectively), at the time of first measurement. After 48 hours, the NO_2^- concentration in incubations of the wild-type was 0.58±0.71 mmol l^{-1}, i.e. much larger than in incubations with NORB-deficient cells (0.07±0.11 mmol l^{-1}). Since the pH was similar, these large NO_2^- concentrations should also have led to chemodenitrification and large N_2O production in incubations of the wild-type if chemodenitrification was an important source of N_2O in incubations of NORB-deficient cells. Thus, chemodenitrification was probably not a major source of N_2O in incubations of NORB-deficient cells.

Consumption of NO_2^- could also be explained by nitrifier denitrification. In the NORB-deficient cells with a corrupted nitric oxide reductase, NO should be the end-product of nitrifier denitrification (Figure 11C). However, NO production could not be detected when measured with a chemiluminescence NO-NO_2-NO_x analyser (Model 42C, Thermo Environmental Instruments Inc., USA, detection limit for NO: 1 nl l^{-1}; results not shown). This could have two reasons: NO might have reacted further or the decrease in NO_2^- concentration was not due to a reduction by nitrite reductase. NO is a very reactive species and might indeed have reacted further, e.g. to nitrogen dioxide (NO_2). However, we also have to consider alternative explanations, especially since the N_2O production was very large in these cells.

The observation that a net consumption of NO_2^- occurred in NORB-deficient cells simultaneously with ammonia oxidation and production of large amounts of N_2O suggests that ammonia oxidation proceeds in the direction of N_2O in the absence of a functional nitric oxide reductase. Hence, the enzyme nitric oxide reductase seems to be necessary to force ammonia oxidation in the wild type strain of *N. europaea* towards NO_2^- as major end product (Figure 12). It can be argued that the pathway towards NO_2^- is slightly profitable with respect to the energy yield from ammonia oxidation when NH_3 is limiting:

$NH_3 + 1.5\ O_2 \rightarrow NO_2^- + H^+ + H_2O$ $\quad\quad$ $\Delta G_o' = -275$ kJ / mol NH_3

$NH_3 + O_2 \rightarrow 0.5\ N_2O + 1.5\ H_2O$ $\quad\quad$ $\Delta G_o' = -264$ kJ / mol NH_3

Probably more important than this profit in energy yield is the total number of electrons produced during the ammonia oxidation towards N_2O. In contrast to the oxidation of NH_2OH to NO_2^-, which yields 4 electrons, the oxidation of NH_2OH to N_2O yields only a net production of 2 electrons per atom of nitrogen. These 2 electrons are just sufficient for the enzyme ammonia monooxygenase to convert NH_3 to NH_2OH, leaving no additional electrons for the generation of energy.

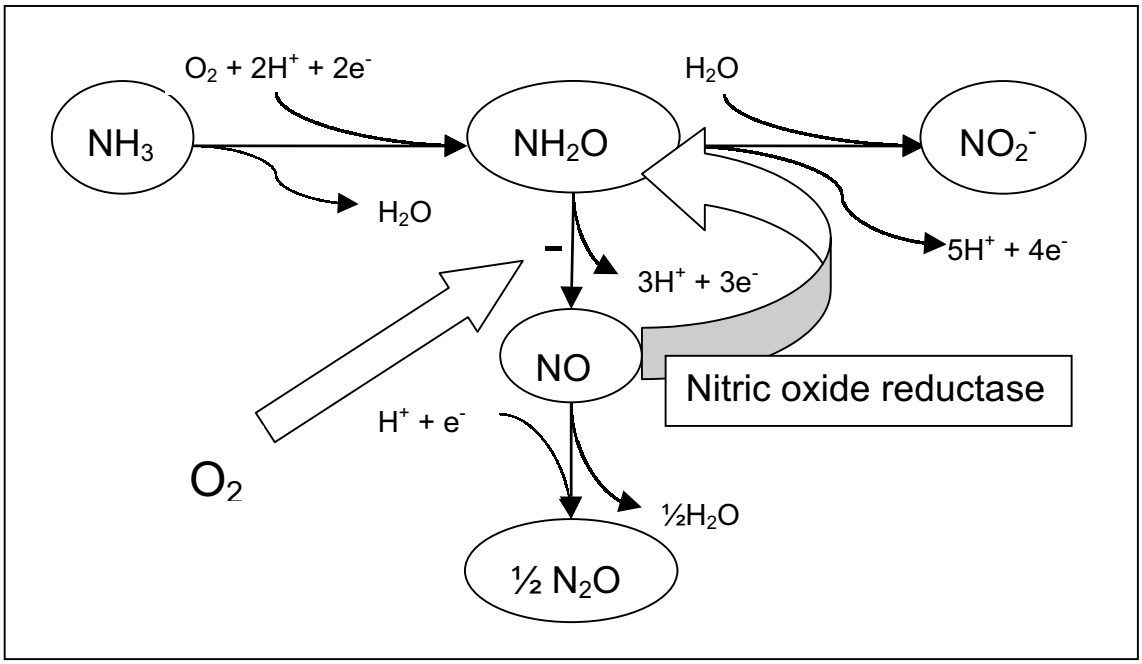

Figure 12: Hypothetic role of nitric oxide reductase and large oxygen concentrations (100 kPa) in directing ammonia oxidation towards nitrite. Thin arrows: pathways, thick arrows: suggested effects of O_2 and nitric oxide reductase, respectively.

Surprisingly, the application of large O_2 concentrations nullified the effect of the lack of a functional nitric oxide reductase in the NORB-deficient cells. Under these conditions, N_2O and NO_2^- production rates of this mutant became comparable again with those of the wild-

type and the NirK-deficient cells. Apparently, a large supply of O_2, like a functional nitric oxide reductase in the wild-type, directs the oxidation reaction towards NO_2^- (Figure 12).

It is very intriguing that the NORB-deficient cells were insensitive to C_2H_2 in terms of N_2O production. This could mean that ammonia oxidation in this transformant was not influenced by C_2H_2. However, in the presence of C_2H_2, NO_2^- production and O_2 consumption were inhibited (Tables 15 and 16). Hence, we conclude that ammonia oxidation was also inhibited by C_2H_2 in NORB-deficient cells. This seems to suggest another source of N_2O in the NORB-deficient cells. Recently, we reported that N_2O production by *Nitrosospira briensis* was also not affected by C_2H_2 although NO_2^- production ceased in the presence of this gas (Wrage et al., Chapter 4). Maybe the insensitivity of N_2O production in *N. briensis* for C_2H_2 is due to a mechanism that is similar to that in the NORB-deficient cells.

Summarizing, we cannot yet explain the large N_2O production in NORB-deficient cells. We can speculate that the enzyme nitric oxide reductase plays a role in directing ammonia oxidation towards NO_2^- rather than N_2O as end product. The insensitivity of NORB-deficient cells to C_2H_2 might hint at a so far unidentified way of N_2O production. There are indications from the incubations with the NirK-deficient cells that the known way of nitrifier denitrification, where the first step is catalysed by nitrite reductase, is not so important in terms of N_2O production by ammonia oxidizers. The experiments suggest that 100 kPa O_2 is not suitable as an inhibitor of nitrifier denitrification as it also affects ammonia oxidation.

CHAPTER SIX NITRIFIER DENITRIFICATION IN PERSPECTIVE

not published previously

Introduction

In this final chapter, I first present the main results of my thesis up to this point. Then, a sensitivity analysis will be carried out to identify the most probable reasons for the negative fluxes calculated for N_2O production by nitrifier denitrification measured with the inhibition method by Webster and Hopkins (1996a) in soil studies (Chapter 3). This sensitivity analysis should provide new insights into the impact of possible inhibition problems on the measured N_2O production. I will continue this chapter with a discussion of the advantages and disadvantages of alternatives used to distinguish between N_2O production by nitrifiers and denitrifiers. Other inhibitors, stable isotopes and pure cultures will be considered. The insights gained from the sensitivity analysis and from the discussion of other methods will be used to estimate the consequences for our understanding of N_2O production in soils and the importance of nitrifier denitrification for N_2O production. A summary of the most important results of this chapter and an outlook on possibilities for further research conclude this thesis.

Main results obtained so far

In the beginning, the objectives of this study were to quantitatively assess N_2O production by nitrifier denitrification under a range of conditions and to come up with a best estimate for N_2O produced by nitrifier denitrification in The Netherlands. The review of existing knowledge of nitrifier denitrification and related processes in soils (Chapter 2, Wrage et al., 2001) revealed how important it is to get to know more about this poorly studied pathway that might lead to substantial N_2O production in some soils. However, the first results of this study gave rise to questions concerning the prevailing measurement method for nitrifier denitrification (Chapter 3). Therefore, the objectives of this study were adapted and now became i) to test the prominent methodology for quantifying the N_2O production by nitrifier denitrification, and ii) to assess the importance of nitrifier denitrification for N_2O production in pure cultures of *Nitrosomonas europaea* and *Nitrosospira briensis*.

The first objective has been addressed in Chapter 3, 4 and 5. We have seen that the prevailing measurement method using C_2H_2 (0.02 kPa) and O_2 (100 kPa) in different combinations as inhibitors to quantify the N_2O production by nitrifier denitrification was not suitable for all soils. In some conditions, the addition of inhibitors seemed to stimulate the production of N_2O compared to the controls. Furthermore, negative fluxes were calculated for some sources of N_2O, especially for nitrifier denitrification (Chapter 3). Pure culture studies revealed some reasons for the observed problems. O_2 was not suitable as an inhibitor of nitrifier denitrification, since it also had a negative effect on ammonia oxidation (Chapter 4 and 5). C_2H_2 only inhibited the N_2O production by *N. europaea*, but not that by *N. briensis* (Chapter 4). Furthermore, C_2H_2 did not inhibit the N_2O production by a transformant of *N. europaea* lacking nitric oxide reductase (Chapter 5). While it is not clear yet whether the reason for the insensitivity to C_2H_2 was the same in the transformant and in *N. briensis*, we can conclude that C_2H_2 was not reliable as an inhibitor of N_2O production by all nitrifiers.

Due to the consistent results of soil studies and pure culture experiments, we reach the conclusion that the method using C_2H_2 and O_2 is not suitable for differentiating reliably between N_2O produced by different soil sources. In the past, especially C_2H_2 has been used extensively to differentiate between nitrification and denitrification in soils (e.g. Blackmer et al., 1980, Klemedtsson et al., 1988, Granli and Bøckman, 1994, Bollmann and Conrad, 1998). What are the consequences if C_2H_2 does not reliably inhibit N_2O production by nitrifiers? Have we underestimated the share of nitrifiers in N_2O production?

This leads to the second objective of this study, the assessment of the importance of nitrifier denitrification in pure culture studies of *N. europaea* and *N. briensis*. This objective has been addressed in Chapter 4 and 5. In Chapter 4, the production of N_2O by pure cultures of *N. europaea* and *N. briensis* was measured. Large concentrations (100 kPa) of O_2 were used to inhibit nitrifier denitrification. The results suggest that nitrifier denitrification is the most important pathway of N_2O production in *N. europaea* and *N. briensis*. However, we have to bear in mind that nitrification might have been underestimated due to adverse effects of O_2 on nitrification itself. In Chapter 5, the N_2O production by mutants of *N. europaea* that are deficient in either nitrite reductase (NirK) or nitric oxide reductase (NORB) was studied. The mutants should not be able to form N_2O via the known pathway of nitrifier denitrification. Nevertheless, the NirK-deficient cells produced as much N_2O as the wild-type, and the NORB-deficient cells produced even more N_2O. This suggests that nitrifier denitrification is not so important for N_2O production in these mutants. We will further discuss the importance of nitrifier denitrification below. The results of Chapter 5 also point at the possibility of an

unknown, alternative route of N_2O production in nitrifiers. This challenges our understanding of the pathways of N_2O production by ammonia oxidizers.

The pitfalls - A sensitivity analysis

Introduction

In studies of the N_2O production by nitrification, nitrifier denitrification and denitrification, 0.02 kPa C_2H_2 and 100 kPa O_2 are used to differentiate between the sources of N_2O (Robertson and Tiedje, 1987, Webster and Hopkins, 1996a). C_2H_2 in such small concentrations should inhibit ammonia oxidation and therefore stop N_2O production by both nitrification and nitrifier denitrification (Klemedtsson et al., 1990). O_2 should suppress N_2O production by nitrifier denitrification and by denitrification (Robertson and Tiedje, 1987). Previous studies (Chapters 3-5) have shown that 0.02 kPa C_2H_2 and 100 kPa O_2 do not always have the desired effect on pathways of N_2O production. In Chapter 3, five possible reasons were already briefly discussed. In this sensitivity analysis, these will be further investigated to see which of them might have caused the observed effects. Due to the results of Chapters 4 and 5, two more possibilities are added, so that in total, the following possibilities are considered:

a. the inhibitors act as expected with respect to repression of nitrification, nitrifier denitrification and denitrification;

b. ammonia oxidation was stimulated by 100 kPa O_2;

c. ammonia oxidation was partially inhibited by O_2;

d. nitrifier denitrification was incompletely suppressed by O_2;

e. denitrification was incompletely suppressed by O_2;

f. nitrification and nitrifier denitrification were incompletely inhibited by 0.02 kPa C_2H_2;

g. N_2O reductase in denitrifiers was inhibited by C_2H_2.

h. a combination of partial inhibition of ammonia oxidation by O_2, incomplete suppression of nitrification and nitrifier denitrification by C_2H_2, and inhibition of N_2O reductase of denitrifiers by C_2H_2.

Method

To estimate the importance of the different factors evaluated, four hypothetical soils were considered where the N_2O production is: i) dominated by nitrification, ii) dominated by nitrifier denitrification, iii) dominated by denitrification, and iv) not dominated by a single source, but equally caused by nitrification, nitrifier denitrification and denitrification (Table 17).

Table 17: N_2O production rates in four hypothetical soils: a nitrification-dominated one, a nitrifier-denitrification-dominated one, a denitrification-dominated one and a balanced[2] one. Since the production rates add up to 100, the production per source can be interpreted as a percentage of the total production.

Soils	Nitrification	Nitrifier Denitrification	Denitrification	Other Sources[1]	Total
Nitrification dominated	60	20	15	5	100
Nitrifier denitrification dominated	20	60	15	5	100
Denitrification dominated	5	10	80	5	100
Balanced[2]	33	33	33	1	100

[1]Other sources are for example chemodenitrification or heterotrophic nitrification.

[2]Balanced: nitrification, nitrifier denitrification and denitrification contribute equally to the N_2O production.

It was assumed for the calculations that a stimulation of nitrification by O_2 (possibility b) caused a doubling of the N_2O production. A stimulation of nitrification by O_2 has not been measured yet (Chapter 4 and 5). On the contrary, investigations have shown that ammonia oxidation is negatively influenced by large O_2 concentrations (possibility c, Chapter 4 and 5). Therefore, a stimulation of the N_2O production by nitrifiers due to O_2 is not very likely and we suggest that a doubling will be about the maximal effect.

For a partial inhibition of ammonia oxidation by O_2 (possibility c), it is assumed that one third of the N_2O produced by nitrification without inhibitors is suppressed by O_2. Previously, indications of an inhibition of ammonia oxidation by O_2 were found in pure culture studies, but could not be quantified (Chapter 4). In studies with transformants of *Nitrosomonas europaea* lacking nitrite reductase or nitric oxide reductase, O_2 caused a 20-75% reduction of ammonia oxidation (Chapter 5).

In situations of incomplete suppression (possibilities d, e and f), only one third of the N_2O produced by the process in question without inhibitors was assumed to be suppressed by the inhibitor. C_2H_2 (possibility f) sometimes does not affect N_2O production from nitrifier denitrification (Chapter 4 and 5). In that light, an inhibition of one third of the N_2O production from nitrifiers by C_2H_2 seems a rather conservative assumption. There are no data yet that allow to quantify a possible effect of incomplete suppression of nitrifier denitrification and denitrification by O_2 on N_2O production (possibilities d and e). Therefore, the same factor as for the incomplete inhibition of N_2O production of nitrifiers by C_2H_2 was applied, i.e. an inhibition of one third of the N_2O produced by nitrifier denitrification and denitrification by O_2.

For the inhibition of the N_2O reductase of denitrifiers by C_2H_2 (possibility g), it was assumed that the N_2O production by denitrifiers was increased by a factor three. This assumption was based on results of investigations of total denitrification using large concentrations (1-10 kPa) of C_2H_2. In these studies, the N_2O production has been found to be 2 to 6 times larger than in controls without C_2H_2 (e.g. Duxbury and McConnaughey, 1986, Colbourn, 1992, Dendooven et al., 1999, Estavillo et al., 2002).

There was supposed to be no interaction between inhibitors. Thus, the inhibitors' effect was assumed to be the same whether used in combination with another inhibitor or alone. For a combination of incomplete inhibition of nitrification by C_2H_2 plus partial inhibition of nitrification by O_2 (possibility h), N_2O production by nitrification was assumed to be fully inhibited in incubations with both C_2H_2 and O_2.

Equation 7: $N_2O_{Nitrification} = N_2O_O - N_2O_{AO}$

Equation 8: $N_2O_{Denitrification} = N_2O_A - N_2O_{AO}$

Equation 9: $N_2O_{Nitrifier\ Denitrification} = N_2O_C - N_2O_O - N_2O_A + N_2O_{AO}$

Equation 10: $N_2O_{Other} = N_2O_{AO}$

N_2O_C: N_2O production in control incubations;

N_2O_A: N_2O produced in incubations with 0.02 kPa C_2H_2;

N_2O_O: N_2O produced in incubations with 100 kPa O_2;

N_2O_{AO}: N_2O produced in incubations with 0.02 kPa C_2H_2 and 100 kPa O_2.

The calculations of the amounts of N_2O produced by the different sources were done following Equations 7-10 (textbox; see Chapter 3, Equations 1-4 for further details).

Results and discussion

The results of the analysis are shown in Table 18. It becomes obvious that all sources of N_2O can be over- or underestimated, depending on the supposed effect of the inhibitor. The

four different soils -dominated by either nitrification, nitrifier denitrification or denitrification, or with equal N_2O production by nitrification, nitrifier denitrification and denitrification- showed different sensitivities to artefacts in O_2 or C_2H_2 treatments (Table 18).

A stimulation of nitrification by O_2 would have the largest consequences in nitrification-dominated systems (Table 18, column b). The overestimation of nitrification causes here a severe underestimation of nitrifier denitrification. It could even lead to the calculation of negative values for nitrifier denitrification. However, as already pointed out, such a stimulation of nitrification by O_2 is not very likely since no indications for it could be found in pure culture studies (see Chapters 4 and 5).

The more likely partial inhibition of nitrification by O_2 (Table 18, column c) would result in large overestimations of nitrifier denitrification in the nitrification-dominated system. In Chapter 3, mostly negative or small numbers were calculated for N_2O production by nitrifier denitrification. Therefore, it can be speculated that either nitrification was not very important in these soils under the conditions studied or that O_2 did not have a large negative effect on nitrification. In studies with pure cultures of *N. europaea* and *N. briensis* (Chapter 4), indications of a partial inhibition of nitrification by O_2 were found. However, the production of NO_2^- did not differ significantly between control incubations and those with 100 kPa O_2. In studies with transformed nitrifiers (Chapter 5), O_2 caused a 20 to 75% inhibition of ammonia oxidation. The larger heterogeneity of the soil compared to the pure culture solutions might have led to a less uniform distribution of O_2 and thus to a reduction of the inhibition effect.

An incomplete inhibition of nitrifier denitrification by O_2 (Table 18, column d) has the largest effects in systems where the N_2O production is dominated by nitrifier denitrification. The relative error made in the calculations is also large in the denitrification-dominated system. The N_2O production by nitrification would be overestimated to the same extent that the production by nitrifier denitrification would be underestimated. In Chapter 3, small or negative values for nitrifier denitrification were not always connected with large values for nitrification. Therefore, incomplete inhibition of nitrifier denitrification by O_2 might play a role, but cannot be the only explanation for the calculated data.

Table 18: Sensitivity analysis with hypothetical soils where the N_2O production is dominated by different processes. The considered artefacts caused by the inhibitors are shown in the Textbox. It is supposed that the effect of every inhibitor* was the same, whether used alone or in combination with the other inhibitor. N: nitrification, ND: nitrifier denitrification, D: denitrification, O: other sources of N_2O. Changes relative to the controls are indicated in bold.

	Ideal	Artefacts by O_2*				Artefacts by C_2H_2*		Combination
Soils	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**
Nitrification dominated								
N	60	**120**	**40**	**73**	60	**20**	60	**40**
ND	20	**-40**	**40**	**7**	20	**7**	**-10**	**-43**
D	15	15	15	15	**5**	**28**	**45**	**98**
O	5	5	5	5	**15**	**45**	5	5
Nitrifier denitrification dominated								
N	20	**40**	**13**	**60**	20	**7**	20	**13**
ND	60	**40**	**67**	**20**	60	**20**	**30**	**-17**
D	15	15	15	15	**5**	**55**	**45**	**98**
O	5	5	5	5	**15**	**18**	5	5
Denitrification dominated								
N	5	**10**	**3**	**12**	5	**2**	5	**3**
ND	10	**5**	**12**	**3**	10	**3**	**-150**	**-158**
D	80	80	80	80	**27**	**87**	**240**	**250**
O	5	5	5	5	**58**	**8**	5	5
Nitrification, nitrifier denitrification and denitrification contribute equally								
N	33	**66**	**22**	**55**	33	**11**	33	**22**
ND	33	**0**	**44**	**11**	33	**11**	**-33**	**-66**
D	33	33	33	33	**11**	**55**	**99**	**143**
O	1	1	1	1	**23**	**23**	1	1

*As inhibitors, 0.02 kPa C_2H_2 and 100 kPa O_2 were used.

a) the ideal situation: all inhibitors work as supposed;

b) O_2 stimulates ammonia oxidation;

c) O_2 partly inhibits ammonia oxidation;

d) O_2 does not totally inhibit nitrifier denitrification;

e) O_2 does not totally inhibit denitrification;

f) C_2H_2 does not totally inhibit nitrification and nitrifier denitrification;

g) C_2H_2 inhibits the N_2O reductase of denitrifiers;

h) O_2 partly inhibits ammonia oxidation, C_2H_2 does not totally inhibit nitrification and nitrifier denitrification, and C_2H_2 inhibits the N_2O reductase of denitrifiers (combination of c, f and g)

An incomplete inhibition of denitrification by O_2 (Table 18, column e) would be most important in denitrification-dominated systems. The underestimation of denitrification would be matched by an overestimation of the other sources of N_2O production. Incomplete inhibition of denitrification could for example be explained by aerobic denitrification that might have taken place. In Chapter 3, values for N_2O production by other sources were generally quite large. However, negative values for denitrification were only occasionally calculated. Therefore, incomplete inhibition of denitrification by O_2 was probably not an important problem in these soils.

More important might have been an incomplete inhibition of nitrification and nitrifier denitrification by C_2H_2. As shown in Table 18 (column f), this would have consequences for the calculation of all sources of N_2O in all types of model soils. Nitrification and nitrifier denitrification would be underestimated and denitrification and other sources overestimated as sources of N_2O. It is very likely that this played a role in the soil studies, since incomplete inhibition of N_2O production by nitrifiers by C_2H_2 was also found in pure culture studies (Chapter 4 and 5).

Of all considered artefacts, the inhibition of N_2O reductase of denitrifiers by C_2H_2 (Table 18, column g) had the largest consequences on the calculated N_2O production rates. This was of course coupled to the assumption that this inhibition increased the N_2O production by denitrifiers threefold, while stimulation of nitrification by O_2 was supposed to have only a doubling effect. It is noteworthy that an inhibition of N_2O reductase of denitrifiers by C_2H_2 led to equally large, but opposite changes in nitrifier denitrification and denitrification. In Chapter 3, the largest negative values for N_2O production by nitrifier denitrification were coupled to approximately equally large, but positive values for denitrification. This hints at the possible practical importance of the inhibition of N_2O reductase of denitrifiers by C_2H_2. Former studies have shown that partial inhibition of N_2O reduction by denitrifiers can occur in soils at C_2H_2 concentrations as small as 0.01 kPa (Ryden et al., 1979).

A combination of partial inhibition of nitrification and nitrifier denitrification by C_2H_2, inhibition of N_2O reductase of denitrifiers by C_2H_2, and inhibition of nitrification by O_2 (Table 18, column h) causes an underestimation of nitrification, a large underestimation of nitrifier denitrification and a large overestimation of denitrification. Such a combination of effects is very likely and could have caused some of the values calculated in Chapter 3. Especially the relationship between large underestimations of nitrifier denitrification and large overestimations of denitrification fits in well with many values calculated in Chapter 3.

The different factors can lead to negative values resulting from the calculations. In the considered example, negative values were only calculated for nitrifier denitrification. This fits in with the results from Chapter 3, where most negative values were derived for nitrifier denitrification.

Conclusions

It can be concluded that an inhibition of the N_2O reductase of denitrifiers alone or in combination with a partial inhibition of nitrification by O_2 plus an incomplete inhibition of nitrification and nitrifier denitrification by C_2H_2 was the most probable factor causing under- and overestimations in the calculated N_2O production by the different sources. These factors can have caused the calculated negative values found in Chapter 3. The influence of the factors varies dependent on the largest source of N_2O in the studied system.

Alternatives for the differentiation between sources of N_2O

Different alternatives are known for the differentiation between soil sources of N_2O. In the following, other inhibitors, stable isotopes and pure culture studies will be considered. This will enable us to validate and compare the results of other studies that have differentiated between sources of N_2O from soils. Furthermore, it will help to outline opportunities for further research.

Other inhibitors

Inhibitors usually provide an easy to use and comparably cheap method to measure different sources of N_2O. A lot of research has been carried out on nitrification inhibitors, since they were regarded as possible solutions for problems with leaching and denitrification losses of N from agricultural soils. Both leaching and denitrification depend on the availability of NO_3^-. Besides possible additions as fertilizer, NO_3^- is supplied by nitrification. So it was concluded that if nitrification could be inhibited, this should substantially decrease the losses of N (Kurtz, 1980). The ideal compound would specifically block ammonia oxidation but not nitrite oxidation, would not adversely affect other soil organisms and higher plants, was not toxic to animals and humans in the amounts needed to effectively inhibit nitrification, would remain effective in soil for several weeks after application and was economical to use (Hauck, 1980).

Besides potential uses in agriculture, nitrification inhibitors are also used to differentiate between N_2O production by nitrification and denitrification in soils. For this differentiation, two treatments are needed: one control and one treatment with addition of the inhibitor. The

contribution of nitrification is then derived as the part inhibited, while the contribution of denitrification is the reciprocal part. Normally, these inhibition studies do not differentiate between nitrification and nitrifier denitrification.

A whole range of chemicals has been discussed for possible uses as nitrification inhibitors. As can be seen from Table 19, most of these nitrification inhibitors have side effects. Nitrapyrin, for example, also known as the chemical substance 2-chloro-6-(trichloromethyl)-pyridine or under the brand name 'N-Serve', chelates the copper of the cytochrome oxidase component involved in ammonia oxidation (Hauck, 1980). Its effect can be reversed by addition of Cu^{2+} (Hooper and Terry, 1973). However, nitrapyrin has also been shown to inhibit denitrification in pure culture experiments (Henninger and Bolag, 1976). On the contrary, in soil experiments, a stimulation of denitrification could be observed (Klemedtsson et al., 1988). Thus, nitrapyrin is not specific for only nitrification. The same is true for methyl fluoride (CH_3F). It is converted to formaldehyde by ammonia monooxygenase (Hyman et al., 1994), but also blocks consumption of methane and the oxidation of methane to CO_2. In general, many inhibitors of ammonia oxidation also inhibit methane oxidation due to the similarities between the enzymes ammonia monooxygenase and methane monooxygenase (McCarty, 1999).

Table 19: Nitrification inhibitors used in studies of the sources of N_2O, and possible side effects.

Nitrification inhibitor	Side effect	Reference
Methyl fluoride	inhibitor of aerobic methane oxidation	Oremland and Culbertson, 1992
Dimethyl ether	inhibitor of aerobic methane oxidation	Oremland and Culbertson, 1992
Nitrapyrin (N-Serve, 2-chloro-6-(trichloromethyl)-pyridine	inhibits denitrification by *Pseudomonas* sp.	Henninger and Bollag, 1976
	stimulates denitrification in soil	Klemedtsson et al., 1988
	binds largely indiscriminately to membrane proteins	McCarty, 1999
1,1,1-trichloroethylene	production product (epoxide) can bind polypeptides	McCarty, 1999
dicyandiamide (DCD)		Skiba et al., 1993

So far, only nitrification inhibitors have been discussed. To differentiate between sources of N_2O in soils, including nitrifier denitrification, it would be helpful if denitrification could also be selectively inhibited. However, so far no inhibitors are known to selectively affect

denitrification. Denitrification is carried out by a diverse group of microorganisms (Firestone, 1982). They switch to denitrification if O_2 becomes limiting for respiration. However, some denitrifiers are also known to carry out denitrification in oxic environments (Robertson and Kuenen, 1984). Thus, O_2 might not inhibit all denitrifiers in soils. Furthermore, O_2 has in large concentrations negative effects on nitrification (Chapter 4 and 5).

Since so far no inhibitor is known to specifically and reliably inhibit denitrification, a differentiation between nitrification, nitrifier denitrification, denitrification and other sources of N_2O in soils is not possible with inhibitors. One intrinsic problem of inhibitors is that they change the conditions in the soil. By inhibiting one process, they might influence the other processes taking place. Thus, denitrification might for example be limited by low availability of NO_3^- if nitrification is inhibited. As shown in Table 19, many nitrification inhibitors also affect other processes in soils. Thus, they are not completely specific. Furthermore, inhibitors cannot normally be applied directly to the soil in the field. They are used in incubation studies. While this can provide good opportunities to study a given soil under controlled conditions in the laboratory and also to manipulate these conditions, care has to be taken when the results are extrapolated to processes in the field. The soil has been removed from its natural surroundings and placed in artificial conditions, mostly without a crop. This change of system could influence the responses of the soil. The results need not necessarily be related to the processes in the undisturbed soil.

Stable isotopes

Stable isotopes have the advantage over inhibitors that they enable a study of N_2O producing processes directly in the field. Stable isotope analyses have already provided important new insights into many natural systems (e.g. Durka et al., 1994, Gebauer and Schulze, 1991, Hopkins et al., 1998). Isotopes are natural variants of the same element differing in their number of neutrons. Therefore, they differ in weight. Stable isotopes are contrasted to radioactive isotopes, which disintegrate over time. For N, the stable isotopes are ^{14}N and ^{15}N. The more frequent isotope is ^{14}N, which makes up 99.63% of the N pool. The remaining 0.37% is ^{15}N (Ehleringer and Rundel, 1989). In the study of N_2O, also two of the three stable isotopes of oxygen, ^{16}O and ^{18}O, are used. ^{16}O is the more common isotope (99.76%, Ehleringer and Rundel, 1989).

The study of the natural variation of stable isotopes in different pools uses the fact that most reactions prefer the use of the lighter isotope over the heavier (Moore, 1974, Krankowsky and Mauersberger, 1996, Gellene,

Equation 11 (for explanations see text):

$$\delta \mathrm{x} = \left(\frac{\mathrm{R_{Sample}}}{\mathrm{R_{Standard}}} - 1 \right) \cdot 1000 \left[‰ \right]$$

1996). Therefore, reaction products are normally depleted when the substrate is not fully consumed, i.e. they contain less heavy isotope (^{15}N and ^{18}O) than their substrate. These small changes in the composition are recorded in delta (δ) values (Equation 11). In these δ values, the ratio of the heavy to light isotope (e.g. $^{15}N/^{14}N$) of the sample (R_{Sample}) is compared to that of a standard ($R_{Standard}$). Since the changes in composition are small, δ values are given in per mille (Equation 11). A δ value of 0‰ means that this sample has the same composition as the standard. Positive δ values indicate enrichment, i.e. the sample contains more of the heavy isotope than the standard. Negative δ values indicate depletion. The standard for nitrogen is atmospheric nitrogen and for oxygen Vienna Standard Mean Ocean Water (V-SMOW).

Two different methods are used in the study of stable isotopes: the natural abundance method and the tracer method. As the name implies, the natural abundance method uses the natural abundances of the stable isotopes. Since different reactions discriminate differently between the isotopes, one can differentiate between different reactions leading to the same end product. In nitrification and denitrification, different sources of N and O are used for N_2O production. This makes it in theory possible to differentiate between N_2O from these sources. The N incorporated into N_2O from nitrification comes from NH_3 and should be more depleted than this source due to a strong fractionation by nitrifying bacteria and a strong fractionation in the NH_4^+/NH_3 equilibrium. Denitrification uses NO_3^- as an N pool. Thus, N_2O from denitrification should be more depleted in ^{15}N than the NO_3^- that is used for denitrification. Nitrifiers use soil O_2 to oxidize NH_3 to hydroxylamine (NH_2OH, Schmidt and Voerkelius, 1989). N_2O from nitrification should thus have δ values related to this source ($\delta^{18}O_{atmospheric\ oxygen}$ = 23.5‰, Durka et al., 1994). Denitrifiers use the O in NO_3^- for N_2O production. The NO_3^- can either come from a fertilizer source or from nitrification. In the latter case, the $\delta^{18}O$ value of the NO_3^- is originally between that for soil O_2 and that for soil water ($\delta^{18}O_{soil\ water}$ = -10.5 to -3‰), since nitrifiers use soil water for the oxidation of NH_2OH to NO_3^- (Durka et al., 1994). N_2O from nitrification has been reported to have a depletion of more than 60‰ against the substrate in pure culture experiments (Yoshida, 1988, Webster and Hopkins, 1996b). In soils, the isotope signatures of N_2O from nitrification yet remain to be measured accurately (Tilsner et al., 2002). Generally, nitrification has a larger isotope fractionation for ^{15}N than denitrification (median isotope fractionation: β=1.0185 for denitrification, β=1.0250 for nitrification; Bedard-Haughn, 2002). N_2O from denitrification in soils has been found to have δ values of about -40‰ for $\delta^{15}N$ and +3‰ for $\delta^{18}O$ (Tilsner et al., 2002, Voerkelius, 1990, Webster and Hopkins, 1996a). Further reduction of N_2O to N_2 can lead to an enrichment of the remaining N_2O (Wahlen and Yoshinari, 1985).

The tracer method introduces a tracer material that is labelled, i.e. artificially enriched, into the system. As the tracer makes its way through the system, different products will become enriched. Thus, by finding the enrichment in different pools, one can trace back the way the 'tracer' has taken. In the study of N_2O production, usually ^{15}N-labelled NO_3^- is added. Thus, ^{15}N-enriched N_2O will originate from denitrification. In tracer studies, the artificial enrichment exceeds any fractionation by several orders of magnitude. Therefore, fractionation dynamics do not have to be taken into account, and ^{15}N concentrations are often reported in atom% rather than in δ values.

While the differentiation between N_2O from nitrification and denitrification is relatively well established, especially in soils with large N_2O production (Tilsner et al., 2002), studies with stable isotopes have not often tried to also account for nitrifier denitrification. There are reasons for this. The sources of N and O for N_2O from nitrifier denitrification are very similar to those for N_2O from denitrification. Denitrifying nitrifiers use NO_2^- as N and O source, while denitrifiers use NO_3^- or NO_2^-. Since the pathways are also very similar, the fractionation should not be too different either. Thus, the δ values for N_2O from nitrifier denitrification and from denitrification will probably be too similar in both N and O to distinguish between these sources with stable isotopes. Therefore, the N_2O production from denitrification could be overestimated in stable isotope studies when the possibility of nitrifier denitrification is not taken into account. The production measured for nitrification is probably N_2O purely from nitrification, without nitrifier denitrification.

Pure cultures

Pure culture studies are not used to investigate the situation in soils, but to get to know more about the involved pathways and enzymes. They allow to manipulate the conditions in a controlled environment. Thus, they offer the possibility to study the complex soil processes in a simplified artificial system. In the study of nitrifier denitrification, the investigation of the amount of N_2O produced by nitrifiers in different conditions (e.g. low O_2, high NO_2^-) has led to new insights and provides opportunity for further studies. The use of transformants can also deepen our understanding of the involved pathways of N_2O production (Chapter 5). The indications found for a possible role of nitric oxide reductase in directing the reactions to NO_2^- production and for a possible unknown pathway of N_2O production by nitrifiers need to be evaluated in further studies.

One problem of pure culture studies is that the results are not directly related to what is happening in soil. The artificial environment with only one species, for example, eliminates influences that come from interactions with other organisms. For instance, NO_2^- accumulation

is normal in pure culture studies with ammonia oxidizers, but does not normally occur in soils. Furthermore, the complexity of the soil offers different microhabitats next to each other. Thus, reaction can occur simultaneously that require totally different conditions. Maybe constructed microcosm experiments could help to bridge some of the gap. The use of sterilized sand or soil as a substrate for pure culture studies could introduce some of the heterogeneity of the natural habitat. A mixture of different pure cultures could increase the interactions and make the results somewhat better comparable to the conditions in soil.

Our understanding of N_2O production in soils

I am now going to address the question of the consequences for our understanding of the N cycle. Have nitrifiers been underestimated as producers of N_2O?

The results of some studies investigating N_2O production by different sources are shown in Table 20. It becomes obvious that nitrifiers potentially produce substantial parts of the total N_2O. Most of the studies used nitrification inhibitors to differentiate between sources of N_2O. Thus, they consider denitrification as opposed to nitrification plus nitrifier denitrification. Other sources of N_2O production, like chemodenitrification or heterotrophic nitrification, are not distinguished and are –since they are not affected by the inhibitor– counted as part of the N_2O produced by denitrifiers. Due to these simplifications and to the use of nitrification inhibitors that might not necessarily have totally inhibited nitrification, denitrification might have been overestimated and the share of nitrifiers underestimated in some of these studies. Nitrapyrin has been shown to either stimulate of partially inhibit denitrification (Henninger and Bollag, 1976, Klemedtsson et al., 1988, Table 19). Therefore, nitrification plus nitrifier denitrification might have been either over- or underestimated in the study with nitrapyrin (Table 20). The study using the tracer method (Stevens et al., 1997) probably gives the most accurate idea of N_2O production by nitrification, without accounting for nitrifier denitrification. Thus, we see that nitrification can have a large influence on the N_2O production of a soil.

In Chapters 4 and 5, the question of how important nitrifier denitrification is as a source of N_2O by nitrifiers has been addressed. Different conclusions were drawn from the results of the two chapters. That was due to the different methodologies used. The results of Chapter 4 rely on the inhibition of nitrifier denitrification by O_2. In Chapter 5, O_2 was shown to also adversely affect nitrification. Therefore, nitrification was probably underestimated as a source of N_2O in Chapter 4. In Chapter 5, we came to the conclusion that nitrification might be more important for N_2O production than nitrifier denitrification. We saw also indications of another route of N_2O production in nitrifiers. This needs to be investigated in future studies. We have

to bear in mind, however, that in the transformed nitrifiers investigated in Chapter 5, some pathways that might be of minor importance in the wild-type might be enhanced as a way of compensating for the pathway that had been blocked. Generally, it is difficult to relate the results of pure culture experiments conducted under controlled laboratory conditions to the soil environment, as discussed in the previous section.

Table 20: Total N_2O production by different soils and percentage derived from nitrifiers (i.e. nitrification plus nitrifier denitrification).

Site description	Methodology	Total N_2O emission [different units]	N_2O emission from nitrifiers [% of total emission]	References
early successional forest	C_2H_2 inhibition	0.024-0.074 g N kg_{soil}^{-1} a^{-1}	3-40	Robertson and Tiedje, 1987
freely drained sandy loam, ryegrass-chickweed (greenhouse)	DCD inhibition	0.3-2.3 kg N ha^{-1} a^{-1}	40	Skiba et al., 1993
Douglas fir stand	nitrapyrin inhibition	0.005-0.04 g N kg_{soil}^{-1} a^{-1}	40-96	Martikainen and DeBoer, 1993
agricultural acid brown earth	^{15}N tracer method	0.005-0.051 g N kg_{soil}^{-1} a^{-1}	70	Stevens et al., 1997

Clearly, we do not know enough about N_2O production by nitrifiers yet to reach a definite conclusion of which pathway is the most important one for N_2O production. The contribution of nitrifiers to N_2O production has probably been underestimated rather than overestimated. The importance of nitrification and nitrifier denitrification as sources of N_2O will vary with the environmental conditions. Earlier studies have indicated that nitrifier denitrification was the most important pathway of N_2O production in nitrifiers subject to suboxic conditions (Ritchie and Nicholas, 1972, Poth and Focht, 1985). NO_2^- also is an important factor regulating N_2O production by nitrifiers (Chapter 3, Kester et al., 1997). Larger NO_2^- concentrations will most probably favour nitrifier denitrification (Ritchie and Nicholas, 1972, Poth and Focht, 1985).

Conclusions and outlook

To sum up, there are indications that nitrifiers might have been underestimated so far as producers of N_2O. While we cannot yet conclude whether nitrification or nitrifier denitrification is more important for N_2O production by nitrifiers, it is probable that both pathways are influenced by different conditions and will vary in importance in interaction with their environment. A measurement method is needed that enables a differentiation between nitrification, nitrifier denitrification and denitrification in soils.

Maybe it would be possible to combine the strong points of stable isotope measurements and nitrification inhibitors in one methodology. I have discussed above that stable isotopes probably give a good estimate of N_2O production by nitrification, while the part identified as N_2O from denitrification is probably a combination of N_2O from nitrifier denitrification and denitrification. If nitrification and nitrifier denitrification could be reliably inhibited, it would be possible to identify N_2O from denitrification alone. Then, one would have a good estimate of the amount and the δ values of N_2O from nitrification and from denitrification. The total amount of N_2O produced minus the parts produced by nitrification and denitrification would be the maximum amount that could have been produced by nitrifier denitrification and other sources like chemodenitrification. Shortcomings of the method are the need for soil incubations and inhibition, with all the involved disadvantages discussed above. Furthermore, a reliable inhibitor of nitrification and nitrifier denitrification would have to be found.

Further studies with pure cultures of nitrifiers are needed to understand the different pathways of N_2O production in these organisms. We found indications for another, so far unknown, route of N_2O production. This has to be further investigated. Studies of the enzymes of nitrifiers should provide new insights into the pathways. Genetically modified nitrifiers can be a strong tool in these studies. However, we have to keep in mind that the modification might have more effects than the ones intended.

While molecular biologists and microbiologists should try to gain more insights into the mechanisms of N_2O production by nitrifiers, further field studies should enhance our understanding of the factors influencing N_2O production in the field. Maybe from a global point of view, the differentiation between sources of N_2O is less important than the understanding of the factors and conditions favouring N_2O production. The use of models might provide new insights. So far, I am not aware of models that incorporate a term for nitrifier denitrification in the calculations. Of course, this is related to the many unknowns of this pathway. Since nitrifier denitrification can potentially be an important source of N_2O in

some soils, process-based models should at least try to incorporate N_2O produced by nitrifier denitrification into the calculations.

In general, a better cooperation and communication between scientists from different fields working on N_2O production should offer great opportunities to enhance our understanding of the production of this gas that has so strong implications on different areas of human well-being.

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SUMMARY

Nitrous oxide (N_2O) is an important greenhouse gas. At present, it causes 6% of global warming. The atmospheric concentration of N_2O continues to increase at a rate of 0.8 ppb per year. The main known sink of N_2O is its destruction in the stratosphere to nitric oxide (NO). Via that destruction product, N_2O contributes to the decomposition of stratospheric ozone.

The most important sources of N_2O are the microbial soil processes nitrification and denitrification. Especially after fertilization of the soil, large amounts of N_2O can be emitted. Nitrifiers produce N_2O by nitrification and by nitrifier denitrification. In nitrification, N_2O develops during the oxidation of hydroxylamine (NH_2OH). In nitrifier denitrification, nitrifiers reduce nitrite (NO_2^-) via N_2O to N_2. Not much is known about nitrifier denitrification yet. The discovery of several intermediates and enzymes is in line with a suspected similarity between nitrifier denitrification and denitrification. Denitrifiers reduce nitrate (NO_3^-) to N_2. N_2O is an intermediate in that process. It is important to be able to differentiate between N_2O produced by the different processes in soils, since they are influenced by different factors. Only with a profound knowledge of the sources is a mitigation of N_2O emission from soils possible.

The objectives of this study were to quantitatively assess N_2O production by nitrifier denitrification under a range of conditions and to come up with a best estimate for N_2O produced by nitrifier denitrification in The Netherlands. A review of nitrifier denitrification and related processes in soils (Chapter 2) revealed how important it is to get to know more about this poorly studied pathway. Up to 30% of the total N_2O production in soils has been attributed to nitrifier denitrification. Especially low oxygen (O_2) conditions coupled with low organic carbon contents might favour this pathway. It was concluded that there was a need to quantify the N_2O production by nitrifier denitrification under different conditions. Therefore, a soil study was carried out with different soils in a range of conditions. Rather than leading to new quantitative insights, this study gave rise to questions concerning the prevailing measurement method for nitrifier denitrification (Chapter 3). In this method, the differentiation between nitrification, nitrifier denitrification, denitrification and other soil sources of N_2O is based on incubations with combinations of 0.02 kPa acetylene (C_2H_2) and 100 kPa O_2. C_2H_2 is supposed to inhibit nitrification and nitrifier denitrification without

influencing denitrification, and O_2 is supposed to inhibit nitrifier denitrification and denitrification, without affecting nitrification. However, this method did not seem to be suitable for all soils. In some conditions, the addition of inhibitors seemed to stimulate the production of N_2O compared to the controls. Furthermore, negative fluxes were calculated for some sources of N_2O, especially for nitrifier denitrification (Chapter 3). Due to these methodological difficulties, the objectives of this study were adapted and became i) to test the prominent methodology for quantifying the N_2O production by nitrifier denitrification, and ii) to assess the importance of nitrifier denitrification for N_2O production in pure cultures of *Nitrosomonas europaea* and *Nitrosospira briensis*. *N. europaea* is often used as a model organism in laboratory studies. It has frequently been found in environments high in N like water treatment plants. *N. briensis* is better adapted to environments less abundant in N and is common in a number of fertilized arable soils of neutral pH.

The first objective has been addressed in Chapter 3, 4 and 5. We have seen in Chapter 3 that the prevailing measurement method using the inhibitors C_2H_2 (0.02 kPa) and O_2 (100 kPa) in different combinations to quantify the N_2O production by nitrifier denitrification was not suitable for all soils. Pure culture studies revealed some reasons for the observed problems (Chapter 4 and 5). O_2 was not suitable as an inhibitor of nitrifier denitrification, since it also had a negative effect on ammonia oxidation, the first step of nitrification (Chapter 4 and 5). C_2H_2 only inhibited the N_2O production by *N. europaea*, but not that by *N. briensis* (Chapter 4). C_2H_2 did furthermore not inhibit the N_2O production by a transformant of *N. europaea* lacking nitric oxide reductase, an enzyme catalyzing the reduction of nitric oxide to N_2O in the nitrifier denitrification pathway (Chapter 5). While it is not clear yet whether the reason for the insensitivity to C_2H_2 was the same in the transformant and in *N. briensis*, we can conclude that C_2H_2 was not reliable as an inhibitor of N_2O production by all nitrifiers.

Due to the consistent results of soil studies and pure culture experiments, we reach the conclusion that the method using C_2H_2 and O_2 is not suitable for differentiating reliably between sources of N_2O in soils. In the past, especially C_2H_2 has been used extensively to differentiate between nitrification and denitrification in soils. If C_2H_2 does not inhibit N_2O production by nitrifiers reliably, the share of nitrifiers in N_2O production might have been underestimated in these studies.

The importance of nitrifier denitrification for N_2O production has been studied in pure culture experiments (Chapter 4 and 5). In Chapter 4, a study of the production of N_2O by pure cultures of *N. europaea* and *N. briensis* is described. Large concentrations (100 kPa) of O_2 were used to inhibit nitrifier denitrification. The results suggested that nitrifier denitrification was the most important pathway in this respect, causing about 80% of the N_2O production by

N. europaea and about 65% of that by *N. briensis*. However, there were indications that nitrification might have been underestimated due to adverse effects of O_2 on ammonia oxidation. In Chapter 5, the N_2O production was studied in mutants of *N. europaea* that were deficient in either nitrite reductase (NirK) or nitric oxide reductase (NORB), two enzymes of the nitrifier denitrification pathway. The NirK-deficient cells produced similar amounts of N_2O as the wild-type. Since the NirK-deficient cells could not have produced this N_2O via the known pathway of nitrifier denitrification, this result suggests that nitrifier denitrification is not so important for N_2O production in this mutant. The NORB-deficient cells produced even more N_2O, about 60 times as much as the wild-type. At the same time, the NORB-deficient cells consumed NO_2^-. While side-effects of the mutation on pathways of N_2O production cannot be excluded, there are indications for a role of the enzyme NORB in directing ammonia oxidation towards NO_2^- rather than N_2O. Large concentrations of O_2 inhibited the N_2O production and NO_2^- consumption in this mutant and might therefore be able to fulfil a role similar to NORB in directing the reaction to NO_2^-. The N_2O production of the NORB-deficient cells was not inhibited by C_2H_2. This could hint at an unknown pathway of N_2O production in nitrifiers (Chapter 5).

A sensitivity analysis (Chapter 6) revealed that an inhibition of the N_2O reductase of denitrifiers by C_2H_2 most likely caused some of the observed over- and underestimations of sources of N_2O in the soil survey. Furthermore, it is likely that C_2H_2 only inhibited part of nitrification and nitrifier denitrification and that O_2 also partly inhibited nitrification in the soil. This suggests that nitrifiers have probably been underestimated as producers of N_2O in studies using C_2H_2 and O_2 as inhibitors. Future studies should further investigate the pathways of N_2O production, including the indicated possible unknown pathway of nitrifiers. A combination of stable isotope studies of N and O and incubation studies with inhibitors might enable the differentiation between sources of N_2O in soils. Since this study shows that 0.02 kPa C_2H_2 and 100 kPa O_2 are not suitable as inhibitors of different N_2O producing processes, alternatives need to be found.

SAMENVATTING

Lachgas (N_2O) is een belangrijk broeikasgas. Het veroorzaakt tegenwoordig 6% van de globale opwarming. De concentratie van N_2O in de atmosfeer blijft onverminderd met 0.8 ppb (parts per billion, 10^{-9}) per jaar groeien. Het belangrijkste bekende mechanisme om N_2O te verwijderen is zijn destructie in de stratosfeer tot stikstofmonoxide (NO). Via dit product is N_2O ook verantwoordelijk voor de afbraak van de stratosferische ozonlaag.

De meest belangrijke bronnen van N_2O zijn de microbiële bodemprocessen nitrificatie en denitrificatie. Vooral na bemesting van de grond kunnen grote hoeveelheden N_2O vrij komen. Nitrificeerders produceren N_2O door nitrificatie en door nitrificeerder-denitrificatie. Bij nitrificatie wordt N_2O tijdens de oxidatie van hydroxylamine (NH_2OH) ontwikkeld. In nitrificeerder-denitrificatie reduceren nitrificeerders nitriet (NO_2^-) via N_2O tot N_2. Over nitrificeerder-denitrificatie is nog niet veel bekend. De ontdekking van verschillende tussenproducten en enzymen ondersteunt een veronderstelde similariteit van nitrificeerder-denitrificatie en denitrificatie. Denitrificeerders reduceren nitraat (NO_3^-) tot N_2. N_2O is een tussenproduct van dit process. Het is belangrijk dat er onderscheid gemaakt kan worden tussen N_2O-productie van de verschillende bronnen, omdat de bronnen door verschillende factoren beïnvloed worden. Alleen met een solide kennis van de bronnen kan N_2O-emissie uit de bodem worden voorkomen.

De doelstellingen van dit onderzoek waren de N_2O-productie door nitrificeerder-denitrificatie in uiteenlopende omstandigheden te kwantificeren en een schatting te maken van de N_2O-productie door nitrificeerder-denitrificatie in Nederland. Een literatuurstudie over nitrificeerder-denitrificatie en gerelateerde processen in de bodem (Hoofdstuk 2) maakte duidelijk hoe belangrijk het is om meer over deze weinig bestudeerde bron te weten te komen. Tot aan 30% van de totale N_2O-productie in de grond werdt toegeschreven aan nitrificeerder-denitrificatie. Vooral lage zuurstofconcentraties gekoppeld met lage organische koolstofconcentraties zouden deze bron kunnen bevorderen. De conclusie was dat het nodig was de N_2O-productie door nitrificeerder-denitrificatie in verschillende omstandigheden te kwantificeren. Hiervoor werd een bodemonderzoek uitgevoerd met verschillende grondsoorten in combinatie met uiteenlopende omstandigheden. In plaats van nieuwe kwantitatieve kennis leverde dit onderzoek echter vragen op over de tot dusver algemeen geaccepteerde meetmethode voor nitrificeerder-denitrificatie (Hoofdstuk 3). In deze methode

wordt onderscheid gemaakt tussen nitrificatie, nitrificeerder-denitificatie, denitrificatie en andere bronnen van N_2O in de grond door middel van incubaties met combinaties van 0.02 kPa acetyleen (C_2H_2) en 100 kPa zuurstof (O_2). Verondersteld wordt dat C_2H_2 de nitrificatie en nitrificeerder-denitrificatie remt zonder invloed te hebben op de denitrificatie en dat O_2 nitrificeerder-denitrificatie en de denitrificatie remt zonder invloed te hebben op de nitrificatie. Deze methode bleek echter niet in alle gronden te werken. Onder sommige omstandigheden leek de toevoeging van remmers de N_2O-productie ten opzichte van de controle te stimuleren. Bovendien werd voor sommige bronnen van N_2O een negatieve flux berekend, en dit gold in het bijzonder vaak voor nitrificeerder-denitrificatie (Hoofdstuk 3). Vanwege deze analytische moeilijkheden werden de doelstellingen van dit onderzoek aangepast. De nieuwe doelstellingen werden i) het testen van de gangbare meetmethode waarmee N_2O-productie door nitrificeerder-denitrificatie kan worden gekwantificeerd en ii) het schatten van het belang van nitrificeerder-denitrificatie voor N_2O-productie in reincultures van *Nitrosomonas europaea* en *Nitrosospira briensis*. *N. europaea* wordt vaak als model in laboratoriumstudies gebruikt. Deze nitrificeerder wordt vaak in stikstofrijke milieus gevonden zoals bijvoorbeeld waterzuiveringsinstallaties. *N. briensis* is meer aangepast aan een stikstofarmere omgeving en komt vaak voor in bemeste landbouwgronden met neutrale pH.

De eerste doelstelling werd in de hoofdstukken 3, 4 en 5 behandeld. We hebben in Hoofdstuk 3 gezien dat de gangbare meetmethode die gebruik maakt van verschillende combinaties van de remmers C_2H_2 (0.02 kPa) en O_2 (100 kPa) om de N_2O-productie door nitrificeerder-denitrificatie te meten, niet geschikt is voor alle gronden. Studies met reincultures leverden inzichten op over vastgestelde problemen (Hoofdstuk 4 en 5). O_2 was niet geschikt als remmer van nitrificeerder-denitrificatie, omdat het ook een negatief effect had op ammonia-oxidatie, de eerste stap van nitrificatie (Hoofdstuk 4 en 5). C_2H_2 remde alleen de N_2O-productie van *N. europaea*, maar niet die van *N. briensis* (Hoofdstuk 4). Bovendien remde C_2H_2 ook niet de N_2O-productie van een mutant van *N. europaea* die geen stikstofmonoxidereductase had, een enzym dat de reductie van stikstofmonoxide na N_2O in nitrificeerder-denitrificatie katalyseert (Hoofdstuk 5). Hoewel nog niet duidelijk is of de redenen voor de ongevoeligheid voor C_2H_2 in de mutant en in *N. briensis* dezelfde waren, kunnen we toch concluderen dat C_2H_2 niet betrouwbaar is als remmer van de N_2O-productie door alle nitrificeerders.

Op grond van de consistente resultaten van de studies met grond enerzijds en die met reincultures anderzijds concluderen we dat de methode die gebruik maakt van C_2H_2 en O_2 niet geschikt is om betrouwbaar onderscheid te kunnen maken tussen bronnen van N_2O in de grond. Tot nu toe werd vooral C_2H_2 vaak gebruikt om nitrificatie van denitrificatie in de

grond te onderscheiden. Als C_2H_2 de N_2O productie door nitrificeerders niet betrouwbaar remt, zijn nitrificeerders in deze studies mogelijk onderschat als bron van N_2O.

Het belang van nitrificeerder-denitrificatie voor de N_2O-productie werd in experimenten met reincultures bestudeerd (Hoofdstuk 4 en 5). In Hoofdstuk 4 wordt een studie over de N_2O-productie door reincultures van *N. europaea* en *N. briensis* beschreven. Hoge concentraties (100 kPa) O_2 werden gebruikt om de nitrificeerder-denitrificatie te remmen. De resultaten suggereren dat nitrificeerder-denitrificatie de meest belangrijke bron voor N_2O productie was. Ongeveer 80% van de N_2O-productie van *N. europaea* en ongeveer 65% van die van *N. briensis* werd veroorzaakt door nitrificeerder-denitrificatie. Er waren echter ook indicaties dat nitrificatie hier door tegenwerkende effecten van O_2 op de ammonia-oxidatie onderschat werd. In Hoofdstuk 5 werd de N_2O-productie in mutanten van *N. europaea* bestudeerd die geen nitrietreductase (NirK) of stikstofmonoxidereductase (NORB) hadden; twee enzymen aangetroffen bij de nitrificeerder-denitrificatie. De NirK-deficiënte cellen produceerden vergelijkbare hoeveelheiden N_2O als het wildtype. Omdat de NirK-deficiënte cellen dit N_2O niet via de bekende weg van nitrificeerder-denitrificatie gevormd kunnen hebben, suggereren deze resultaten dat nitrificeerder-denitrificatie niet zo belangrijk is voor de N_2O-productie door deze mutant. De NORB-deficiënte cellen produceerden zelfs nog meer N_2O, ongeveer 60 keer zoveel als het wildtype. Tegelijkertijd consumeerden de NORB-deficiënte cellen NO_2^-. Hoewel we nevenverschijnselen van de mutatie op de N_2O-productie door nitrificeerders niet kunnen uitsluiten, zijn er indicaties dat het enzym NORB ammonia-oxidatie in de richting van NO_2^- stuurt en niet naar N_2O. Hoge concentraties O_2 remden de N_2O-productie en de NO_2^--consumptie in deze mutant en zouden dus mogelijk ook in staat kunnen zijn de reactie net als NORB in richting van NO_2^- te sturen. De N_2O-productie van de NORB-deficiënte cellen werd niet door C_2H_2 geremd. Dit zou kunnen wijzen op een tot nog toe onbekende weg van N_2O-productie door nitrificeerders (Hoofdstuk 5).

Een gevoeligheidsanalyse (Hoofdstuk 6) maakte duidelijk dat de over- en onderschattingen van bronnen van N_2O die in de proeven met grond geconstateerd werden, waarschijnlijk veroorzaakt werden door een remming van de N_2O-reductase van denitrificeerders door C_2H_2. Bovendien is het waarschijnlijk dat C_2H_2 maar een deel van nitrificatie en nitrificeerder-denitrificatie remde en dat O_2 ook een deel van de nitrificatie in de grond remde. Dit doet vermoeden dat in studies die gebruik maakten van C_2H_2 en O_2 als remmers de nitrificeerders waarschijnlijk onderschat werden als produceerders van N_2O. Toekomstige studies zouden de verschillende wegen van N_2O-productie verder moeten bestuderen, ook de aangewezen mogelijke onbekende weg bij nitrificeerders. Met behulp van een combinatie van metingen van de stabiele isotopen van N en O en incubatieproeven met remmers kan er misschien

onderscheid gemaakt worden tussen bronnen van N_2O in de grond. Aangezien deze studie laat zien dat 0.02 kPa C_2H_2 en 100 kPa O_2 niet geschikt zijn als remmers van verschillende N_2O-produceerende processen, moeten alternatieve methoden gevonden worden.

ZUSAMMENFASSUNG

Lachgas (N_2O) ist ein wichtiges Treibhausgas. Es verursacht momentan 6% der globalen Erwärmung. Die N_2O-Konzentration in der Atmosphäre steigt mit einer Rate von 0.8 ppb (parts per billion, 10^{-9}) pro Jahr. Die wichtigste bekannte Senke von N_2O ist sein Abbau in der Stratosphäre zu Stickstoffmonoxid (NO). Über dieses Produkt ist N_2O auch an der Zerstörung der stratosphärischen Ozonschicht beteiligt.

Die wichtigsten Quellen von N_2O sind die mikrobiellen Bodenprozesse Nitrifikation und Denitrifikation. Besonders nach Düngung des Bodens können hier große Mengen N_2O frei kommen. Nitrifizierer produzieren N_2O bei der Nitrifikation und der Nitrifizierer-Denitrifikation. Bei der Nitrifikation wird N_2O bei der Oxidation von Hydroxylamin (NH_2OH) produziert. Bei der Nitrifizierer-Denitrifikation reduzieren Nitrifizierer Nitrit (NO_2^-) über N_2O zu N_2. Bisher ist nicht viel über die Nitrifizierer-Denitrifikation bekannt. Die Entdeckung verschiedener Zwischenprodukte und Enzyme bestätigt die Vermutung, daß Nitrifizierer-Denitrifikation und Denitrifikation ähnlich verlaufen. Denitrifizierer reduzieren Nitrat (NO_3^-) zu N_2. N_2O ist ein Zwischenprodukt in diesem Prozess. Es ist wichtig, zwischen den verschiedenen Quellen von N_2O im Boden unterscheiden zu können, da sie von unterschiedlichen Faktoren beeinflußt werden. Die Emission von N_2O aus dem Boden kann nur mit solider Kenntnis der Quellen vermieden werden.

Die Ziele dieser Arbeit waren es, die N_2O-Produktion durch Nitrifizierer-Denitrifikation unter verschiedenen Bedingungen quantitativ zu erfassen und die Höhe der N_2O-Produktion durch Nitrifizierer-Denitrifikation in den Niederlanden abzuschätzen. Eine Literaturübersicht über Nitrifizierer-Denitrifikation und verwandte Bodenprozesse (Kapitel 2) machte deutlich, wie wichtig es ist, mehr über diesen wenig erforschten Weg zu erfahren. Bis zu 30% der gesamten N_2O-Produktion im Boden wurden bisher der Nitrifizierer-Denitrifikation zugeschrieben. Insbesondere könnten niedrige Sauerstoffkonzentrationen gekoppelt mit niedrigem Kohlenstoffgehalt diesen Weg der N_2O-Produktion fördern. Es wurde gefolgert, daß es notwendig ist, die N_2O-Produktion durch Nitrifizierer-Denitrifikation unter unterschiedlichen Bedingungen zu quantifizieren. Daher wurde eine Studie mit verschiedenen Böden unter unterschiedlichen Bedingungen durchgeführt. Statt der erhofften neuen Kenntnis gab diese Studie jedoch Anlaß dazu, die vorherrschende Meßmethode für Nitrifizierer-Denitrifikation kritisch zu hinterfragen (Kapitel 3). In dieser Methode wird zwischen Nitrifikation,

Nitrifizierer-Denitrifikation, Denitrifikation und anderen Bodenquellen von N_2O unterschieden, indem unterschiedliche Inkubationen mit 0.02 kPa Azetylen (C_2H_2) und 100 kPa Sauerstoff (O_2) durchgeführt werden. C_2H_2 soll die Nitrifikation und Nitrifizierer-Denitrifikation stoppen, ohne die Denitrifikation zu beeinflussen, und O_2 die Nitrifizierer-Denitrifikation und Denitrifikation, ohne die Nitrifikation zu beeinflussen. Diese Methode schien jedoch nicht für alle Böden geeignet zu sein. Unter bestimmten Bedingungen schien die Zugabe der Inhibitoren die N_2O-Produktion gegenüber den Kontrollen zu stimulieren. Im übrigen wurden für einige N_2O-Quellen negative Flüsse berechnet, besonders häufig für Nitrifizierer-Denitrifikation (Kapitel 3). Aufgrund dieser methodischen Schwierigkeiten wurden die Ziele dieser Arbeit angepaßt. Die neuen Zielsetzungen wurden i) das Testen der vorherrschenden Meßmethode für die Quantifikation der N_2O-Produktion durch Nitrifizierer-Denitrifikation und ii) die Abschätzung der Bedeutung von Nitrifizierer-Denitrifikation für die N_2O-Produktion in Experimenten mit Reinkulturen von *Nitrosomonas europaea* und *Nitrosospira briensis*. *N. europaea* wird oft als Modell in Laborstudien verwendet. Dieser Nitrifizierer wurde häufig in einem stickstoffreichen Milieu angetroffen, wie zum Beispiel Wasseraufbereitungsanlagen. *N. briensis* ist besser an Umgebungen angepaßt, die weniger Stickstoff enthalten und kommt häufig in gedüngten Ackerböden mit neutralem pH vor.

Das erste Ziel dieser Arbeit wurde in den Kapiteln 3, 4 und 5 behandelt. Wir haben in Kapitel 3 gesehen, daß die vorherrschende Meßmethode, die die Inhibitoren C_2H_2 (0.02 kPa) und O_2 (100 kPa) in verschiedenen Kombinationen verwendet, um die N_2O-Produktion von Nitrifizierer-Denitrifikation zu quantifizieren, nicht für alle Böden geeignet ist. Studien mit Reinkulturen zeigten einige Gründe für die beobachteten Probleme auf (Kapitel 4 und 5). O_2 war ungeeignet als Inhibitor der Nitrifizierer-Denitrifikation, da es auch einen negativen Effekt auf die Ammoniumoxidation, den ersten Schritt der Nitrifikation, hatte (Kapitel 4 und 5). C_2H_2 stoppte nur die N_2O-Produktion von *N. europaea*, nicht jedoch die von *N. briensis* (Kapitel 4). Im übrigen hatte C_2H_2 keinen Einfluß auf die N_2O-Produktion in einer Mutante von *N. europaea*, die keine Stickstoffmonoxidreduktase enthält, ein Enzym, das die Reduktion von NO zu N_2O in der Nitrifizierer-Denitrifikation katalysiert (Kapitel 5). Während noch nicht klar ist, ob die Gründe für die Unwirksamkeit von C_2H_2 in der Mutante dieselben waren wie bei *N. briensis*, können wir doch schließen, daß C_2H_2 kein verläßlicher Inhibitor der N_2O-Produktion von allen Nitrifizierern ist.

Durch die übereinstimmenden Resultate der Experimente mit Boden und mit Reinkulturen kommen wir zu dem Ergebnis, daß die Methode, die C_2H_2 und O_2 verwendet, um zwischen verschiedenen Quellen von N_2O in Böden zu unterscheiden, nicht verläßlich ist. Bisher wurde vor allem C_2H_2 oft verwendet, um zwischen Nitrifikation und Denitrifikation in Böden zu

unterscheiden. Wenn C_2H_2 die N_2O-Produktion durch Nitrifizierer nicht verläßlich hemmt, könnte der Anteil der Nitrifizierer an der N_2O-Produktion in diesen Studien unterschätzt worden sein.

Die Bedeutung der Nitrifizierer-Denitrifikation für die N_2O-Produktion wurde in Reinkultur-Experimenten erforscht (Kapitel 4 und 5). In Kapitel 4 wird eine Studie zur N_2O-Produktion in Reinkulturen von *N. europaea* und *N. briensis* beschrieben. Hohe Konzentrationen (100 kPa) von O_2 wurden angewandt, um die Nitrifizierer-Denitrifikation zu stoppen. Die Ergebnisse deuten darauf hin, daß Nitrifizierer-Denitrifikation hier die wichtigste Quelle von N_2O war. Sie verursachte etwa 80% der N_2O-Produktion von *N. europaea* und etwa 65% der von *N. briensis*. Es gab jedoch auch Hinweise darauf, daß die Nitrifikation durch nachteilige Einflüsse von O_2 auf die Ammoniumoxidation unterschätzt wurde. In Kapitel 5 wurde die N_2O-Produktion von Mutanten von *N. europaea* untersucht, bei denen ein Enzym der Nitrifizierer-Denitrifikation ausgeschaltet worden war, und zwar entweder Nitritreduktase (NirK) oder Stickstoffmonoxidreduktase (NORB). Die NirK-defizienten Zellen produzierten ähnliche Mengen N_2O wie der Wildtyp. Da die NirK-defizienten Zellen N_2O nicht über den bekannten Weg der Nitrifizierer-Denitrifikation produziert haben können, legen diese Ergebnisse nahe, daß Nitrifizierer-Denitrifikation in dieser Mutante für die N_2O-Produktion nicht so wichtig war. Die NORB-defizienten Zellen produzierten sogar mehr N_2O, etwa 60 mal so viel wie der Wildtyp. Gleichzeitig konsumierten die NORB-defizienten Zellen NO_2^-. Während Nebenwirkungen der Mutation auf die Wege der N_2O-Produktion nicht ausgeschlossen werden können, gab es Hinweise dafür, daß das Enzym NORB die Ammoniumoxidation in die Richtung von NO_2^- lenkt, statt zu N_2O. Hohe Konzentrationen von O_2 hemmten die N_2O-Produktion und den Verbrauch von NO_2^- und könnten daher in der Lage sein, eine ähnliche Rolle wie NORB in der Ausrichtung der Reaktion auf NO_2^- zu spielen. Die N_2O-Produktion der NORB-defizienten Zellen wurde nicht durch C_2H_2 gestoppt. Dies könnte auf einen unbekannten Weg der N_2O-Produktion in Nitrifizierern weisen (Kapitel 5).

Eine Sensitivitätsanalyse (Kapitel 6) machte deutlich, daß die Über- und Unterschätzung von N_2O-Quellen, die in den Experimenten mit Boden konstatiert wurde, wahrscheinlich durch eine Hemmung der N_2O-Reduktase von Denitrifizierern verursacht wurde. Außerdem ist es wahrscheinlich, daß C_2H_2 nur einen Teil der Nitrifikation und Nitrifizierer-Denitrifikation hemmte und daß auch O_2 nur einen Teil der Nitrifikation im Boden hemmte. Dies läßt vermuten, daß Nitrifizierer in Studien, die C_2H_2 und O_2 als Inhibitoren genutzt haben, als N_2O-Produzenten unterschätzt worden sind. Weiterführende Studien sollten die Wege der N_2O-Produktion weiter beleuchten, auch den aufgezeichneten möglicherweise unbekannten

Weg bei Nitrifizierern. Eine Kombination der Messung der stabilen Isotope von N und O mit Inkubationsexperimenten mit Inhibitoren ermöglicht eventuell die Unterscheidung zwischen N_2O-Quellen im Boden. Da die vorliegende Studie zeigt, daß 0.02 kPa C_2H_2 und 100 kPa O_2 für die Hemmung verschiedener N_2O-produzierender Prozesse im Boden nicht geeignet sind, müssen alternative Methoden hierfür gefunden werden.

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CURRICULUM VITAE

Nicole Wrage werd geboren op 3 oktober 1973 in Hagen (Duitsland). In 1993 behaalde zij haar Abitur aan de Hildegardis Schule te Hagen. Van 1993 tot 1995 studeerde zij Biologie aan de Heinrich-Heine-Universiteit te Düsseldorf. Van 1995 tot 1996 studeerde zij aan de universiteit van Plymouth (Engeland) het programma 'Environmental Biology with Resources, Manufacturing and the Environment'. Zij vervolgde haar studie Biologie aan de Universiteit Bayreuth (Duitsland), waar zij eind januari 1999 afstudeerde met als hoofdvak Plantenecologie en de bijvakken Dierecologie en Milieumanagement. Vanaf 1 februari 1999 was Nicole aangesteld als assistent in opleiding bij de sectie Bodemkunde en Plantenvoeding (nu Bodemkwaliteit) van Wageningen Universiteit. Hier deed zij onderzoek naar de productie van lachgas door nitrificeerder denitrificatie. De belangrijkste resultaten van dit onderzoek staan in dit proefschrift beschreven. Naast haar promotieonderzoek is Nicole in 2000 een MSc opleiding Politieke Wetenschappen, Rechten en Economie aan de Open Universiteit Hagen (Duitsland) begonnen.

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