On the origin of nitrous oxide and its oxygen

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Abstract

Nitrous oxide (N_2O) is a greenhouse gas that contributes to global warming and the destruction of stratospheric ozone. To reduce N_2O emissions to the atmosphere it is important to understand how and where it is produced. This research aimed to identify the presence and importance of an hitherto elusive N_2O production pathway named 'nitrifier denitrification'. The potential of this pathway had been identified in pure cultures, but experimental proof of the presence of nitrifier denitrification in actual soils remained inconclusive due to the lack of adequate methodology.

A promising approach to distinguish nitrifier denitrification from other N_2O production pathways (nitrification and denitrification) was based on tracing the stable isotopes of oxygen (O) and nitrogen (N) in N_2O. However, this approach did not account for the effect of O exchange between H_2O and intermediates of the N_2O production pathways. Our literature review suggests that such O exchange may likely be present in soil and aquatic environments. In soil incubation experiments using O and N tracing, we showed that O exchange can indeed strongly determine the O isotopic composition of N_2O. We quantified O exchange for denitrification of NO_3^- to N_2O, and deduced that O can occur during nitrifier pathways of N_2O production as well. Next to N_2O, we demonstrated that the O isotopic signature of NO_3^- in soil could also be affected by O exchange.

Accounting for O exchange, we subsequently developed a novel dual isotope approach to study N_2O production pathways in soil. We therewith showed for the first time that nitrifier denitrification can indeed be a production pathway of N_2O *in soils*. We further studied how environmental controls of N_2O may affect the individual pathways differently, and showed that nitrifier denitrification responds idiosyncratically to soil moisture content.

In conclusion, the revealed significance of O exchange between H_2O and intermediates of N_2O production in soil has serious implications for source determination of N_2O and NO_3^- in ecosystems. The acknowledgement of nitrifier denitrification as distinct N_2O production pathway in soil is an important step forward in our understanding of N_2O production to ultimately obtain accurate inventories and effective mitigation strategies for N_2O emissions.

Contents

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Chapter 1

General Introduction

Nitrous oxide in our environment

1

Despite its popular name (laughing gas), nitrous oxide (N_2O) is a serious matter. The contribution of atmospheric N_2O to global warming and its ability to breakdown stratospheric ozone (Crutzen, 1981; Duxbury et al., 1993) are of great concern to our environment. Alarmingly, atmospheric concentrations of N_2O have been and still are steadily rising since the start of the industrial era. Nitrous oxide has now become the prime ozone depleting emission (Ravishankara et al., 2009) and the third most important anthropogenic greenhouse gas (IPCC, 2007), with a global warming potential approximately 300 times higher than CO_2 (Ramaswamy et al., 2001). Evidently, the continuous increase in N_2O emissions to our atmosphere constitutes a major environmental concern. To stabilize the current atmospheric concentrations of N_2O, emissions would need to be reduced by about 50% (IPCC, 2007). Adequate mitigation of these emissions is only possible if we understand the processes that produce N_2O. In other words, studying the origin of N_2O is of global environmental concern.

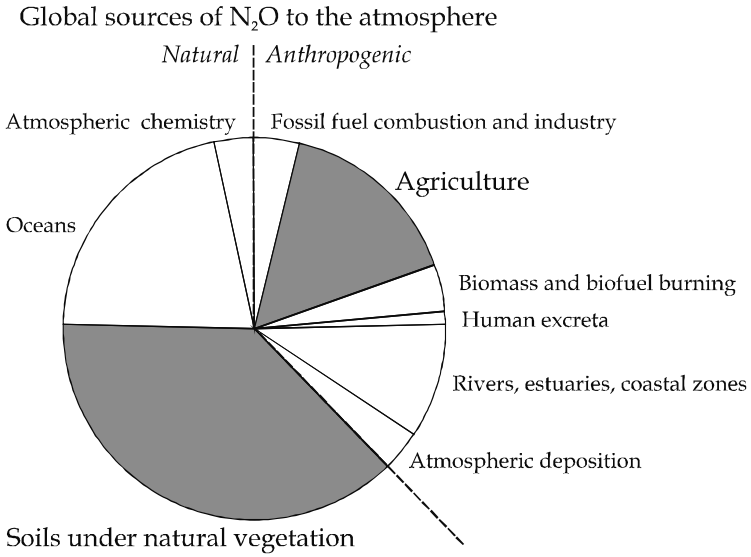


Figure 1.1: Global anthropogenic and natural sources of nitrous oxide (N_2O) to the atmosphere (total budget: 17.7 Tg N yr^{-1}). Soils (in grey compartments) globally comprise the largest source of N_2O (IPCC, 2007; Ravishankara et al., 2009).

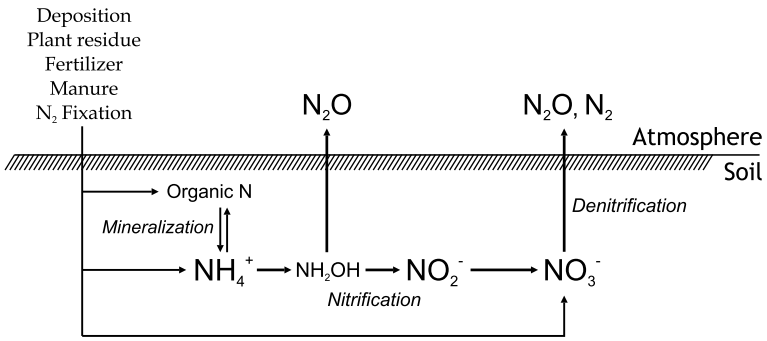


Figure 1.2: Schematic presentation of soil nitrogen (N) cycling and the main pathways of N_2O production in soil.

Nitrogen cycling and N_2O production in soils

Nitrous oxide is produced through several processes in the nitrogen (N) cycle, related to the cycling of reactive N. Reactive N refers to organic and inorganic forms of N that are biologically, photochemically and/or radiatively active, in contrast to the vast but inert atmospheric dinitrogen (N_2) pool (Galloway et al., 2008). Globally, **soils** comprise the largest of all anthropogenic and natural sources of N_2O to the atmosphere (Figure 1.1) (IPCC, 2007; Ravishankara et al., 2009). Reactive N enters the soil through atmospheric deposition, fertilizer and manure applications, plant residues, and biological nitrogen (N_2) fixation (Figure 1.2). Organic N can be broken down by microorganisms to (inorganic) ammonium (NH_4^+) through 'mineralization'. This is an important step in making organic N available for plants and microorganisms. Microorganisms can take up NH_4^+ and convert it to nitrite (NO_2^-) and nitrate (NO_3^-) by 'nitrification'. Through 'denitrification', microorganism turn NO_3^- again to (gaseous) N_2 (Figure 1.2).

Nitrous oxide can be formed through several biochemical processes. In soils, nitrification and denitrification are conventionally considered as the prime N_2O production processes (Figure 1.2) (Mosier et al., 1998b; Pérez et al., 2001). Nitrification is carried out by autotrophic bacteria that (i) oxidize ammonia (NH_3, in equilibrium with NH_4^+) via hydroxylamine (NH_2OH) to nitrite (NO_2^-) (ammonia oxidizers), and (ii) oxidize NO_2^- further to nitrate (NO_3^-) (nitrite oxidizers) (Paul et al., 1996). During the first step of nitrification, N_2O can be released as a by-product of ammonia oxidation (Hooper et al., 1979).

Denitrification is performed by heterotrophic bacteria that use NO_3^- as electron acceptor when O_2 is not available. They reduce NO_3^- via NO_2^-, nitric oxide (NO), and N_2O to N_2 (Knowles, 1982; Zumft, 1997). Nitrous oxide can be the end-product, and/or escape to the atmosphere as an intermediate compound before it is completely reduced to N_2.

To enable the development of accurate N_2O emission inventories and effective mitigation strategies for N_2O emissions, we need to understand how both total N_2O emissions and its individual production processes are affected by environmental factors, soil properties and land use. Main factors controlling total N_2O production in soil include aerobicity and related moisture content, carbon and nitrogen availability and pH (Knowles, 1982; Firestone et al., 1989; Paul et al., 1996; Robertson et al., 2007). However, the different pathways of N_2O production respond differently to these environmental factors. For example, where oxygen (O_2) is needed for nitrification, denitrification is inhibited by its presence (Knowles, 1982; Paul et al., 1996). Denitrification, as a heterotrophic process, requires an organic carbon source, and is therefore strongly dependent on the soil organic carbon (SOC) quality and C:N ratio (Knowles, 1982; Paul et al., 1996; Robertson et al., 2007). Autotrophic nitrifiers do not need organic C for their own metabolism. However, they are indirectly affected by C:N ratio and SOC quality through mineralization and immobilization rates that affect NH_4^+ availability, the most important factor regulating nitrification in soil (Paul et al., 1996; Robertson et al., 2007). Both nitrification and denitrification are favored by a relatively high pH with an optimum in the range of 7 to 8, but appear to respond to low pH differently (Knowles, 1982; Paul et al., 1996).

Unconventional pathways of N_2O production: Nitrifier Denitrification

However, the 'conventional' paradigm that considers (autotrophic) nitrification and (heterotrophic) denitrification as the two principal production pathways of N_2O is a simplified presentation of reality. It has long been acknowledged that a wide range of biological processes has the potential to produce N_2O as (by-) product, and that similar pathways may be carried out by various organisms. For example, nitrification may be carried out by heterotrophic organisms and methanotrophic bacteria as well; NO_3^- may be subject to co-denitrification and

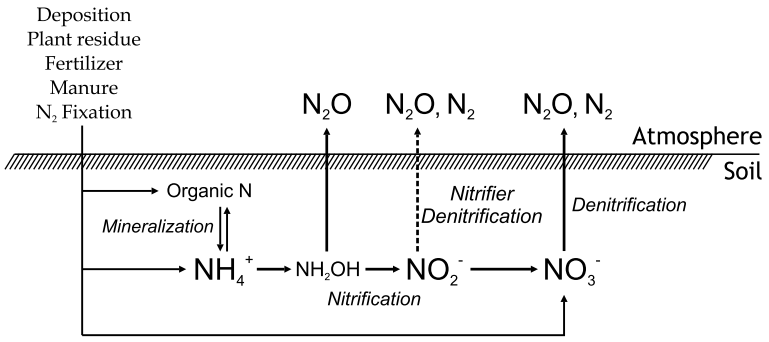


Figure 1.3: Soil N cycling and N_2O production in soil, including nitrifier denitrification as potential N_2O production pathway.

dissimilatory NO_3^- reduction to ammonia; and several fungi and Archaea also capable of denitrification. In soils however, the relative contribution of most above mentioned N_2O production pathways is thought to be minor. Yet there is one other potential pathway that receives increasing interest in soil-based studies: the potential of autotrophic ammonia oxidizing bacteria (AOB) to produce N_2O through reduction of NO_2^- (Figure 1.3). The terminology in literature has previously been inconsistent, but this process is now commonly labeled 'nitrifier denitrification' (Wrage et al., 2001).

Nitrifier denitrification was identified already four decades ago in pure culture studies (Hooper, 1968; Ritchie et al., 1972). However, despite this early discovery and continued pure culture studies (Poth et al., 1985; Remde et al., 1990; Zart et al., 1998; Colliver et al., 2000; Schmidt et al., 2004; Shaw et al., 2006), the proof that nitrifier denitrification can be a significant N_2O producing pathway *in soil* has remained elusive. Evidently, unraveling the potential of N_2O production through nitrifier denitrification in soil is vital to proper understanding of total N_2O production, as this distinct pathway will likely respond idiosyncratically to soil environmental conditions. Although literature acknowledges that 'conventional' nitrification would implicitly cover nitrifier denitrification as well (Granli et al., 1994; Mosier et al., 1998a; Hayatsu et al., 2008), nitrifier denitrification has not been experimentally distinguished from 'nitrification' in soil. Soil-based experimental studies increasingly suggest that nitrifier denitrification could contribute significantly to N_2O production in soil (Granli et al., 1994; Webster et al., 1996; Hütsch et al., 1999; Wrage et al., 2004b;

McLain et al., 2005; Ma et al., 2007; Venterea, 2007; Sánchez-Martín et al., 2008). However, conclusive proof of its actual occurrence in soil remains pending due to the lack of reliable analytical techniques.

Established methods to distinguish between sources of N_2O from soil have typically made use of nitrogen (N) isotope tracing (Stevens et al., 1997; Baggs et al., 2003; Tilsner et al., 2003; Bateman et al., 2005), and of specific inhibitors (Yoshinari et al., 1977; Robertson et al., 1987; Klemedtsson et al., 1988; Webster et al., 1996). Nitrogen isotope labeling techniques differentiate N_2O production from nitrification and denitrification in soil by applying and tracing ^{15}N enrichment from NH_4^+ and NO_3^-. However, ^{15}N labeling alone can not distinguish the N_2O that results from NO_2^- reduction (i.e. nitrifier denitrification) from the N_2O generated as by-product from ammonia oxidation (i.e. 'conventional' nitrification), as in both processes the N originates from NH_4^+ (Wrage et al., 2005; Hayatsu et al., 2008). Acetylene (C_2H_2) and O_2 have been used as inhibitors for specific (steps in) N_2O production processes, but unfortunately these inhibition techniques are not reliable as the targeted inhibition is not always complete and/ or selective (Tilsner et al., 2003; Beaumont et al., 2004a; Beaumont et al., 2004b; Wrage et al., 2004b; Wrage et al., 2004a).

The origin of N_2O and its oxygen

To quantify the contribution of nitrifier denitrification to N_2O production, Wrage et al. (2005) proposed a novel 'dual isotope approach', based on tracing both the N and oxygen (O) isotopes in N_2O. The principle of this approach is that the origin of the O atom in the N_2O molecule would differ between production pathways (Figure 1.4). In the first step of ammonia oxidation (to NH_2OH), O is obtained from molecular oxygen (O_2). Nitrous oxide released as by-product of nitrification is therefore assumed to contain O derived from O_2. In the following oxidation steps of nitrification to NO_2^- and NO_3^-, the added oxygen comes from water (H_2O). Correspondingly, N_2O from nitrifier denitrification would obtain 50% of its O from O_2 and the other 50% from H_2O, reflecting NO_2^-. The O in N_2O from 'conventional' denitrification would reflect that of NO_3^-, which in the case of nitrification-derived NO_3^- would originate for 1/3rd from O_2 and 2/3rd from H_2O (Figure 1.4). Based on this principle, studying the origin of the O in N_2O could

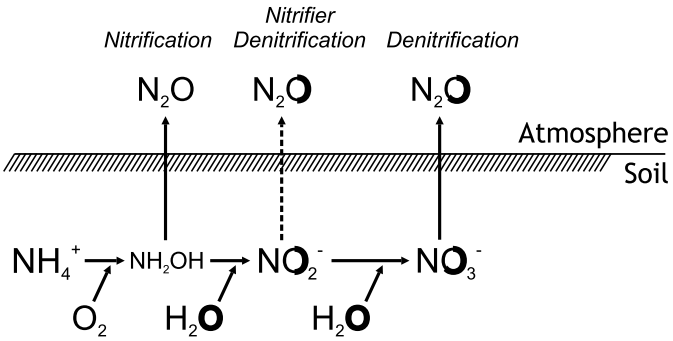


Figure 1.4: Illustration of the incorporation of oxygen (O) from O_2 and H_2O during the production of N_2O from nitrification, nitrifier denitrification and denitrification. The thicker part of the O indicates the relative contribution of O from H_2O.

thus improve our understanding of the processes responsible for its production.

In the novel dual isotope approach, soils are treated with ^{15}N enriched NH_4^+ and NO_3^- and ^{18}O enriched H_2O. Evaluation of the isotopic enrichment of the produced N_2O will then identify the relative contribution of the different pathways to total N_2O production.

The origin and organization of this thesis

The general aim of the research presented in this thesis is to improve our understanding of the pathways of N_2O production in soil, and the pathway of nitrifier denitrification in particular. The dual isotope approach as proposed by Wrage et al. (2005) would be a fundamental tool for this work, developed to identify the contribution of nitrifier denitrification in soil for the first time. At the start, the **main objectives** were:

(i) To further develop the dual isotope labeling approach; which would enable
(ii) To quantify the relative contribution of nitrifier denitrification as pathway of N_2O production in soil; and
(iii) To study the idiosyncratic response to environmental controls of N_2O production through nitrifier denitrification.

However, shortly after the start of my PhD research, I found that the origin of the O in N_2O is more complex than assumed in the dual isotope approach of

Wrage et al. (2005). The assumption that reaction stoichiometry determines the proportion of O in N_2O that is derived from O_2 and H_2O (Kendall, 1998; Pérez, 2005; Wrage et al., 2005) underestimated the significance of another process: O exchange between H_2O and intermediate compounds of the N_2O production pathways. Throughout this thesis, 'oxygen (or O) exchange' is used as short for the exchange of O between nitrogen oxides and H_2O. This finding necessitated closer investigation of the origin of the O in N_2O, which became a priority of my **revised research objectives**:

(i) To study, identify and quantify the process of O exchange between H_2O and intermediate compounds of the N_2O production pathways, and its effect on the O isotopic signature of N_2O produced in soil;

(ii) To develop and apply an advanced stable isotope tracing approach that accounts for the effect of O exchange and subsequently *can* identify nitrifier denitrification in soil-based studies; and

(iii) To study the significance and idiosyncratic character of nitrifier denitrification as production pathway of N_2O in soil.

This thesis presents my research on O and N isotope tracing to identify the origin of N_2O from soil. In chapter 2, I start with a literature review on O exchange between H_2O and intermediates of N_2O production processes. Subsequently, I present a series of experimental studies to unravel the process of O exchange and its effect on the origin of the O in N_2O. First, I identify the general presence of O exchange in soil, and quantify O exchange during denitrification (chapter 3). In chapter 4, I evaluate the occurrence of O exchange during the different pathways of N_2O production. Using the acquired knowledge on the process of O exchange, I then develop and apply an advanced dual isotope tracing approach. With this approach I show that, in soil, N_2O can indeed be produced through nitrifier denitrification (chapter 5). In chapter 6, I identify that nitrifier denitrification can contribute significantly to total N_2O production and that its relative contribution is idiosyncratically affected by moisture conditions. With an exploratory study presented in chapter 7, I demonstrate that O exchange may affect the isotopic signature of NO_3^- as well. In the final chapter 8, I summarize my main findings and discuss their implications for current and future research on the origin of N_2O.

1

Chapter 2

Oxygen exchange between (de)nitrification intermediates and H_2O and its implications for source determination of NO_3^- and N_2O: a review

Abstract Stable isotope analysis of oxygen (O) is increasingly used to determine the origin of nitrate (NO_3^-) and nitrous oxide (N_2O) in the environment. The assumption underlying these studies is that the ^{18}O signature of NO_3^- and N_2O provides information on the different O sources (O_2 and H_2O) during production of these compounds by various biochemical pathways. However, exchange of O atoms between H_2O and intermediates of the (de)nitrification pathways may change the isotopic signal and thereby bias its interpretation for source determination. Chemical exchange of O between H_2O and various nitrogenous oxides has been reported in the literature, but the probability and extent of its occurrence in terrestrial ecosystems remain unclear. Biochemical O exchange between H_2O and nitrogenous oxides, NO_2^- in particular, has been reported for monocultures of many nitrifiers and denitrifiers that are abundant in nature, with exchange rates of up to 100%. Therefore, biochemical O exchange is likely to be important in most soil ecosystems, and should be taken into account in source determination studies. Failing to do so might lead to (i) an overestimation of nitrification as NO_3^- source, and (ii) an overestimation of nitrifier denitrification and nitrification-coupled denitrification as N_2O production pathways. A method to quantify the rate and controls of biochemical O exchange in ecosystems is needed, and we argue this can only be done reliably with artificially enriched ^{18}O compounds. We conclude that in N source determination studies, the O isotopic signature of especially N_2O should only be used with extreme caution.

D.M. Kool, N. Wrage, O. Oenema, J. Dolfing, J.W. Van Groenigen. 2007. Oxygen exchange between (de)nitrification intermediates and H_2O and its implications for source determination of NO_3^- and N_2O: a review. Rapid Communications in Mass Spectrometry 21: 3569-3578

Introduction

2

Increased anthropogenic emissions of nitrate (NO_3^-) contribute to contamination of ground water and eutrophication of surface waters (Howarth et al., 1996; Galloway et al., 2003). Rising concentrations of nitrous oxide (N_2O) are of environmental concern because of its contribution to the greenhouse effect (Crutzen, 1981). Environmental legislation aimed at mitigating these emissions has resulted in increased attention on the study of their origin.

Stable isotope analyses of nitrogen (N) and oxygen (O) are increasingly used to quantify N transformations and to determine the original N sources of anthropogenic and naturally derived NO_3^- and N_2O in the environment. Both the natural abundance variation of ^{15}N and ^{18}O and artificially enriched compounds are used to trace the sources of NO_3^- or N_2O (Wahlen et al., 1985; Durka et al., 1994; Webster et al., 1996; Kendall, 1998; Mayer et al., 2002; Pérez et al., 2006).

Natural abundance studies utilize the fact that the various natural sources of NO_3^- have distinct isotopic signatures (Figure 2.1). A wide range of $\delta^{18}O$ values for atmospheric NO_3^- have been reported (+15 to +75‰$_{SMOW}$, as summarized by Kendall). The $\delta^{18}O$ of microbially produced NO_3^- is partially determined by that of O_2, which contributes one O atom during ammonia oxidation to hydroxylamine (NH_2OH), and partially by that of H_2O, which contributes the other two O atoms during the further oxidation to nitrite (NO_2^-) and NO_3^- (Figure 2)(Aleem et al., 1965; Hollocher et al., 1981; Andersson et al., 1983; Hollocher, 1984; Voerkelius, 1990; Kendall, 1998). Assuming that the $\delta^{18}O$ of soil O_2 is approximately equivalent to atmospheric O_2 (+23.5‰$_{SMOW}$), and with $\delta^{18}O$ of soil H_2O usually in the range of -25 to +4‰$_{SMOW}$ (Amberger et al., 1987), the $\delta^{18}O$ of NO_3^- formed by nitrification will range from -10 to +10‰$_{SMOW}$ (Pardo et al., 2004). However, the $\delta^{18}O$ of O_2 in soil may be increased relative to atmospheric O_2 due to fractionation by respiration in soil (Lane et al., 1956; Guy et al., 1993; Kendall, 1998), resulting in higher $\delta^{18}O$ values for NO_3^- formed by nitrification (Kendall, 1998). Isotope fractionation during denitrification results in relative enrichment in $\delta^{15}N$ and $\delta^{18}O$ of the remaining NO_3^- (Amberger et al., 1987; Böttcher et al., 1990; Aravena et al., 1998; Mengis et al., 1999; Mengis et al., 2001; Sebilo et al., 2006).

Microbial processes that produce and consume N_2O all tend to fractionate in favor of the lighter isotopes, leaving the residual compounds relatively enriched

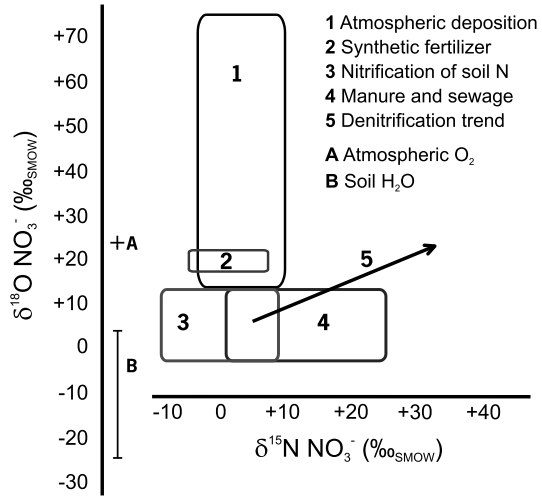
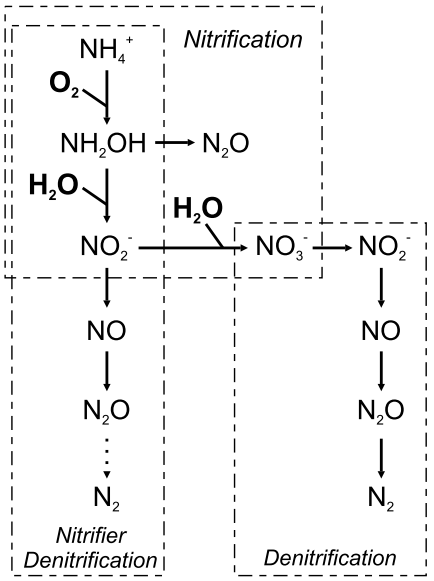


Figure 2.1: Schematic overview of the N and O isotopic signature in NO_3^- from different sources, after Kendall (1998). The arrow indicates the change in isotopic composition upon denitrification due to isotopic fractionation, of which the slope, $\delta^{15}N:\delta^{18}O$, is approximately 1:2 (Böttcher et al., 1990). Along the Y-axis the $\delta^{18}O$ of atmospheric O_2 and (soil) H_2O are indicated.

in $\delta^{15}N$ and $\delta^{18}O$ (Handley et al., 1992; Bedard-Haughn et al., 2003). Various sources and pools of N_2O (soil, oceanic, tropospheric and stratospheric) therefore show distinct O isotopic signatures. Based on this natural abundance variation, the $\delta^{18}O$-N_2O signature is increasingly used in addition to $\delta^{15}N$-N_2O to characterize the source, production and consumption of N_2O (Wahlen et al., 1985; Kim et al., 1990; Kim et al., 1993; Yoshinari et al., 1997; Schmidt et al., 2004a; Van Groenigen et al., 2005a; Pérez et al., 2006).

Besides natural abundance methods, combinations of NH_4^+ and NO_3^- that are artificially enriched with ^{15}N are routinely used to study the processes of nitrification and denitrification and their relative contribution to N_2O production (Stevens et al., 1997; Panek et al., 2000; Baggs et al., 2003; Bateman et al., 2005). The additional use of ^{18}O-enriched H_2O has been suggested to enable the distinction between N_2O from nitrification and nitrifier denitrification (the reduction of NO_2^- to N_2O by nitrifiers) (Wrage et al., 2005). Similar to natural abundance studies, in this approach by Wrage et al. (2005) the different contributions of O_2-O and H_2O-O to N_2O-O in the various pathways is used for



N_2O-O reflects:	Preceding compound	O_2	H_2O	Fertilizer NO_3^-
Nitrification	NH_2OH	100%	0%	0%
Nitrifier denitrification	NO_2^-	50%	50%	0%
Nitrification-coupled denitrification	NO_3^-	33%	67%	0%
Fertilizer denitrification	NO_3^-	0%	0%	100%

Figure 2.2: Overview of nitrification, denitrification and nitrifier denitrification pathway, including the incorporation of oxygen atoms from O_2 and H_2O into NO_2^- and NO_3^- (after Wrage et al. (2005)). The table shows how the O isotopic signal of N_2O is determined by the O isotopic signal of O_2 and H_2O, without any O exchange between H_2O and intermediate compounds. Fertilizer denitrification: denitrification of applied NO_3^-.

this determination (Figure 2.2). Although the exact pathway of N_2O formation from nitrification is unknown, the O isotopic signature of nitrification-N_2O is assumed to be identical to that of hydroxylamine (NH_2OH) and 100% determined by O_2-O (Wrage et al., 2005). For nitrifier denitrification, the O isotopic signature of N_2O will equal that of NO_2^-, with half of the O derived from O_2 and half of H_2O. The O isotopic signature of denitrification-N_2O will be identical to that of NO_3^-. For denitrification of nitrification-derived NO_3^-, 1/3rd of the O will be from O_2 and 2/3rd from H_2O-O. Denitrification of fertilizer-NO_3^- will produce N_2O with an O isotopic signature determined by that of the fertilizer (Figure 2.2). These differences in the O origin and the resulting isotopic composition of N_2O from the various pathways are then used to distinguish between their relative contributions.

One complication in the interpretation of $\delta^{18}O$ values of N_2O is that the exact pathway resulting in N_2O as a byproduct of nitrification (Figure 2.2) is still

unknown. It is thought to be formed as by-product of incomplete oxidation of NH_2OH to NO_2^- (Arp et al., 2003; Stein et al., 2003). If the N_2O is produced before the incorporation of the second O atom, the O isotopic signature of nitrification-N_2O will indeed be identical to that of NH_2OH, and thereby of O_2. However, if the second O atom has already been incorporated before the release of N_2O, the O isotopic signature of the N_2O would also be partly determined by that of H_2O. This uncertainty could form a source of error for approaches that assume the O in nitrification-N_2O to be 100% derived from O_2.

Another complication in the interpretation of $\delta^{18}O$ signatures of N_2O and NO_3^- is the assumption that no significant O exchange will take place between H_2O and other compounds involved in NO_3^- or N_2O formation (either sources, intermediates or end-products). Such exchange could be a mechanical process, physically exchanging the O of H_2O with that of another compound. It could also occur during reactions that involve the incorporation of O from water, or the release of O to water, when such a reaction is reversible. In that case, alternating occurrence of the forward and reverse reaction would induce O exchange between H_2O and the nitrogenous oxides involved in the reaction. If such an exchange would take place at significant rates, the differences in O isotopic signatures of the respective compounds, on which both natural abundance and artificially enriched studies are based, would blur. This would complicate the interpretation of the $\delta^{18}O$-N_2O values, possibly leading to incorrect conclusions about the origin of NO_3^- or N_2O. The possibility of such an exchange is occasionally mentioned in source determination studies, and in those cases is assumed to be of minor importance under the specific experimental conditions (Wahlen et al., 1985; Toyoda et al., 2005; Wrage et al., 2005; Menyailo et al., 2006a). However, the available literature on O exchange is extensive but largely confined to chemical and microbiological studies on monocultures rather than soils. Its potential impact was mentioned in a few natural abundance studies on marine ecosystems (Casciotti et al., 2002; Sigman et al., 2005), but overall there have been few efforts to evaluate its relevance to source determination studies in terrestrial ecosystems.

With this review paper, we aim to provide a better understanding of the processes of O exchange and their implications for source determination studies. We first summarize the literature on O exchange. We then discuss processes

likely to cause such exchange, and speculate on factors controlling the extent of O exchange in natural ecosystems. Finally, we identify the implications of O exchange for source determination studies of NO_3^- and N_2O, and discuss research needs and possibilities to be addressed in the future.

Oxygen exchange reported in literature

Oxygen exchange has been reported to occur through both chemical and biochemical processes. The following text presents the current level of understanding on both.

Chemical oxygen exchange

Table 2.1 lists studies on chemical O exchange between H_2O and various nitrogenous oxides. The exact reactions are unclear, but most studies report that the rate of O exchange was affected by pH and nitrous acid (HNO_2) concentrations. Acidic conditions promote O exchange, and both first and second order rate laws are reported (for $[H^+]$ as well as $[HNO_2]$). Bonner and Jordan (1973) showed the rate of O exchange to decrease with increasing NO_2^- concentrations. Furthermore, a catalytic effect of chloride ions was found by Anbar and Guttmann (1961).

Chemical oxygen exchange was in these studies investigated in aqueous solutions, often under conditions that are not commonly encountered in

Table 2.1: Studies reporting on chemical O exchange between H_2O and nitrogen oxides.

Ion or compound	Exchange effected by	Reference
HNO_3	Chloride	Anbar et al. (1961)
NO	pH, nitrous acid; no exchange in pure water	Bonner (1970)
NO	pH, nitrite & nitrous acid	Bonner et al. (1963)
HNO_2	pH	Bothner-By et al. (1952)
HNO_2	pH, nitrous acid	Bunton et al. (1959)
HNO_2	pH, nitrous acid	Bunton et al. (1959)
NO_2^-	pH	Van Etten et al. (1981)
HNO_2	pH, nitrite / nitrous acid, hydrogen peroxide	Anbar et al. (1954)
KNO_2, KNO_3	No oxygen exchange, no effect of pH	Hall et al. (1940)

ecosystems. Solutions were sometimes highly acidic (e.g. pH<1 in Bunton and Stedman (1959a)), and temperatures frequently higher or lower than they would normally be in most soils. Temperatures were 0°C or lower in Bothner-By and Friedman (1952), Bunton et al. (1959b), and Bunton and Stedman (1959a); around 25°C in Anbar and Taube (1954), Bonner (1970), Bonner and Jordan (1973) and Van Etten and Risley (1981); and 60 to 100°C in Anbar and Guttmann (1961). Also, substrate concentrations were often much higher than in terrestrial ecosystems. For example, NO_2^- and HNO_2 concentrations were up to 3 M and 1.13 M, respectively, in Bunton et al. (1959b).

Little is known about chemical exchange at near-neutral pH. Some indication may be derived from Casciotti et al. (2007) who evaluated the effect of storage conditions on the O isotopic signature of NO_2^- in freshwater and seawater samples at various pH values, temperatures and NO_2^- concentrations. In particular at pH 6 and 8 at 4°C, they found substantial exchange of O between NO_2^- and H_2O, totaling 10-30% within 3 weeks of storage. This may indicate a potential for O exchange in soil moisture under normal conditions. However, it is doubtful whether the exchange observed was strictly chemical since the samples analyzed were derived from freshwater and marine ecosystems. Microorganisms present in the samples may likely be responsible for the observed exchange.

Based on our examination of the current literature we conclude that the possibility for chemical O exchange in soils can not be excluded. However, we consider chemical O exchange unlikely to be significant in soils, as there is currently no proof of its occurrence or extent under conditions that normally prevail in soil ecosystems.

Biochemical oxygen exchange

While proof is lacking on the occurrence and significance of chemical O exchange in soil ecosystems, this is not the case when considering biochemical exchange. The remainder of this study we focus on biochemical exchange. The processes during which biochemical O exchange is reported to occur are all enzymatically catalyzed. We will discuss these processes, their thermodynamics and the enzymes involved. Finally, we review ecosystem studies that may indirectly provide support for the occurrence of substantial O exchange in soil ecosystems.

Nitrification pathway

Biochemical O exchange has been associated with several nitrifiers (Table 2.2), including both ammonia (NH_3) and nitrite oxidizers. Andersson et al. (1982) analyzed the $\delta^{18}O$ of NO_2^- derived from NH_4^+ and hydroxylamine (NH_2OH) oxidation by the NH_3 oxidizer *Nitrosomonas europaea* in the presence of ^{18}O enriched H_2O. They reported O exchange between NO_2^- and H_2O. The NO_2^- oxidizer *Nitrobacter agilis* grown on $^{15}N^{16}O_2^-$ in the presence of ^{18}O enriched H_2O produced zero, single and double ^{18}O labeled NO_3^- (Kumar et al., 1983). This double ^{18}O labeled NO_3^- could only have been formed as a result of O exchange during NO_2^- oxidation to NO_3^- (Kumar et al., 1983). DiSpirito et al. (1986) reported O exchange between NO_3^- molecules was catalyzed by *Nitrobacter winogradskyi* grown on NO_2^-. Albeit to a smaller extent than between NO_3^- molecules, O exchange also occurred between H_2O and NO_3^- (DiSpirito et al., 1986).

Ammonia oxidation takes place in two steps: first NH_3 is oxidized to NH_2OH, which is then oxidized to NO_2^-. The former process is catalyzed by the membrane bound enzyme ammonia mono-oxygenase, and requires O_2 and the input of electrons (Arp et al., 2002; Arp et al., 2003; Fiencke et al., 2006) (Figure 2.3). The required electrons are provided by the second step, the conversion of NH_2OH to NO_2^-, which is catalyzed by the enzyme hydroxylamine oxidoreductase (HAO) that is located in the periplasm (Arp et al., 2002; Arp et al., 2003) (Figure 2.3). This conversion yields additional reducing equivalents that are needed to gain energy from this reaction. This is an important point with respect to the possibility of O exchange; as this second step involves the incorporation of O from H_2O (Figure 2.4(a)), the reverse of the process could thus allow the exchange of O. However, NH_3 oxidizers are mainly obligatory lithoautotrophic organisms and gain energy

Table 2.2: ^{15}N-NMR studies reporting biochemical oxygen exchange between H_2O and intermediate compounds of the nitrification and denitrification pathways by nitrifiers.

Species	Substrate	Measured Ion	Reference
Nitrosomonas europaea	NH_4^+, NH_2OH	NO_2^-, NO_3^-	Andersson et al. (1982)
Nitrobacter agilis	NO_2^-	NO_3^-	Kumar et al. (1983)
Nitrobacter winogradskyi	NO_2^-	NO_3^-	DiSpirito et al. (1986)

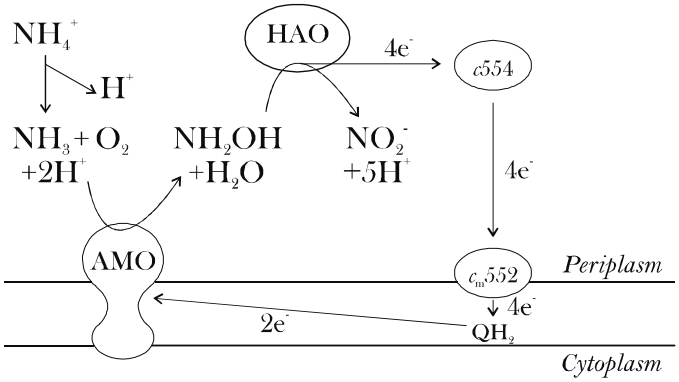
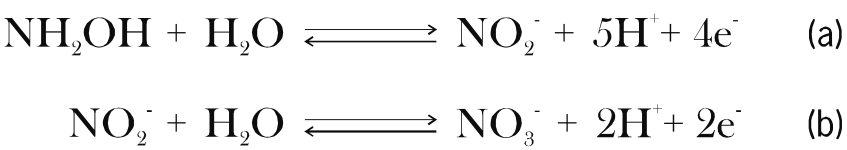


Figure 2.3: Arrangement of the enzymes involved in oxidation of ammonia to nitrite in cells of ammonia oxidizing bacteria (AOB) (Arp et al., 2002; Arp et al., 2003; Fiencke et al., 2006). AMO = ammonia monooxygenase; HAO = hydroxylamine oxidoreductase; QH_2 = quinol; c554 and c_m552 = cytochromes involved in (part of) the electron flow.

from NH_3 oxidation for their growth. The conversion of NH_2OH to NO_2^- is essential to provide electrons for NH_3 oxidation to NH_2OH. It would thus be unbeneficial and therefore unlikely that the reverse of the reaction of NH_2OH oxidation to NO_2^- would take place and allow for O exchange by NH_3 oxidizing nitrifiers. In addition, *Nitrosomonas europaea* is the only NH_3 oxidizer studied with regard to O exchange (Andersson et al., 1982), but it is not a very common nitrifier in the soil. It is rather unrepresentative of this group of nitrifiers in a variety of traits (Casciotti et al., 2001; Kowalchuk et al., 2001; Arp et al., 2003; Wrage et al., 2004b; Shaw et al., 2006), and this possibly extends to its behavior regarding O exchange. Recently, it has been reported that Archaea may also be capable of NH_3 oxidation (Könneke et al., 2005), and that such Archaea constitute a significant part of the NH_3 oxidizing community in soil (Leininger et al., 2006). However, little is known about (dis-)similarities between these Archaea and the better-known bacterial NH_3 oxidizers. Altogether, we can only speculate about O exchange induced by NH_3 oxidation in soil systems.

In nitrification, NH_3 oxidation is followed by the oxidation of NO_2^- to NO_3^-. This process is catalyzed by the membrane-bound enzyme nitrite-oxidoreductase (Bock et al., 1986). Again, the reversibility of this step could allow for the exchange of O atoms between H_2O and NO_2^- or NO_3^- (Figure 2.4(b)) (Aleem, 1968; Sundermeyer-Klinger et al., 1984; Wood, 1986). Removal of reducing equivalents



$$NH_2OH + H_2O \rightleftharpoons NO_2^- + 5H^+ + 4e^- \quad (a)$$

$$NO_2^- + H_2O \rightleftharpoons NO_3^- + 2H^+ + 2e^- \quad (b)$$

Figure 2.4: Reversible processes in the pathway of nitrification including the incorporation/release of H_2O, thus allowing for O exchange. (a) The oxidation of hydroxylamine (NH_2OH) to NO_2^-; a reversible reaction catalyzed by hydroxylamine oxidoreductase (HAO). (b) The oxidation of NO_2^- to NO_3^-; a reversible reaction catalyzed by nitrite oxidoreductase (Aleem, 1968; Sundermeyer-Klinger et al., 1984; Wood, 1986).

(H_2), by burning with O_2, is needed to pull this reaction forward (towards production of NO_3^-). Because of these thermodynamics of the reaction, the reaction is probably close to equilibrium and the reverse may occur as well, thereby allowing for O exchange.

In addition it should be noted that the two NO_2^- oxidizing nitrifiers studied and reported to catalyze O exchange between NO_2^- and H_2O are of the genus *Nitrobacter* (Kumar et al., 1983; DiSpirito et al., 1986). These form an exceptional group of nitrifiers in that they are not strictly autotrophic and aerobic. *Nitrobacter* can grow heterotrophically (Bock et al., 1986) while repressing the nitrite-oxidizing system (Steinmüller et al., 1977), and they are also capable of anaerobic growth, converting NO_3^- to NO_2^- with pyruvate, acetate or glycerol as electron donors (Aleem et al., 1981; Sundermeyer-Klinger et al., 1984). Compared to strictly autotrophic NO_2^- oxidizers, these organisms are thus less dependent on the energy derived from NO_2^- oxidation. The argument that the reverse reaction would not take place because it is energetically unfavorable may therefore not hold for these NO_2^- oxidizers. The reverse reaction, and thereby O exchange, is likely to occur in this case. For strictly autotrophic NO_2^- oxidizers, the occurrence and extent of the reverse reaction remains speculative.

Denitrification pathway

Oxygen exchange between H_2O and nitrogen oxides (NO_3^-, NO_2^- and nitric oxide (NO)) has been quantified for a number of denitrifiers, and was observed to take place at least to some extent in all denitrifiers studied (Table 2.3) (Garber et al., 1982; Aerssens et al., 1986; Shearer et al., 1988; Ye et al., 1991; Casciotti et al., 2002). The denitrifiers studied were all bacteria, so the discussion below will

consider the processes and enzymes involved in bacterial denitrification only (as opposed to fungal denitrification).

The O exchange is quantified by measuring the incorporation of O from artificially ^{18}O enriched H_2O into either N_2O or NO_2^-. The extent of O exchange differed both between species and substrates (Table 2.3). Casciotti et al. (2002) found O exchange during NO_3^- reduction to N_2O to be small for *Pseudomonas aureofaciens* (< 10% incorporation of H_2O-O into N_2O), but approximately 30% for *Corynebacterium nephridii* and up to 78% for *Pseudomonas chlororaphis*. Ye et al. (1991) studied O exchange during NO_2^- and NO reduction to N_2O for eight

Table 2.3: Studies reporting on biochemical oxygen exchange between H_2O and intermediate compounds of the denitrification pathway by denitrifiers.

Species	Substrate	Measured ion or compound	Percentage of exchange[$]	NiR type	Reference
Paracoccus denitrificans	NO_2^-	N_2O	59	heme-cd1	Ye et al. (1991)
	NO	N_2O	11		
Pseudomonas aeruginosa	NO_2^-	N_2O	76	heme-cd1	Ye et al. (1991)
	NO	N_2O	19		
Pseudomonas stutzeri	NO_2^-	N_2O	58	heme-cd1	Ye et al. (1991)
	NO	N_2O	4		
Pseudomonas fluorescens	NO_2^-	N_2O	39	heme-cd1	Ye et al. (1991)
	NO	N_2O	15		
Alcaligenes eutrophus	NO_2^-	N_2O	94	copper	Ye et al. (1991)
	NO	N_2O	84		
Achromobacter cycloclastes	NO_2^-	N_2O	4	copper	Ye et al. (1991)
	NO	N_2O	30		
Pseudomonas aureofaciens	NO_2^-	N_2O	6	copper	Ye et al. (1991)
	NO	N_2O	37		
Rhodopseudomonas sphaeroides	NO_2^-	N_2O	90	copper	Ye et al. (1991)
	NO	N_2O	61		
Paracoccus denitrificans	NO_2^-	NO_2^-; N_2O	32; 100	heme-cd1	Garber & Hollocher (1982)
Pseudomonas denitrificans	NO_2^-	NO_2^-; N_2O	12; 70	heme-cd1	
Pseudomonas stutzeri	NO_2^-	N_2O	8 - 35	heme-cd1	Aerssens et al. (1986)
	NO	N_2O	13 - 31		
Pseudomonas stutzeri	NO_2^-	NO_2^-	5 - 8	heme-cd1	Shearer & Kohl (1988)
Pseudomonas aureofaciens	NO_3^-	N_2O	< 10	copper	Casciotti et al. (2002)
Corynebacterium nephridii	NO_3^-	N_2O	30	copper	
Pseudomonas chlororaphis	NO_3^-	N_2O	61 - 78	heme-cd1	

[$] at the end of incubation; total incubation period differs for different experiments

denitrifiers. They observed exchange rates up to 94% during NO_2^- reduction. During reduction of NO, the amount of O incorporated from H_2O into N_2O ranged between 4 and 84%. No O from H_2O was incorporated in N_2O when pure cultures of *Pseudomonas aureofaciens* were incubated with N_2O, showing that O exchange takes place during reduction of NO_2^- and/or NO to N_2O, and not with N_2O itself (Ye et al., 1991). Aerssens et al. (1986) also determined O exchange during both NO_2^- and NO reduction, by *Pseudomonas stutzeri*. They report the extent of O exchange between H_2O and the produced N_2O to range between 8 and 35% for NO_2^- reduction, and between 13 and 31% for NO reduction. In addition, they found O exchange to decrease with increasing NO_2^- concentrations. Shearer and Kohl (1988) reported the incorporation of O from ^{18}O-labeled H_2O in NO_2^- by *Pseudomonas stutzeri* to range between 5 and 8%. Garber and Hollocher (1982) found the extent of exchange to differ among denitrifiers, but to be present in all species studied.

Most denitrifier studies have concentrated on O exchange during NO_2^- and NO reduction to N_2O. The NO_2^- reduction to NO in the denitrification pathway is an enzyme-bound dehydration step including the incorporation of H^+ and formation of H_2O (Figure 2.5). This step is reversible and hence allows for O exchange (Averill et al., 1982; Garber et al., 1982; Kim et al., 1984; Weeg-Aerssens et al., 1987; Shearer et al., 1988; Weeg-Aerssens et al., 1988). Enzymes responsible for NO_2^- reduction to NO are of two distinct types: cytochrome cd_1 and copper-containing nitrite reductase (heme-cd_1-NiR and copper-NiR, respectively) (Hochstein et al., 1988; Averill, 1996). Kim and Hollocher (1984) have shown that NiRs of the heme-cd_1 type are able to catalyze NO_2^-/H_2O-O exchange. Ye et al. (1994) also found significant rates of O exchange during NO_2^- reduction for all four heme-cd_1-NiR containing denitrifiers studied. They showed O exchange to be possible in copper-NiR containing species as well, but the extent differed considerably between the four species studied (Table 2.3). The latter may be explained by the fact that copper-NiR containing organisms exhibit quite extreme physiological diversity (Coyne et al., 1989; Ye et al., 1991; Averill, 1996). Diversity in functional enzymes may thus result in differences in the extent of exchange. In nature, the heme-cd_1-NiR is present in about two-third of the denitrifying species examined (Hochstein et al., 1988; Averill, 1996). Overall, organisms with the ability to catalyze O exchange during NO_2^- reduction are very likely to be present

$$NO_2^- + E \rightleftharpoons E.NO_2^-$$

$$E.NO_2^- + 2H^+ \rightleftharpoons E.NO^+ + H_2O$$

Figure 2.5: The step of NO_2^- reduction in denitrification to N_2O and N_2 is an enzyme-bound, reversible dehydration step (E = enzyme) (Averill et al., 1982; Garber et al., 1982; Kim et al., 1984; Weeg-Aerssens et al., 1987 and 1988; Shearer et al., 1988).

in both natural and agricultural ecosystems.

For NO reduction, three types of nitric oxide reductase (NOR) have been identified in bacteria; cNOR, qNOR and qCuNOR (Tavares et al., 2006). All three are membrane-bound enzymes. Although structure and composition of their inactive subunits differ, their active site structure is thought to be highly homologous (Tavares et al., 2006). While theoretical studies have pointed out the nature of the enzymatic reaction of NO reduction, the exact mechanism is still unclear (Tavares et al., 2006). With respect to O exchange, Ye et al. (1991) reported differences in the rate of exchange during NO reduction for species differing in their *NiR*-type. Significantly higher extents of exchange were found for the copper-NiR than the heme-cd_1-NiR containing species (Table 2.3). It may be hypothesized that species with distinct NiRs also have different NORs, causing differences in exchange during NO reduction.

Although not as intensively studied as NO_2^- and NO reduction, the possibility of O exchange during the first step of denitrification, the reduction of NO_3^- to NO_2^-, should also be considered. Exchange of O during denitrification of NO_3^- to N_2O has been reported (Casciotti et al., 2002), but the results could be interpreted as a result of O exchange during later stages of the denitrification process. Nitrate reductases appear to be fairly similar among denitrifying bacteria (Averill, 1996). Respiratory NO_3^- reduction involves the nitrate reductase NaR, which is located in the cytoplasmic membrane, with its active site in the cytoplasm (Ye et al., 1994; Averill, 1996; Tavares et al., 2006; Wallenstein et al., 2006). In contrast, NiR is a soluble enzyme in the periplasmic space and NOR is bound to the cytoplasmic membrane, but has its active site in the perisplasm (Figure 2.6). For NO_3^- reduction to NO_2^-, NO_3^- thus first has to pass the cytoplasmic membrane, and NO_2^- needs to be transported back into the periplasm. So, for O exchange with (^{18}O-) H_2O to take place, the H_2O would need to pass the membrane as well,

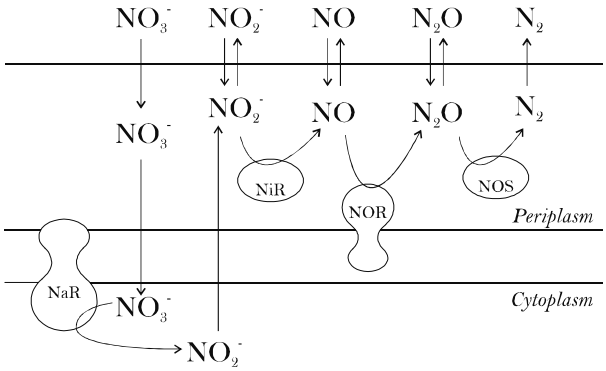


Figure 2.6: Arrangement of the enzymes involved in denitrification in Gram-negative bacteria. NaR = nitrate reductase; NiR = nitrite reductase; NoR = nitric oxide reductase; NoS = nitrous oxide reductase. Modified from Ye et al.(1994), Averill (1996) and Wallenstein (2006).

while this is not needed for O exchange during NO_2^- and NO reduction. The exchange of O may thus be less likely during the first part of the bacterial denitrification pathway. A second type of nitrate reductase is known, NaS, which participates in nitrogen assimilation (Lin et al., 1998; Richardson et al., 2001). This enzyme is located in the cytoplasmic space (Lin et al., 1998; Richardson et al., 2001) (as is the active site of NaR), so the H_2O would also need to pass the cytoplasmic membrane for O exchange during NO_3^- reduction to NO_2^- (as part of N assimilation) catalyzed by this enzyme. Yet another respiratory nitrate reductase is known which is involved in nitrate respiration that is coupled to quinol oxidation (Richardson et al., 2001; Tavares et al., 2006). This NaP is located in the periplasmic space (Berks et al., 1994; Berks et al., 1995a; Berks et al., 1995b; Richardson et al., 2001; Tavares et al., 2006). So for O exchange with H_2O to take place upon NO_3^- reduction by microorganisms that possess this enzyme, the H_2O (and NO_3^-) does not need to pass the cytoplasmic membrane.

The last compound left in the denitrification pathway that may be subject to O exchange is N_2O. As N_2O formation is chemically (Bonner et al., 1952) and enzymatically (St. John et al., 1977) irreversible, O exchange between H_2O and N_2O is very unlikely. Results of Ye et al. (1991) also confirm this, since no ^{18}O from ^{18}O-H_2O was incorporated into N_2O when pure cultures of *Pseudomonas aureofaciens* were incubated with N_2O.

To summarize, O exchange during the process of denitrification seems to be

mainly associated with NO_2^- and NO reduction. All the reactions in the stepwise reduction of NO_3^- to N_2 are exergonic (Averill, 1996), but the energy gain is smallest for NO_2^- reduction. Reduction of NO_2^- could therefore be argued to be the major contributor to O exchange since this step is most likely to be reversed. Garber and Hollocher (1982) even argued, based on required functional traits and kinetic considerations, that NiR is the only reasonable candidate as the enzyme responsible for the catalysis of O exchange during denitrification.

Nitrifier denitrification pathway

The only organism studied with respect to O exchange that is capable of nitrifier denitrification is *Nitrosomonas europaea*. However, O exchange in this microorganism was studied with respect to the nitrification pathway (Andersson et al., 1982). As *N. europaea* is not very common in the soil and because N_2O production through the nitrifier denitrification pathway by this organism differs from that in other nitrifiers (Casciotti et al., 2001; Kowalchuk et al., 2001; Arp et al., 2003; Wrage et al., 2004b; Shaw et al., 2006), there is effectively no literature on O exchange during the nitrifier denitrification pathway in soil. Current understanding on the enzymes involved in this pathway may provide some insight. The enzyme responsible for NO_2^- reduction by nitrifiers has been identified as a copper-containing NiR that seems very similar to that found in denitrifiers (Casciotti et al., 2001; Chain et al., 2003; Cantera et al., 2007), possibly as a result of lateral gene transfer (Garbeva et al., 2007). It could thus be hypothesized to behave similarly to copper-NiR in denitrifiers. However, the behavior of copper-NiR containing denitrifiers with respect to O exchange was very diverse (Table 2.3), which suggests that the extent of O exchange during nitrifier denitrification could be similarly diverse. Literature suggests that the NOR in denitrifying ammonia oxidizers may be similar to that in denitrifiers as well. Casciotti and Ward (Casciotti et al., 2005) identified the widespread occurrence of genes encoding NOR in strains of ammonia oxidizing bacteria, including *Nitrosomonas* and *Nitrosococcus* spp., similar to *norB* gene sequences from denitrifiers. Garbeva et al. (Garbeva et al., 2007) found gene sequences of *norB* (and *nirK*) from several *Nitrosospira* spp. not phylogenetically different from those of denitrifiers. If the NOR in ammonia oxidizers is indeed similar to that in denitrifiers, nitrifiers capable of carrying out the nitrifier denitrification pathway

will be also able to catalyze O exchange with NO as substrate. Although future research needs to explore this further, we can hypothesize that O exchange may occur during nitrifier denitrification.

Soil studies

The studies summarized above show that O exchange has been reported in many major groups of nitrifiers and denitrifiers. Temperatures and pH in these experiments were not unrealistic for soil. This suggests that O exchange catalyzed by such microorganisms can be expected in soil as well. However, these were all studies on monocultures incubated in a nutrient medium. The importance of O exchange in functioning soil ecosystems has not been established yet.

Several soil incubation studies indirectly suggest the occurrence of O exchange. Wrage et al. (2005) incubated soil with ^{18}O enriched H_2O at 1.0 atom% excess. In the presence of acetylene (C_2H_2; inhibiting nitrification and the reduction of N_2O to N_2), the $\delta^{18}O$ values of N_2O did not increase above background levels (Figure 2.7). Soil was also incubated without C_2H_2 to determine N_2O production and its O isotopic signature. In the absence of O exchange, the maximum ^{18}O enrichment that could be reached in the produced N_2O would then be 67% of the atom% in H_2O (Figure 2.2); or 0.67 atom% excess in this case. This theoretical maximum could only be reached if all N_2O would be produced through nitrification-coupled denitrification of the applied NH_4^+, without any N_2O resulting from nitrification, nitrifier denitrification or denitrification of applied NO_3^- (Figure 2.2). This was unlikely as considerable amounts of NH_4^+ and NO_3^- had been applied to the soil. However, within 24 hours the O isotopic signature of N_2O became identical to that of the ^{18}O enriched H_2O (Figure 2.7, Wrage et al. (2005)). A reinterpretation of these results therefore seems to suggest (i) extensive and rapid O exchange between H_2O and intermediate compounds of the N_2O producing pathway(s); and (ii) a biochemical rather than a chemical controlled exchange process, as this exchange is inhibited by C_2H_2.

Menyailo and Hungate (2006a) applied ^{18}O enriched NO_3^- to forest soils and measured the $\delta^{18}O$ of the produced N_2O. Under circumstances where both nitrification and the reduction of N_2O to N_2 were inhibited, the O isotopic signature of N_2O produced should be identical to that of NO_3^- present in the soil.

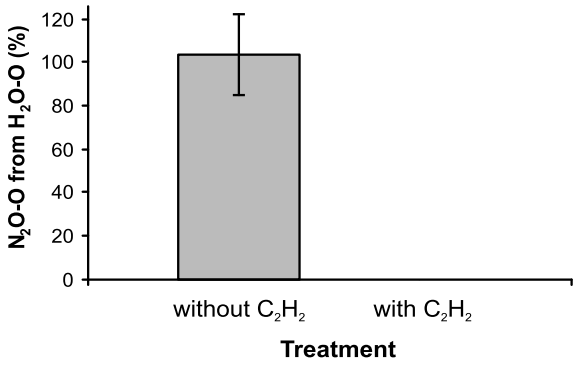


Figure 2.7: ^{18}O-N_2O enrichment relative to the applied ^{18}O-H_2O enrichment (in %), after 24 h incubation with and without C_2H_2. Data from Wrage et al. (2005), averaged over 0.1, 0.5 and 1.0 atom% excess ^{18}O-H_2O. At 100%, the N_2O thus obtained the same enrichment as the applied H_2O. The theoretical maximum enrichment of N_2O possible if no O exchange would take place would be 67%. Both average and error bars for the treatment with C_2H_2 are too small compared to the thickness of the x-axis to be visible.

However, the maximum ^{18}O-N_2O was 1.13 atom% excess, while that of the NO_3^- applied was 1.40 atom% excess. Incomplete inhibition of nitrification and the reduction of N_2O to N_2 should be considered as possible explanations for this lower enrichment. However, O exchange between the enriched NO_3^- and unlabeled H_2O could be an alternative explanation.

Discussion - Interference with stable isotope tracing studies

To our knowledge, O exchange between H_2O and nitrogenous oxides during nitrification and denitrification has not been quantified in terrestrial ecosystems, neither in field studies nor in laboratory incubations. As O isotopic analyses are now increasingly used to study N turnover processes in these ecosystems, information about the occurrence of O exchange is crucial to evaluate the reliability of these studies. Below, we discuss the possible implications of the process of O exchange for such studies.

Source determination of NO_3^-

Biological processes have a significant effect on the natural abundance isotopic composition of NO_3^-. Analyses of $\delta^{15}N$- and $\delta^{18}O$-NO_3^- are commonly used to discriminate between NO_3^- sources and to evaluate the residence time of NO_3^- in

the soil-plant environment (Amberger et al., 1987; Durka et al., 1994; Mengis et al., 2001; Williard et al., 2001; Burns et al., 2002; Pardo et al., 2004). In many of these studies the $\delta^{18}O$ of the NO_3^- is low compared to atmospheric NO_3^- (+15 to +75‰$_{SMOW}$), but closer to the range expected from nitrification of soil N (-5 to +15‰$_{SMOW}$) (Figure 2.1). It is consequently reasoned that most NO_3^- in e.g. groundwater or drainage water is derived from microbial nitrification of soil-N within the soil system, with little direct contribution of atmospheric deposition. However, soil water has an even lower $\delta^{18}O$ (about -25 to +4‰$_{SMOW}$, Figure 2.1). Therefore, exchange of O atoms between NO_3^- and H_2O (as a result of reversible processes of NO_3^- transformation where O is incorporated from or released to H_2O) would decrease the $\delta^{18}O$ signature of the NO_3^-. Depending on the extent of this exchange, the resulting net $\delta^{18}O$-NO_3^- could be any intermediate between the $\delta^{18}O$ of H_2O and that of the actual source(s). The contribution of nitrification of soil N to the NO_3^- pool could therefore be overestimated if O exchange is not taken into account. However, at equilibrium NO_2^- and NO_3^- appear to be slightly enriched in ^{18}O compared to water (equilibrium isotope effect) (Bohlke et al., 2003; Casciotti et al., 2007). Reliable quantitative knowledge on this effect remains unavailable as only these two studies have addressed this issue.

Analyses of $\delta^{18}O$- (and $\delta^{15}N$-) NO_3^- are also used to evaluate the progress of denitrification (Böttcher et al., 1990; Wassenaar, 1995; Aravena et al., 1998; Mengis et al., 1999; Mengis et al., 2001; Groffman et al., 2006; Panno et al., 2006; Sebilo et al., 2006). During denitrification, isotope fractionation leaves the residual NO_3^- relatively enriched in the heavier isotopes ^{18}O and ^{15}N, which is used to assess the role of denitrification. However, if O exchange with H_2O takes place, the $\delta^{18}O$-NO_3^- would decrease again, leading to a possible underestimation of the rate of denitrification.

Source determination of N_2O

Measurements of $\delta^{18}O$ and $\delta^{15}N$ of N_2O are used to study the production and consumption of N_2O, and to distinguish between pools of N_2O (Wahlen et al., 1985; Kim et al., 1990; Kim et al., 1993; Yoshinari et al., 1997; Tilsner et al., 2003; Schmidt et al., 2004a; Wrage et al., 2004c; Van Groenigen et al., 2005a; Pérez et al., 2006). For such studies, the occurrence of O exchange upon processes of N_2O formation and consumption may cause similar problems as for NO_3^- source

determination. Interpretation of the isotopic signatures for source determination is based on distinct isotopic signatures from different N_2O pools (soil, oceans, stratosphere), and isotopic fractionation is again used to evaluate the process of denitrification to N_2O and further reduction (consumption) to N_2 in soil. In this process of N_2O reduction, isotope fractionation causes a relative enrichment of the heavier isotopes in N_2O. However, O exchange during reduction of NO_3^- and/or NO_2^- to N_2O with H_2O would dilute the O pool of these nitrogenous oxides with ^{16}O, and lead to a lower $\delta^{18}O$-N_2O signature. If such an exchange effect is not accounted for, this would lead to incorrect interpretation of the $\delta^{18}O$-N_2O signature, and to an underestimation of the contribution of denitrification.

In the study of Wrage et al. (2005), soil treated with ^{18}O enriched H_2O in the presence of C_2H_2 produced N_2O of which the O isotopic signature did not differ from background levels, suggesting the absence of O exchange. However, the data of the non-acetylene incubations indicated the presence of biochemical O exchange; the O isotopic signature of the N_2O produced often reached values close to that of the ^{18}O enriched H_2O of the treatment. This methodology aims at distinguishing N_2O production from nitrification, nitrifier denitrification and denitrification. O exchange interferes with such experiments since it increases the ^{18}O value of the N_2O. It thereby leads to an overestimation of the contribution of nitrifier denitrification and nitrification-coupled denitrification to total N_2O production.

To summarize, the presence of O exchange between H_2O and nitrogenous oxides and the uncertainty about its extent is relevant to the interpretation of ^{18}O data from studies on (i) source determination of NO_3^-; (ii) evaluation of soil processes and residence time of NO_3^- and soil-N in general (cycling through biota and nitrification); (iii) estimates on the progress of denitrification; (iv) source determination of N_2O; and (v) distinction between pathways of N_2O production. Considering previous studies on O exchange and plausible processes that facilitate such exchange, major concerns are especially associated with N_2O source determination studies. Published literature mainly emphasizes O exchange with intermediates (NO_2^- and NO) of denitrification. Therefore, the O isotopic signature of N_2O is likely to be more affected by O exchange than that of NO_3^- and its ^{18}O analysis thus more susceptible to misinterpretation.

Discussion - Research recommendations

As discussed above, the presence of O exchange can interfere with NO_3^- and N_2O source determination studies. To enable correct interpretation of O isotopic signatures in such studies, quantitative knowledge on O exchange in soil is required. We suggest that future research should, next to extending monoculture studies, focus on developing methods to quantify O exchange in soil. Below, we will discuss some challenges and possible experimental approaches.

At natural abundance levels it is impossible to make a reliable distinction between ^{18}O fractionation and ^{18}O exchange. Therefore, we postulate that quantification of O exchange needs to be done with artificially enriched ^{18}O compounds. The use of ^{18}O enriched H_2O in combination with O isotopic analyses of NO_3^- and N_2O could provide such information.

Measuring the incorporation of ^{18}O from labeled H_2O into N_2O may already identify the *presence* of O exchange. However, since usually the relative contributions of nitrification, denitrification and nitrifier denitrification to N_2O production are unknown, this does not allow quantification of the *extent* of O exchange. We propose that research should therefore first focus on the few soil conditions where the contribution of these processes is known. The best opportunity for this is soil where denitrification is the sole process of N_2O production. This could be reliably checked using combinations of ^{15}N enriched NH_4^+ and NO_3^- with subsequent ^{15}N-N_2O analyses. If in such a system ^{18}O enriched H_2O is applied, the ^{18}O signal of the produced N_2O would be a direct quantification of O exchange during denitrification.

In addition to ^{18}O enriched H_2O, the use of ^{18}O enriched NO_3^- is an especially promising tool for studying O exchange. Combinations of treatments with ^{15}N and ^{18}O enriched NO_3^- and subsequent isotopic analyses of N_2O would allow the quantification of O exchange during denitrification. Without O exchange, the ratio of the N and O enrichment in the produced N_2O should equal that of the applied NO_3^-. In theory, this would hold in any soil system, regardless of the relative contribution of denitrification to N_2O production.

It is clear that ^{18}O analyses of NO_3^- would provide another useful tool in studying O exchange. Determination of the ^{18}O signature of soil NO_3^- may be done on soil extracts. However, such soil extraction for mineral N analyses is

often done with KCl at relatively high concentrations (1-2 M). Although it is unclear whether significant chemical exchange may take place in soil, chloride ions are reported to catalyze chemical O exchange in aqueous solutions (Anbar et al., 1961). The possibility of the occurrence of O exchange in such soil KCl extraction solutions, prior to O isotopic analyses, should therefore be considered.

Similar to all the other biochemical processes involved, the extent of O exchange is likely to be dependent on soil conditions like pH, moisture content and temperature. Consequently, the effect of these parameters on the extent of O exchange needs to be identified. In particular, in order to assess complications relating to source determination using isotope analysis, it needs to be determined whether ^{18}O exchange is progressive with time or whether it occurs at fixed rates during the process of (de)nitrification.

To summarize, a method needs to be developed to study the extent of O exchange in soil systems, as well as its controlling factors. Such analysis of O exchange should then be included in methodology using ^{18}O analyses of N_2O, and preferably also NO_3^-, to correctly interpret the isotopic data.

Conclusion

The literature reviewed demonstrates that most major groups of nitrifiers and denitrifiers are able to catalyze O exchange between NO_2^- and H_2O. Oxygen exchange, especially during denitrification, is likely to be significant in most soils, but uncertainty about the extent of exchange and its controls remains and needs to be studied. The occurrence of O exchange is a concern for isotope tracer methods using ^{18}O analyses, both at natural abundance and artificially enriched levels. Consequently, quantification of this exchange by microbial communities in the soil is necessary. As it is difficult to make a reliable distinction between ^{18}O fractionation and ^{18}O exchange at natural abundance levels, this needs to be done with artificially enriched ^{18}O compounds. In particular, we suggest that future research should focus on developing methods to quantify O exchange in soil (as opposed to in monocultures). In addition, it needs to be determined whether ^{18}O exchange is progressive with time or occurs at fixed rates (depending on environmental conditions) during the process of (de)nitrification. This is essential for correct interpretation of O isotopic signatures from tracer studies.

If O exchange in natural systems is significant but not corrected for, ^{18}O analysis may lead to an overestimation of microbial nitrification of soil N as the source of both NO_3^- and N_2O compared to fertilizer, manure and atmospheric deposition. We conclude that especially the ^{18}O signature of N_2O should only be used with extreme caution in N source determination studies.

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Chapter 3

The ^{18}O signature of biogenic nitrous oxide is determined by oxygen exchange with water

Abstract To effectively mitigate emissions of the greenhouse gas nitrous oxide (N_2O) it is essential to understand the biochemical pathways by which it is produced. The ^{18}O signature of N_2O is increasingly used to characterize these processes. However, assumptions on the origin of the O atom and resultant isotopic composition of N_2O that are based on reaction stoichiometry may be questioned. In particular, deficient knowledge on O exchange between H_2O and nitrogen oxides during N_2O production complicates the interpretation of the ^{18}O signature of N_2O. Here we studied O exchange during N_2O formation in soil, using a novel combination of ^{18}O and ^{15}N tracing. Twelve soils were studied, covering soil and land-use variability across Europe. All soils demonstrated the significant presence of O exchange, as incorporation of O from ^{18}O enriched H_2O into N_2O exceeded their maxima achievable through reaction stoichiometry. Based on the retention of the enrichment ratio of ^{18}O and ^{15}N of NO_3^- into N_2O, we quantified O exchange during denitrification. Up to 97% (median 85%) of the N_2O-O originated from H_2O instead of from the denitrification substrate NO_3^-. We conclude that in soil, the main source of atmospheric N_2O, the ^{18}O signature of N_2O is mainly determined by H_2O due to O exchange between nitrogen oxides and H_2O. This challenges the assumption that the O of N_2O originates from O_2 and NO_3^- as well, in ratios reflecting reaction stoichiometry.

D.M. Kool, N. Wrage, O. Oenema, D. Harris, J.W. Van Groenigen. 2009. The ^{18}O signature of biogenic nitrous oxide is determined by O exchange with water. Rapid Communications in Mass Spectrometry 23: 102-108

Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas and contributes to the breakdown of stratospheric ozone (Crutzen, 1981). Concerns about rising concentrations of atmospheric N_2O and the need to develop effective mitigation strategies have led to increased interest in its biochemical production pathways. Oxygen isotopic analyses, generally expressed as its ^{18}O signature, are commonly used in NO_3^- source determination and suggested to be a promising tool to study production and consumption of N_2O as well (Yoshinari et al., 1985; Böttcher et al., 1990; Kim et al., 1990; Durka et al., 1994; Cliff et al., 1997; Yoshinari et al., 1997; Naqvi et al., 1998; Pérez, 2005; Wrage et al., 2005; Menyailo et al., 2006b; Oelmann et al., 2007; Rock et al., 2007). Nitrification, nitrifier denitrification, and denitrification have been identified as the major microbial N_2O producing pathways in soils and oceans (Firestone et al., 1989; Granli et al., 1994). Based on their reaction stoichiometry, the relative contribution of O_2 and H_2O as sources of the O in N_2O differs for these processes (Figure 3.1). The O isotopic composition of N_2O is therefore considered to be distinct for these different pathways (Pérez, 2005; Wrage et al., 2005). However, the use of oxygen isotopic analyses to characterize these processes might be impaired by O exchange between H_2O and intermediates (e.g. nitrite, nitrate) in the various N_2O production pathways, which might alter the O isotopic signature (Kool et al., 2007). (Throughout this thesis, 'oxygen exchange' is used as short for the exchange of O between nitrogen oxides and H_2O.) Oxygen exchange between such intermediates and H_2O can be catalyzed by a variety of major groups of nitrifiers and denitrifiers (Kool et al., 2007). However, ecosystem studies using isotopes to determine sources of N_2O rarely consider the possible exchange of O between H_2O and nitrogen oxides. To our knowledge, its significance has never been established for soils, which constitute the main source of atmospheric N_2O (IPCC, 2007).

Here we evaluated the significance of O exchange during N_2O production in soil. We developed novel methodology using ^{18}O enriched H_2O and ^{18}O and ^{15}N enriched NO_3^-, combined with N_2O isotopic analyses, to study the process of O exchange. In a series of laboratory incubation experiments on 12 soils covering European soil and land-use variability, we identified the presence and quantified the extent of O exchange in soil.

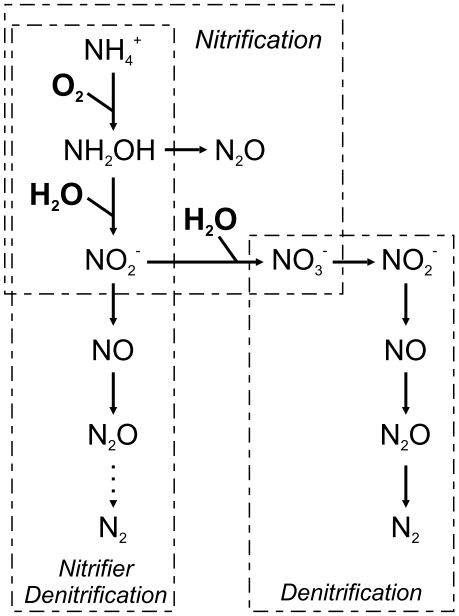

Figure 3.1: Incorporation of oxygen (O) from O_2 and H_2O into nitrogen oxides during nitrification, denitrification and nitrifier denitrification, following reaction stoichiometry.

Methods

Soil incubation

Soil samples were collected from 12 soils across Europe, of which location, soil properties and land-use are summarized in Table 3.1. The upper 10 cm of the soil was sampled after removal of the litter layer. The soil was dried at 40°C, sieved over 2 mm for homogeneity and stored at 4°C until further use.

Soil samples of 75 g dry soil were pre-incubated in glass jars for 7 days, at 16°C and 40% water holding capacity (WHC). Temperature and moisture conditions during incubation were set at 16°C and 80% WHC, respectively. The incubation period for the experiments was 28 h, as preliminary experiments had shown such a period to allow for sufficient N_2O production.

At the start of the incubation, all samples received 100 mg N kg^{-1} soil consisting of 50 mg NH_4^+-N kg^{-1} and 50 mg NO_3^--N kg^{-1} soil. Four different treatments were established, each replicated five times. The different treatments (TR) involved the application of compounds enriched in ^{18}O or ^{15}N, as follows:

^{18}O enriched H_2O (TR1), ^{18}O enriched NO_3^- (TR2), ^{15}N enriched NO_3^- (TR3), or ^{15}N enriched NH_4^+ (TR4). The respective compounds were enriched in ^{18}O at 1.0 atom% excess and at 40.0 atom% excess for ^{15}N. The NH_4^+ (^{15}N enriched and non-enriched) was applied as NH_4Cl; NO_3^- as $Ca(NO_3)_2{\cdot}4H_2O$ (^{15}N enriched and non-enriched) and partially as $NaNO_3$ in TR2 (^{18}O enriched NO_3^-). Demineralized water was used in all treatments to establish the correct moisture content. After treatment application, all jars were closed with septum-equipped lids for the duration of incubation.

At the end of incubation, gas samples were taken from the headspace and transferred to (vacuum) 12mL exetainers, to be analyzed on N_2O content and its ^{18}O signature. Subsequently, the soil dry weight of each replicate sample was determined to calculate its exact moisture content and therewith the exact ^{18}O enrichment of the soil moisture during incubation.

Table 3.1: Description of the 12 soils studied, sampled across Europe. F, G and A denote forest, grassland and arable soils, respectively.

		Location			Vegetation and management	
Code	Soil texture[a]	Latitude/ longitude	Country	pH (H_2O)	Vegetation and crops	Fertilizer[b] (kgN ha^{-1} yr^{-1})
F1	loam	48°30/11°11	GE	3.3[c]	Norway Spruce	0
F2	sandy loam	61°51/24°17	FI	3.6	Scots Pine	0
F3	sandy loam	55°29/11°38	DE	4.2	Beech	0
F4	sandy loam	52°22/05°32	NL	3.8	Douglas fir, Oak	0
G1	sand	46°41/19°36	HU	7.8	Festuca spp.	0
G2	clay	47°17/07°44	SW	6.0	Grass, Clover	150
G3	silt loam	55°52/-03°12	UK	6.2	Lolium perenne	120
G4	silt loam	55°52/-03°12	UK	5.9	Lolium perenne	290
A1	silty clay loam	51°06/10°55	GE	7.1	Sugerbeat, winter Wheat	100
A2	silt loam	48°51/01°58	FR	7.2	Mustard, Maize, Wheat, Barley	175
A3	sandy clay	40°31/14°57	IT	7.5	Maize, Alfalfa, Lolium perenne	500
A4	clay loam	45°12/09°04	IT	7.1	Maize, Rice	400, 100[d]

[a] USDA soil texture classification

[b] approximate, mineral plus organic fertilizer-N

[c] pH measured in $CaCl_2$

[d] fertilizer for maize, rice respectively

Isotopic analyses

Gas samples were analyzed at the UC Davis Stable Isotope Facility. The N_2O concentration and its ^{15}N and ^{18}O signatures were determined using a Sercon Cryoprep trace gas concentration system interfaced to a Sercon 20/20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Isotope ratios were compared with N_2 and N_2O reference gases injected into the mass spectrometer within each sample analysis. Results were normalized using correction factors derived for standard samples containing 1000 µL m^{-3} N_2O balanced with N_2, which were distributed throughout the analytical batch. No international certified isotope standards are available for N_2O; therefore, we calibrated the $\delta^{15}N$ of the N_2O reference gas by comparison with N_2 with known isotopic content (i.e. $\delta^{15}N$= -3.1‰ vs. atmospheric N_2) after reduction of N_2O to N_2 over copper at 600°C. We derived a $\delta^{18}O$ value for the N_2O reference gas by comparison with CO_2 of known isotopic content ($\delta^{18}O$ = 10.41‰ VSMOW) after conversion of both gases to CO over carbon at 1400°C. These measurements showed good precision for $\delta^{15}N$ in N_2O (standard deviation = 0.06‰ (n=8)) and greater variability for ^{18}O in N_2O (standard deviation = 0.96‰ (n=8)). We do not report ^{15}N and ^{18}O signatures when the N_2O concentrations were below 800 and 5000 µL m^{-3}, respectively, as we considered these values to be the lower threshold values for reliable analysis. The above gas concentrations correspond to 0.4 and 2.5 nmol N_2O in the gas samples. At these amounts of N_2O, the typical standard deviation of isotope measurements is approximately 3‰.

Data calculations

The use of enriched compounds allowed us to consider only reaction stoichiometry and O exchange as determinants of the ^{18}O signature of N_2O, as the effect of isotope fractionation would be negligible.

Presence of O exchange would be confirmed when the ^{18}O incorporation from H_2O into N_2O (TR1) exceeded the calculated maximum incorporation that could be achieved through reaction stoichiometry. In the absence of O exchange, only N_2O derived from NH_4^+ would contain ^{18}O originating from H_2O. According to reaction stoichiometry, NO_3^- produced through nitrification of NH_4^+ obtains two of its three O-atoms from H_2O (Figure 3.1). The same 2:3 ratio would hold for the

N_2O subsequently produced by denitrification of this nitrification-derived NO_3^-. When produced through nitrifier denitrification, the N_2O (resulting from NO_2^- reduction) obtains half of its O atoms from O_2 and half from H_2O during oxidation of NH_4^+ to NO_2^- (Figure 3.1). The maximum incorporation based on reaction stoichiometry is therefore calculated by assuming that all NH_4^+ derived N_2O is produced through nitrification-coupled denitrification. As such, the ^{18}O enrichment of N_2O could reach maximally 2/3rd of the ^{18}O enrichment of the applied H_2O:

$$Maximum\ ^{18}O\ incorporation\ (\%) = 2/3 \cdot {}^{18}O(H_2O) \cdot N_2O_{(NH4)} \qquad (eq\ 3.1)$$

where the $^{18}O(H_2O)$ is the O-enrichment of the applied H_2O (atom% excess), and $N_2O_{(NH4)}$ the percentage of NH_4^+-derived N_2O:

$$N_2O_{(NH4)} = 100 \cdot \frac{{}^{15}N\left(N_2O_{(TR4)}\right)}{{}^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \qquad (eq\ 3.2)$$

with $^{15}N(N_2O_{(TR3)})$ and $^{15}N(N_2O_{(TR4)})$ denoting the ^{15}N enrichment (atom% excess) of the N_2O in treatment TR3 and TR4, respectively (Table 3.2).

Application of both ^{18}O and ^{15}N enriched NO_3^- enabled the quantification of O exchange during denitrification. If no ^{18}O from NO_3^- would be exchanged with (non-enriched) H_2O-O during denitrification, the ^{18}O:^{15}N ratio of NO_3^- should be retained in N_2O, and all intermediates. Note that a dilution of the (intermediate) compounds would affect both enrichments equally, and therefore would not change their ratio. The ^{18}O:^{15}N enrichment ratio retention (ERR) in the N_2O compared to NO_3^- should therefore be 100% in the absence of O exchange:

$$ERR\ (\%) = 100 \cdot \frac{{}^{18}O\left(N_2O_{(TR2)}\right)}{{}^{15}N\left(N_2O_{(TR3)}\right)} \Bigg/ \frac{{}^{18}O\left(NO_3^-{}_{(TR2)}\right)}{{}^{15}N\left(NO_3^-{}_{(TR3)}\right)} \qquad (eq\ 3.3)$$

where $^{18}O(N_2O_{(TR2)})$ denotes the ^{18}O enrichment of the N_2O produced in treatment TR2, and $^{18}O(NO_3^-{}_{(TR2)})$ and $^{15}N(NO_3^-{}_{(TR3)})$ the ^{18}O and ^{15}N enrichment of the NO_3^- applied in treatment TR2 and TR3, respectively.

The loss of the ^{18}O enrichment relative to the ^{15}N from NO_3^- into N_2O consequently quantifies the percentage of O that has been exchanged (X_{ERR}):

$$X_{ERR} = 100 - ERR \qquad (eq\ 3.4)$$

Results and Discussion

In all soils the measured incorporation of O from ^{18}O enriched H_2O into N_2O exceeded the calculated maximum based on reaction stoichiometry, thereby confirming the presence and significance of O exchange during denitrification (Figure 3.2). Furthermore, the ^{18}O:^{15}N enrichment ratio retention (ERR) from NO_3^- into N_2O was incomplete for all soils (Figure 3.3), demonstrating O exchange during NO_3^- reduction. The median O exchange was 85%, indicating that substantial O exchange during denitrification readily occurs in most, if not all, soils (Figure 3.3 and 3.4). The extent of exchange was relatively low for soils F3 and F4, where N_2O production was only marginally above background levels (Table 3.2, Figure 3.4). We conclude that in soils exhibiting significant N_2O production, O exchange between H_2O and intermediates of (de)nitrification will be a widespread feature and therefore largely determine the O isotopic composition of the N_2O.

Table 3.2: Average N_2O production and its relevant isotopic enrichment for each treatment (^{18}O or ^{15}N). Production is averaged for samples across all treatments. The standard errors of the mean are given between brackets. F, G and A denote forest, grassland and arable soils, respectively.

	N_2O production[a]	Isotopic enrichment			
Code	$\mu gN_2O\text{-}N\ kg^{-1}$ soil	TR1 (^{18}O at%exc[b])	TR2 (^{18}O at%exc[b])	TR3 (^{15}N at%exc[b])	TR4 (^{15}N at%exc[b])
F1	1.7 (0.3)	0.676 (0.092)	0.073 (0.002)	13.38 (2.02)	0.27 (0.05)
F2	21.6 (2.5)	0.853 (0.015)	0.152 (0.003)	54.44 (0.97)	0.03 (0.01)
F3	0.5 (0.0)	0.164 (0.042)	0.135 (0.049)	6.10 (0.69)	0.25 (0.08)
F4	0.3 (0.0)	0.284 (0.052)	0.106 (0.022)	5.46 (1.23)	0.22 (0.08)
G1	26.2 (9.8)	0.764 (0.014)	0.052 (0.008)	3.04 (0.28)	26.92 (1.35)
G2	1031.0 (74.2)	0.896 (0.003)	0.064 (0.002)	26.50 (1.89)	0.70 (0.05)
G3	46.2 (13.7)	0.634 (0.137)	0.056 (0.011)	10.47 (1.22)	7.99 (2.55)
G4	924.3 (122.9)	0.868 (0.009)	0.090 (0.004)	20.27 (0.24)	0.52 (0.21)
A1	239.1 (20.6)	0.914 (0.011)	0.027 (0.002)	24.41 (0.18)	3.20 (0.15)
A2	219.5 (27.3)	0.995 (0.003)	0.016 (0.001)	19.54 (0.20)	6.06 (0.07)
A3	95.4 (10.8)	0.958 (0.003)	0.017 (0.001)	14.31 (0.07)	6.09 (0.17)
A4	146.3 (14.3)	0.912 (0.004)	0.060 (0.002)	18.82 (0.14)	2.95 (0.04)

[a] average for all treatments

[b] at%exc = atom % excess

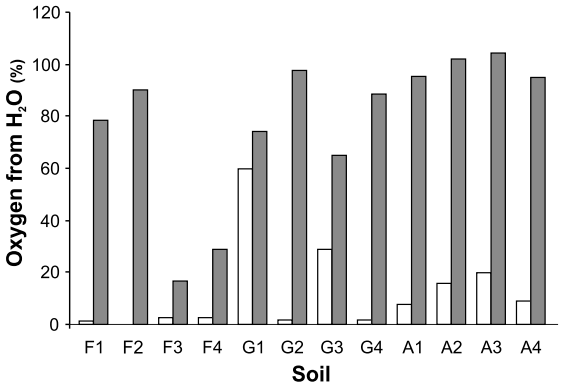

Figure 3.2: Oxygen incorporation from H_2O into N_2O for all soils (%). Grey bars present the actual O incorporation measured, white bars present the theoretical maximum in absence of O exchange. The actual amount of O incorporated from H_2O into N_2O exceeds the theoretical maximum for all soils. These maxima are based on reaction stoichiometry and the relative contribution of NH_4^+ and NO_3^- to total N_2O production, deduced from the ^{15}N enrichment data (Table 3.2).

To be complete, when interpreting the ERR we need to reflect on the potential of N_2O production through co-denitrification as well. N_2O derived through this process may have obtained (maximally) one of its N from another source, while all O may still originate from the (^{18}O enriched) NO_3^-. The measured relative loss in the ^{18}O:^{15}N enrichment ratio could thus be partially due to a dilution of the ^{15}N enrichment, which would imply a lower extent of O exchange than quantified with the current ERR assumptions. However, though the capacity for co-denitrification has been identified for fungi and bacteria (Garber et al., 1982; Kim et al., 1984; Tanimoto et al., 1992b; Morozkina et al., 2007), its significance in total N_2O production in soil has not been identified yet. As we currently quantify a median exchange of 85%, even if part of the loss in ERR should be ascribed to co-denitrification, the extent of O exchange remains highly significant.

Despite the diversity of the soils and land use types, O exchange occurred in all these soils. This suggests that O exchange is a universal feature of biogenic N_2O production. As O isotopic analyses of N_2O are employed in source determination studies outside soil ecosystems as well, we argue that implications of O exchange need to be considered across aqueous and atmospheric ecosystems, as the same microbial processes are responsible for N_2O production in those systems as well.

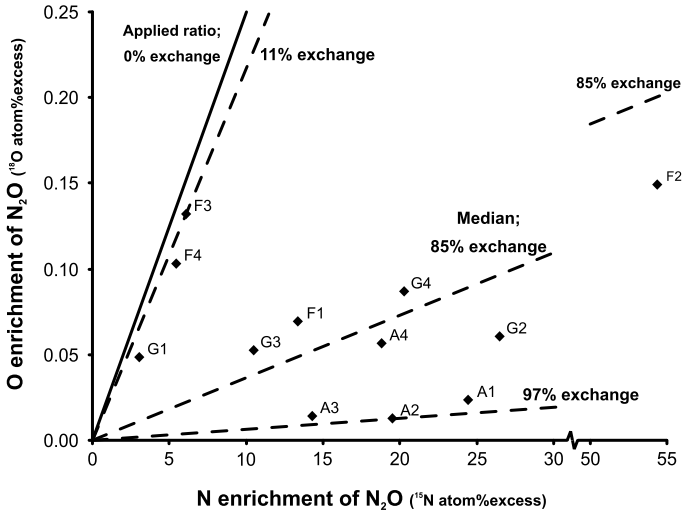

Figure 3.3: The ^{18}O and ^{15}N isotopic enrichment of N_2O produced from denitrification of applied NO_3^- (TR2 and TR3 for ^{18}O and ^{15}N respectively). When no O exchange occurs, the ratio of enrichments of the applied NO_3^-, represented by the solid line, should be retained in the produced N_2O (ERR = 100%). Data points for all soils are positioned below this line, denoting a loss in ^{18}O enrichment relative to ^{15}N in N_2O compared to the NO_3^- and demonstrating the presence of O exchange. The dashed lines indicate the minimum, maximum and median exchange measured in these soils.

In aqueous systems such as lakes, marine environments and wastewater treatment plants, characterization of O isotopic signatures of N_2O has been carried out to study its sources and sinks (Wahlen et al., 1985; Yoshinari et al., 1985; Kim et al., 1990; Yoshinari et al., 1997; Naqvi et al., 1998). The O isotopic composition of N_2O is thereby assumed to depend on the ^{18}O signatures of O_2 and H_2O during N_2O formation through nitrification and denitrification (in ratios reflecting reaction stoichiometry) and on fractionation upon its reduction to N_2 (Kim et al., 1990; Yoshinari et al., 1997). However, if H_2O is effectively the major O source of N_2O through the presence of O exchange as in our study, the interpretation of the O isotopic signatures in such studies needs to be reconsidered.

In the atmosphere, the O isotopic signature of N_2O will scarcely be affected by O exchange *in situ*. Despite the presence of active microorganisms in the atmosphere (Dimmick et al., 1979; Amato et al., 2007), N_2O is thought to be almost exclusively produced at the earth surface. Microbial nitrification and

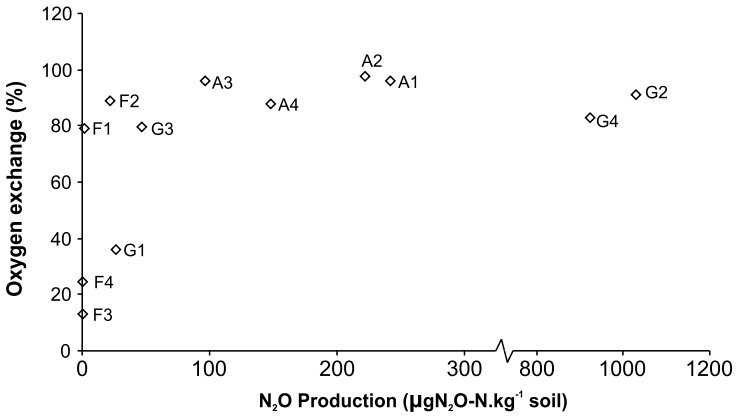

Figure 3.4: The O exchange during denitrification versus N_2O production for all soils. The amount of exchange is calculated using the enrichment ratio retention (ERR) method. The N_2O production is averaged over all replicates of the treatments used for the O exchange calculation (TR2 and TR3).

denitrification in soils and water are considered to be the main sources of atmospheric N_2O, with some minor contribution from biomass burning, industry, combustion in vehicles and power plants (Stein et al., 2003; Kaiser et al., 2005; Bernard et al., 2006; IPCC, 2007; Sorai et al., 2007). In the atmosphere, direct O exchange between H_2O and N_2O is unlikely (Wahlen et al., 1985; Cliff et al., 1997) and is experimentally proven to be negligible for the reaction $N_2O + O(^1D)$ in particular (Kaiser et al., 2005). However, the N_2O emitted from terrestrial and possibly aqueous ecosystems to the atmosphere will have been subject to O exchange during its biochemical production. This corroborates with the observation that the identified ^{17}O anomaly of atmospheric N_2O can be adequately explained by a balanced budget combining biological N_2O emissions and several chemical production sources (Kaiser et al., 2005), under the assumption that H_2O is the only source of O in the microbially produced N_2O, providing the N_2O with the same $^{16}O/^{17}O/^{18}O$-isotope signature as H_2O (Meijer et al., 1998; Kaiser et al., 2004). It remains striking that while these atmospheric studies assume the O in N_2O from the earth surface is exclusively derived from H_2O, terrestrial and aquatic studies generally assume that the N_2O-O is derived from both O_2 and H_2O in ratios reflecting reaction stoichiometry (Kim et al., 1990; Yoshinari et al., 1997; Pérez, 2005). Our results, though soil-derived, corroborate with the former assumption rather than the latter.

In addition, we suggest that O exchange might affect not only the N_2O, but also the intermediate compounds of its production. Future studies should therefore reflect on potential implications of O exchange for NO_3^- source determination based on O isotopic analyses as well.

In summary, our results show that H_2O constitutes the main source of O in N_2O and possibly other nitrogen oxides as well. Nevertheless, such compounds exhibit wide ranges in its O isotopic signature in different pools and sources. For N_2O emissions from soils alone, the reported $\delta^{18}O$ values range from 19.6 to 57.8‰ (Pérez, 2005). However, these large ranges can, partly, be explained by the lack of an international standard. Moreover, the O isotopic signature of H_2O itself (in precipitation, ground water, river water or even tap water) also varies widely across temporal and spatial scales (Dutton et al., 2005; Reddy et al., 2006; Bowen et al., 2007).

Conclusion

We conclude that up to 100% of the O in N_2O can be derived from H_2O through O exchange. Our results prove that general assumptions on the origin of the O and the consequent O isotopic signature of N_2O (Kim et al., 1990; Pérez, 2005) do not hold. O_2 as a source of N_2O-O may often be negligible; in N_2O production by denitrifiers, the O isotopic signature of N_2O does not necessarily reflect that of the substrate NO_3^- at all. The evident significance of O exchange during N_2O production poses a global challenge for the use and interpretation of O isotopic analyses in biogeochemical studies of the N cycle in the biosphere.

Acknowledgements

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Chapter 4

Oxygen exchange between nitrogen oxides and H_2O can occur during nitrifier pathways

Abstract Interpretation of the oxygen isotopic signature of soil-derived N_2O may be flawed when it is based on reaction stoichiometry and fractionation alone. In fact, oxygen (O) exchange between H_2O and intermediates of N_2O production pathways may largely determine this O isotopic signature. Although in our previous work we conclusively proved the occurrence of O exchange during N_2O production by denitrification of NO_3^-, its occurrence in N_2O production pathways by nitrifiers remains unclear. The aim of this study was to examine the likeliness of O exchange during various stages of N_2O production in soil via nitrification, nitrifier denitrification and denitrification. We evaluated a set of scenarios on the presence of such exchange using data from a series of ^{18}O and ^{15}N tracing experiments. The measured actual O incorporation from H_2O into N_2O (AOI) was compared with the theoretical maximum O incorporation (MOI) from various scenarios that differed in their assumptions on the presence of O exchange. We found that scenarios where O exchange was assumed to occur exclusively during denitrification could not explain the observed AOI, as it exceeded the MOI for 9 out of 10 soils. This demonstrates that additional O exchange must have occurred in N_2O production through nitrifier pathways. It remains to be determined in which steps of these pathways O exchange can take place. We conclude that O exchange is likely to be mediated by ammonia oxidizers during NO_2^- reduction (nitrifier denitrification), and that it could possibly occur during NO_2^- oxidation to NO_3^- by nitrite oxidizers as well.

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Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas and contributes to the breakdown of stratospheric ozone (Crutzen, 1981). The rising of its atmospheric concentrations, primarily caused by anthropogenic activities, has led to the demand for measures that adequately mitigate the emissions of N_2O into the atmosphere. Soils comprise the major source of atmospheric N_2O (IPCC, 2007), and accurate understanding of its biochemical production pathways in soil is therefore key to the development of adequate mitigation strategies. Nitrification, nitrifier denitrification, and denitrification have been identified as the major microbial N_2O producing pathways in terrestrial and aquatic ecosystems (Firestone et al., 1989; Granli et al., 1994; Wrage et al., 2001). Given the reaction stoichiometry of these pathways, the relative contribution of O_2 and H_2O as sources of the oxygen (O) in N_2O differs between these production pathways (Figure 4.1). The O isotopic signatures of N_2O are accordingly assumed to be distinct for these different pathways, providing O isotopic analysis of N_2O as a promising and increasingly used tool in studying its sources and production processes (Naqvi et al., 1998; Pérez, 2005; Wrage et al., 2005). Unfortunately, the interpretation of the O isotopic signature based on reaction stoichiometry (and fractionation effects) alone, may be significantly flawed because of O exchange between H_2O and intermediates of the production pathways (Kool et al., 2007; Kool et al., 2009a). In the few studies where it has been considered, O exchange was typically assumed to be negligible (Wahlen et al., 1985; Toyoda et al., 2005; Wrage et al., 2005; Menyailo et al., 2006a). However, based on a literature review we recommended that the O isotopic signature of N_2O should be interpreted with extreme caution because of the probability of O exchange (Kool et al., 2007). We recently showed experimentally, using a combination of O and N isotope tracing, that O exchange during N_2O production was highly significant in a wide range of soils (Kool et al., 2009a). Such exchange between H_2O and intermediates of N_2O production took place in all soils studied, with a median of 85% oxygen exchanged. We therefore concluded that the occurrence of O exchange needs to be taken into account to correctly interpret the O isotopic signature of N_2O (Kool et al., 2009a).

However, it is not clear during what processes and at which stages of N_2O

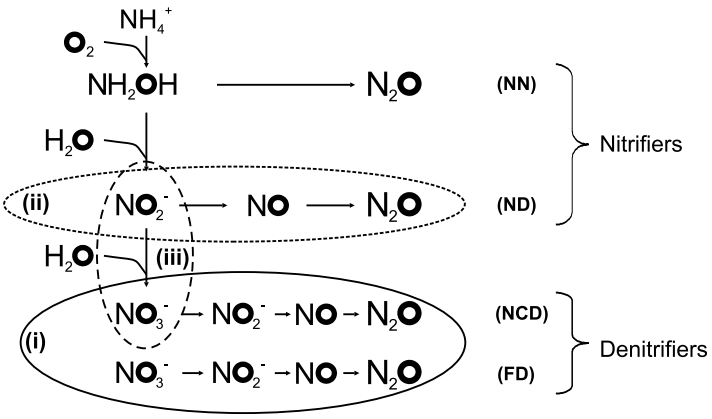

Figure 4.1: Major pathways of N_2O formation as considered in this study; by nitrifiers (ammonia oxidizers) through nitrification (NN) and nitrifier denitrification (ND), and by denitrifiers through reduction of NO_3^- produced by nitrifiers (NCD) and NO_3^- applied as e.g. fertilizer (FD). The O originates from O_2 and H_2O following reaction stoichiometry of the reaction steps of the pathways. Additional O exchange between nitrogen oxides and H_2O may alter the effective origin of N_2O-O. Several reactions in the stepwise production process of N_2O by denitrifiers (i) and nitrifiers (ii and iii) may facilitate O exchange.

production the O exchange occurs. The study by Kool et al. (2009a) confirmed its presence during denitrification of NO_3^- to N_2O (Figure 4.1, i). This will affect the O isotopic signature of N_2O produced through denitrification of NO_3^- generated through nitrification (nitrification-coupled denitrification, NCD), as well as from applied fertilizer NO_3^- (fertilizer denitrification, FD) (Figure 4.1). The widespread occurrence and high rates of O exchange during denitrification raises the question whether such O exchange might also be present during nitrifier-mediated N_2O formation. Oxygen exchange in nitrifier pathways may be anticipated because several reaction steps featuring in these pathways occur in the NO_3^- denitrification pathway as well. Two distinct processes may be eligible to facilitate O exchange: the reduction of NO_2^- to N_2O by ammonium oxidizers (Figure 4.1, ii); and NO_2^- oxidation to NO_3^- by nitrite oxidizers (Figure 4.1, iii). The first process would affect the O in N_2O produced through nitrifier denitrification (ND). Through the latter process, N_2O produced by NCD would be affected, and potentially by ND as well. For both processes, similarities are found between the enzymes that catalyze these reaction steps when carried out by nitrifiers and

denitrifiers. In ammonia oxidizers carrying out nitrifier denitrification (Figure 4.1, ii), the enzyme that reduces NO_2^- to NO is a copper-containing nitrite reductase (NiR) similar to the copper-NiR found in denitrifiers (Casciotti et al., 2001; Chain et al., 2003; Cantera et al., 2007; Garbeva et al., 2007). Also the genes encoding for NO reductase (NOR) involved in NO reduction by these nitrifiers appear analogous to those in denitrifiers (Casciotti et al., 2005; Garbeva et al., 2007). The oxidation of NO_2^- to NO_3^- by nitrite oxidizers (Figure 4.1, iii) is catalyzed by the enzyme nitrite oxidoreductase (Aleem, 1968; Sundermeyer-Klinger et al., 1984; Wood, 1986). This enzyme is found to be a molybdenum iron-sulfur complex, which is also the case for nitrate reductases that catalyze the reverse reaction in denitrifiers (Satoh, 1981; Sundermeyer-Klinger et al., 1984). As the O exchange during these transformations is likely to be a biochemical process, the similarity between the enzymes employed by nitrifiers and denitrifiers suggests that O exchange could occur in these nitrifier pathways as well.

Summarizing, in order to properly interpret the O isotopic signature of N_2O, we should explore the potential of O exchange during all N_2O producing pathways. In this paper we evaluate the likeliness of O exchange between H_2O and intermediates of the major N_2O production pathways. Our evaluation is based on the analysis of the incorporation of O from ^{18}O enriched H_2O and ^{18}O enriched NO_3^- into produced N_2O that was measured during soil incubation experiments. These results are compared with theoretical maxima of O incorporation for a series of scenarios that consider the occurrence of O exchange during the various N_2O producing pathways. The use of enriched compounds in the incubation experiments allowed us to disregard isotope fractionation and to focus on reaction stoichiometry and O exchange.

Methods

Soil incubation

Soil samples from 12 sites across Europe were collected for the soil incubation experiment (Kool et al., 2009a). The soils originated from forest, grassland, and arable fields, the main land uses across Europe (Table 4.1). The experimental units consisted of soil samples (75g) which were pre-incubated at 16°C and 40% water holding capacity (WHC) a week prior to the incubation. At the start of the

incubation, experimental units received different combinations of ^{18}O and ^{15}N labeled compounds. All units received equal total amounts of mineral N (50 mg NH_4^+-N kg^{-1} and 50 mg NO_3^--N kg^{-1} soil), were incubated at 80% WHC by adding appropriate amounts of H_2O, and the temperature was kept at 16°C. The following four treatments with isotopically enriched compounds were implemented; ^{18}O enriched H_2O at 1.0 atom% excess (TR1), ^{18}O enriched NO_3^- at 1.0 atom% excess (TR2), ^{15}N enriched NO_3^- at 40.0 atom% excess (TR3), and ^{15}N enriched NH_4^+ at 40.0 atom% excess (TR4). The experiment was set up as a completely randomized design, with five replicates for each of the four treatments. The jars were closed, by lids equipped with rubber septa, for an incubation period of 28h. At the end of the incubation, gas and soil samples were taken. Gas samples were extracted from the headspace and transferred to 12mL exetainers that were flushed with helium and evacuated before use. The N_2O

Table 4.1: Description of the 12 soils incubated, sampled across Europe. F, G and A denote forest, grassland and arable soils, respectively. Soils F3 and F4 were excluded from the scenario evaluation as their total N_2O production during incubation was only marginally above background levels.

		Location			Vegetation and management	
Code	Soil texture[a]	Latitude/ longitude	Country	pH (H_2O)	Vegetation and crops	Fertilizer[b] (kgN ha^{-1} yr^{-1})
F1	loam	48°30/11°11	GE	3.3[c]	Norway Spruce	0
F2	sandy loam	61°51/24°17	FI	3.6	Scots Pine	0
F3	sandy loam	55°29/11°38	DE	4.2	Beech	0
F4	sandy loam	52°22/05°32	NL	3.8	Douglas fir, Oak	0
G1	sand	46°41/19°36	HU	7.8	Festuca spp.	0
G2	clay	47°17/07°44	SW	6.0	Grass, Clover	150
G3	silt loam	55°52/-03°12	UK	6.2	Lolium perenne	120
G4	silt loam	55°52/-03°12	UK	5.9	Lolium perenne	290
A1	silty clay loam	51°06/10°55	GE	7.1	Sugerbeat, winter Wheat	100
A2	silt loam	48°51/01°58	FR	7.2	Mustard, Maize, Wheat, Barley	175
A3	sandy clay	40°31/14°57	IT	7.5	Maize, Alfalfa, Lolium perenne	500
A4	clay loam	45°12/09°04	IT	7.1	Maize, Rice	400, 100[d]

[a] USDA soil texture classification
[b] approximate, mineral plus organic fertilizer-N
[c] pH measured in $CaCl_2$
[d] fertilizer for maize, rice respectively

concentration and its isotopic signature were measured at the UC Davis Stable Isotope Facility, using a Sercon Cryoprep trace gas concentration system interfaced to a Sercon 20/20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Further details on the gas sampling and analyses for N_2O production and isotopic signature were described previously (Kool et al., 2009a).

Soil samples were taken after gas sampling. Sub-samples of the soil were taken to determine the soil moisture content and the exact ^{18}O enrichment of the soil water. Only minor changes in the moisture content over the incubation period were observed. Other sub-samples of approximately 20 g moist soil were taken for analyses of mineral N (NH_4^+-N and NO_3^--N) and its ^{15}N isotopic signature. Soil mineral N content was determined by extraction with 1M KCl (50 mL $20g^{-1}$ soil) followed by segmented flow analyses (SFA) (Skalar Analytical, Breda, The Netherlands) (Kool et al., 2006).

The ^{15}N enrichments of the mineral N were derived using a microdiffusion method based on Van Groenigen et al. (2005b). In short, for the NH_4^+ isolation a microfilter spiked with $KHSO_4$ (2M) and packed in Teflon was added to the sample, together with ashed MgO to raise the pH to approximately 10, and the sample containers were closed for (at least) 6 days. The filter was removed before the addition of Devarda's alloy, and a new filter for the NO_3^- isolation was added. The samples were left at room temperature (20°C) for both microdiffusion steps. The isotopic analyses were carried out at UC Davis SIF on an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer (EA-IRMS) (Sercon 20/20, Sercon Ltd., Cheshire, UK). Two laboratory standards were analyzed with every 12 samples. The laboratory samples were calibrated against NIST standard reference materials.

Data calculations

The measured N_2O production, its ^{18}O and ^{15}N signatures and the ^{15}N signatures of NH_4^+ and NO_3^- provided the input for our calculations. The isotopic signatures of the soil mineral N used are the average enrichments over the incubation period, calculated by assuming linear changes in enrichment of the pools. Of the 12 soils used in the incubation, two (F3 and F4) were excluded from further analysis as their total N_2O production was only marginally above background levels (Kool et al., 2009a).

The ^{18}O incorporation from H_2O into N_2O, derived from TR1, was calculated previously in Kool et al. (2009a). It is here referred to as the 'actual O incorporation' (AOI, in %), and was calculated as follows:

$$AOI = 100 \cdot \frac{^{18}O\left(N_2O_{(TR1)}\right)}{^{18}O\left(H_2O_{(TR1)}\right)} \qquad (eq\ 4.1)$$

where $^{18}O(N_2O_{(TR1)})$ and $^{18}O(H_2O_{(TR1)})$ denote the O isotopic enrichment (atom% excess) of the produced N_2O and the soil H_2O, respectively, in TR1.

The extent of O exchange (X_{ERR}) for all soils was calculated using the ^{18}O:^{15}N enrichment ratio retention (ERR) method (Kool et al., 2009a). This ERR method quantifies the exchange by comparing the ratio of ^{18}O and ^{15}N enrichment in the produced N_2O with the ratio at which it was applied in NO_3^-. In other words, this ERR is the percentage (%) of the ratio of ^{18}O:^{15}N enrichment in the NO_3^- that is retained in the N_2O:

$$ERR = 100 \cdot \frac{^{18}O\left(N_2O_{(TR2)}\right)}{^{15}N\left(N_2O_{(TR3)}\right)} \Big/ \frac{^{18}O\left(NO_3^-{}_{(TR2)}\right)}{^{15}N\left(NO_3^-{}_{(TR3)}\right)}$$

or;

$$ERR = 100 \cdot \frac{^{18}O\left(N_2O_{(TR2)}\right)}{^{15}N\left(N_2O_{(TR3)}\right)} \cdot \frac{^{15}N\left(NO_3^-{}_{(TR3)}\right)}{^{18}O\left(NO_3^-{}_{(TR2)}\right)} \qquad (eq\ 4.2)$$

where $^{18}O(N_2O_{(TR2)})$ and $^{15}N(N_2O_{(TR3)})$ denote the O and N enrichment of the produced N_2O, and $^{18}O(NO_3^-{}_{(TR2)})$ and $^{15}N(NO_3^-{}_{(TR3)})$ the O and N enrichment of the applied NO_3^- (atom% excess), in TR2 and TR3, respectively. This ratio of the ^{18}O to the ^{15}N enrichments should be conserved in the absence of O exchange, as it is not altered (through dilution) by N_2O production through other sources (i.e. an ERR of 100%). It will however decrease when O exchange occurs, as that would only alter the ^{18}O enrichment and not the ^{15}N. The loss in ^{18}O enrichment relative to ^{15}N from NO_3^- into N_2O quantifies the percentage (%) of O that has been exchanged:

$$X_{ERR} = 100 - ERR \qquad (eq\ 4.3)$$

The relative proportions of total N_2O derived from NH_4^+ and NO_3^-, $N_2O_{(NH4)}$ and $N_2O_{(NO3)}$ respectively, follow from the ^{15}N enrichment of the N_2O established in the treatments TR3 and TR4. They are calculated as percentage (%) of total N_2O production:

$$N_2O_{(NH4)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR4)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \quad (eq\ 4.4)$$

$$N_2O_{(NO3)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR3)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \quad (eq\ 4.5)$$

where $^{15}N(N_2O_{(TR4)})$ refers to the ^{15}N enrichment (atom% excess) of the N_2O in TR4.

Oxygen exchange evaluation

We explore the likeliness of O exchange during the various N_2O production pathways by evaluating the incorporation of O from H_2O into N_2O ($OI(N_2O)$). The actual OI (AOI) was measured in the incubation study. Next, the theoretical maximum OI (MOI) is calculated based on the maximum O incorporation that can be attained through reaction stoichiometry, plus the potential O exchange dependent on various scenarios. If the AOI exceeds MOI this implies that the measured ^{18}O enrichment of the N_2O cannot be fully explained with the assumptions on O exchange under that scenario. Higher MOI that would better fit the observed AOI could be obtained when O exchange is assumed to be more abundant. Accordingly, a series of six scenarios A to F is constructed and evaluated, where O exchange is assumed to take place during one or more of the processes in N_2O production pathways (Figure 4.1).

Oxygen exchange scenarios

In the scenarios we considered the occurrence of O exchange during one or more of the following reaction steps (Figure 4.1):

i. NO_3^- reduction by denitrifiers (denitrification);
ii. NO_2^- reduction to N_2O by ammonia oxidizers (nitrifier denitrification);
iii. NO_2^- oxidation to NO_3^- by nitrite oxidizers (second part of nitrification).

For the process of NO_2^- oxidation (iii), in the scenarios we made the distinction between O exchange that will effect the O isotopic composition of the product NO_3^- only (iii-a), or for the NO_2^- (substrate) as well (iii-b). The occurrence and extent of O exchange has already been established for (i), NO_3^- reduction by denitrifiers (Kool et al., 2009a), and is thus included in all scenarios. Wherever additional O exchange is considered to be present, we assume that it takes place to the same extent as it was quantified for NO_3^- reduction to N_2O (X_{ERR}). We calculate the theoretical maximum O incorporation (MOI) for six scenarios A through F, under which O exchange is assumed to occur as follows (Table 4.2):

A. only during the denitrification of NO_3^- by denitrifiers (i);
B. during (i), and during nitrifier denitrification of NO_2^- (ii)
C. during (i), and during oxidation of NO_2^- to NO_3^- by nitrifiers (iii), affecting only the NO_3^- (iii-a);
D. during (i), and during oxidation of NO_2^- to NO_3^- by nitrifiers (iii), affecting both the NO_2^- and NO_3^- (iii-b);
E. during (i), during nitrifier denitrification of NO_2^- (ii), and during oxidation of NO_2^- to NO_3^- by nitrifiers (iii), affecting only the NO_3^- (iii-a);
F. during (i), during nitrifier denitrification of NO_2^- (ii), and during oxidation of NO_2^- to NO_3^- by nitrifiers (iii), affecting both the NO_2^- and NO_3^- (iii-b).

Note that when all N_2O would be produced by denitrifiers (FD plus NCD), the O incorporation from H_2O into N_2O under scenario B would not differ from A, and that of D, E and F would not differ from that of C.

Table 4.2: Overview of the N-transformation processes during which O exchange is considered to occur under the different scenarios A to F. Figure 4.1 depicts the indicated processes, i.e. denitrification of NO_3^- (i), nitrifier denitrification (ii), and NO_2^- oxidation (iii). For iii, O exchange is assumed to affect only the product NO_3^- under iii-a, and both NO_3^- and the NO_2^- under iii-b. A 'V' indicates that O exchange during the respective processes is included in the particular scenario.

	Processes[a]			
Scenario	i	ii	iiia	iiib
A	V			
B	V	V		
C	V		V	
D	V			V
E	V	V	V	
F	V	V		V

[a] Figure 4.1 depicts the processes indicated as i, ii and iii

Oxygen incorporation calculation

The total O incorporation from H_2O into N_2O ($OI(N_2O)$) is determined by the O incorporation through each pathway ($OI(N_2O_p)$), and by the relative contribution of the different pathways to total production (N_2O_p). The extent of H_2O-O incorporation into N_2O for each pathway ($OI(N_2O_p)$) times their relative contribution to total N_2O production (N_2O_p) sum up to the total O incorporation from the applied enriched H_2O into N_2O:

$$OI(N_2O) = \sum N_2O_p \cdot OI(N_2O_p)$$

$$= N_2O_{FD} \cdot OI(N_2O_{FD}) + N_2O_{NCD} \cdot OI(N_2O_{NCD})$$

$$+ N_2O_{ND} \cdot OI(N_2O_{ND}) + N_2O_{NN} \cdot OI(N_2O_{NN}) \qquad (eq\ 4.6)$$

Each $OI(N_2O_p)$ is a sum of the incorporation of H_2O-O through reaction stoichiometry plus additional incorporation through O exchange. Both differ for the various production pathways, the latter being constrained by the O exchange scenarios. The allocated N_2O_p are constrained by the results of the soil incubation experiment. The different pathways facilitate different amounts of H_2O-O incorporation through reaction stoichiometry. To calculate the theoretical maximum $OI(N_2O)$ (the MOI), the N_2O_p of those pathways providing the highest $OI(N_2O_p)$ through reaction stoichiometry is maximized.

In the appendices, we describe in detail how the partial $OI(N_2O_p)$ and the relative contributions of the different pathways (N_2O_p) are calculated for the respective scenarios (appendices A4-1 and A4-2, respectively). To evaluate the impact of maximizing the stoichiometric O incorporation, we explored two additional sub-evaluations S1 and S2 (appendix A4-2.3) where the contribution of the pathways providing highest $OI(N_2O_p)$ is not maximized. In the calculations, all $OI(N_2O_p)$ and the X_{ERR} are inserted as fractions (range 0 to 1); the N_2O_p, AOI and MOI are in percentages.

Sensitivity Analyses

In our evaluation it is assumed that O exchange takes place to the same extent as quantified for the denitrification pathway. We believe that this provides the most reasonable estimate of the extent of exchange. However, to further evaluate the

implications of this assumption, we carried out a sensitivity analysis of the parameter X_{ERR} varying it by ± 10% (maximum value 100%) to evaluate resulting changes in the MOI.

Results

Table 4.3 lists the actual O incorporation from H_2O into N_2O (AOI) derived from treatment 1 (TR1), the extent of O exchange during denitrification (X_{ERR}) derived from TR2 and TR3, and the proportions of N_2O derived from NH_4^+ and NO_3^- (derived from TR3 and TR4). All those parameters were calculated directly from data provided in Table 4.4, which presents the N_2O production and the relevant ^{18}O and ^{15}N signatures of N_2O and soil mineral N.

Averaged over all soils considered, 89.6% of O in the N_2O originated from H_2O. These levels of AOI confirm the presence of O exchange for all soils (Kool et al., 2009a). In general the data showed low levels of variation between replicates. However, two soils (F1 and G3) had relatively large SE values which could

Table 4.3: Actual O incorporation from H_2O (AOI), the extent of O exchange during NO_3^- reduction to N_2O (X_{ERR}), and the proportion of N_2O derived from NH_4^+ and NO_3^- (%). These parameters are calculated directly from the measured isotopic data, listed in Table 4.4.

	AOI		X_{ERR}	Nmin[a] contribution (%)	
Soil	%	(SE)	%	$N_2O_{(NO3)}$	$N_2O_{(NH4)}$
F1	79.0	(11.1)	78.3	98.02	1.98
F2	90.7	(1.8)	88.8	99.95	0.05
G1	74.6	(1.2)	32.2	10.16	89.84
G2	98.2	(0.4)	90.4	97.44	2.56
G3	65.4	(14.0)	78.7	56.71	43.29
G4	89.0	(1.0)	82.2	97.50	2.50
A1	95.9	(1.1)	95.6	88.43	11.57
A2	102.5	(0.3)	96.8	76.33	23.67
A3	104.9	(0.4)	95.3	70.14	29.86
A4	95.4	(0.4)	87.3	86.46	13.54

[a] mineral N

Table 4.4: Total N_2O production (µg N kg^{-1} soil) and relevant O and N isotopic signatures (atom% excess) of N_2O and soil mineral N (NH_4^+, NO_3^-) of all soils considered in the assessment. Standard errors are given between brackets (n=20 for N_2O production, n=5 for all other data). In TR1, TR2, TR3 and TR4, soils received ^{18}O enriched H_2O, ^{18}O enriched NO_3^-, ^{15}N enriched NO_3^-, and ^{15}N enriched NH_4^+, respectively.

	N_2O production	N_2O isotopic enrichment (atom% excess)				Soil mineral N isotopic enrichment (atom% excess)[a]			
	µgN kg^{-1} soil	^{18}O-N_2O		^{15}N-N_2O		^{15}N-$NH4^+$		^{15}N-NO_3^-	
		TR1	TR2	TR3	TR4	TR3	TR4	TR3	TR4
Soil		H_2O -^{18}O	NO_3^--^{18}O	NO_3^--^{15}N	NH_4^+-^{15}N	NO_3^--^{15}N	NH_4^+-^{15}N	NO_3^--^{15}N	NH_4^+-^{15}N
F1	1.7 (0.3)	0.68 (0.09)	0.07 (0.00)	13.38 (2.02)	0.27 (0.05)	0.21 (0.01)	30.81 (0.52)	33.34 (0.39)	1.71 (0.16)
F2	21.6 (2.5)	0.85 (0.01)	0.15 (0.00)	54.44 (0.97)	0.03 (0.01)	0.20 (0.00)	31.40 (0.06)	34.63 (0.20)	2.20 (0.11)
G1	26.2 (9.8)	0.76 (0.01)	0.05 (0.01)	3.04 (0.28)	26.92 (1.35)	0.20 (0.00)	32.93 (0.06)	26.23 (0.04)	2.93 (0.02)
G2	1031.0 (74.2)	0.90 (0.00)	0.06 (0.00)	26.50 (1.89)	0.70 (0.05)	0.22 (0.00)	33.10 (0.12)	30.37 (0.02)	1.61 (0.03)
G3	46.2 (13.7)	0.63 (0.14)	0.06 (0.01)	10.47 (1.22)	7.99 (2.55)	0.19 (0.00)	30.19 (0.21)	25.33 (0.03)	2.56 (0.06)
G4	924.3 (122.9)	0.87 (0.01)	0.09 (0.00)	20.27 (0.24)	0.52 (0.21)	0.20 (0.00)	28.42 (0.21)	27.43 (0.15)	1.68 (0.08)
A1	239.1 (20.6)	0.91 (0.01)	0.03 (0.00)	24.41 (0.18)	3.20 (0.15)	0.20 (0.00)	35.14 (0.08)	29.81 (0.04)	2.40 (0.08)
A2	219.5 (27.3)	1.00 (0.00)	0.02 (0.00)	19.54 (0.20)	6.06 (0.07)	0.20 (0.00)	34.27 (0.12)	29.17 (0.05)	2.12 (0.03)
A3	95.4 (10.8)	0.96 (0.00)	0.02 (0.00)	14.31 (0.07)	6.09 (0.17)	0.20 (0.00)	36.68 (0.03)	27.77 (0.02)	2.26 (0.02)
A4	146.3 (14.3)	0.91 (0.00)	0.06 (0.00)	18.82 (0.14)	2.95 (0.04)	0.20 (0.00)	17.59 (0.07)	27.96 (0.07)	0.77 (0.01)

[a] average over the incubation period

complicate further interpretation. The extent of O exchange during NO_3^- reduction (X_{ERR}) was significant for all soils, ranging between 32% (G1) to almost 100% (A1, A2, A3; Table 4.3). The N_2O was mainly derived from NO_3^--N (fertilizer-N) for most soils. On average $N_2O_{(NO3)}$ was 77.2%; for 4 out of the 10 soils it was more than 95% (so $N_2O_{(NH4)}$ less than 5%). In soil G3, N_2O was almost evenly derived from NO_3^--N and NH_4^+-N, and only in soil G1 most N_2O-N originated from NH_4^+ (Table 4.3).

The partial OI and relative contributions to N_2O production of the different pathways are presented in Table 4.5, and the therewith calculated MOIs under all scenarios in Table 4.6. The ranges in the MOI obtained from the sensitivity analyses on the X_{ERR} are included in Table 4.6. As a result of the high rates of O exchange quantified for denitrification (X_{ERR}), the pathways in general facilitated high rates of O incorporation (except for NN, as defined). In most soils the N_2O was mainly derived from NO_3^-, i.e. the contribution of fertilizer denitrification

Table 4.5: The partial O incorporations (OI_p) and relative contributions to total N_2O production (N_2O_p) of the different pathways under the different scenarios A-F. The OI_p and N_2O_ps are derived as described in appendices 4-1 and 4-2.

	Partial Oxygen Incorporation (OI_p, fractions)								Pathway contributions (%)		
	OI_{FD}	OI_{NCD}		OI_{ND}				OI_{NN}	N_2O_{FD}	N_2O_{NCD}	N_2O_{NN+ND}
Soil	A-F	A,B	C,D,E,F	A,C	B,E	D	F	A-F	A-F	A-F	A-F
F1	0.78	0.93	0.98	0.50	0.89	0.93	0.98	0.00	98.02	1.98	0.00
F2	0.89	0.96	1.00	0.50	0.94	0.96	1.00	0.00	99.95	0.05	0.00
G1	0.32	0.77	0.85	0.50	0.66	0.77	0.85	0.00	10.16	17.96	71.88
G2	0.90	0.97	1.00	0.50	0.95	0.97	1.00	0.00	97.44	2.56	0.00
G3	0.79	0.93	0.98	0.50	0.89	0.93	0.98	0.00	56.71	34.81	8.48
G4	0.82	0.94	0.99	0.50	0.91	0.94	0.99	0.00	97.50	2.50	0.00
A1	0.96	0.99	1.00	0.50	0.98	0.99	1.00	0.00	88.43	11.29	0.28
A2	0.97	0.99	1.00	0.50	0.98	0.99	1.00	0.00	76.33	20.77	2.90
A3	0.95	0.98	1.00	0.50	0.98	0.98	1.00	0.00	70.14	21.51	8.34
A4	0.87	0.96	0.99	0.50	0.94	0.96	0.99	0.00	86.46	12.98	0.57

(N_2O_{FD}) was generally large (Table 4.5). In most soils the majority of the $N_2O_{(NH4)}$ could theoretically be associated with the NCD pathway. In only three soils (G1, G3, A3) a contribution of direct N_2O production by nitrifiers (NN plus ND) of minimally 5% was assigned. As a result, the calculated MOIs were high for nearly all soils under all scenarios (Table 4.6). A comparison between the MOIs and AOI for the soils with a minimum nitrifier contribution of 10% is depicted in Figure 4.2a. Figure 4.2b presents the comparison of the AOI with the OI resulting from the evaluations S1 and S2, where the NCD contribution is not maximized. Differences between AOI and OI-S1 and OI-S2 (Figure 4.2b) were larger than those between AOI and MOI (Figure 4.2a), especially in those scenarios that include O exchange in fewer reaction steps (A,C).

Table 4.6: The maximum O incorporation (MOI, %) for scenario A-F on the presence of O exchange, constrained by the OI_p and N_2O_p. For soils where all NH_4^+ derived N_2O may theoretically be ascribed to NCD, the MOI under B equals A, and D, E and F equal C. Results from the sensitivity analyses (sa) provide the range of the MOI for X_{ERR} ± 10%.

	Maximum Oxygen Incorporation from H_2O (%)					
Soil	A	B	C	D	E	F
F1	78.6	78.6	78.7	78.7	78.7	78.7
sa	86.3-70.9	86.3-70.9	86.4-71.0	86.4-71.0	86.4-71.0	86.4-71.0
F2	88.8	88.8	88.8	88.8	88.8	88.8
sa	97.7-79.9	97.7-79.9	97.7-80.0	97.7-80.0	97.7-80.0	97.7-80.0
G1	53.1	64.7	54.4	74.1	66.0	79.3
sa	53.6-52.6	66.3-63.0	55.0-53.8	75.4-72.7	67.7-64.2	80.9-77.7
G2	90.6	90.6	90.6	90.6	90.6	90.6
sa	99.5-81.7	99.5-81.7	99.5-81.8	99.5-81.8	99.5-81.8	99.5-81.8
G3	81.2	84.5	83.2	86.8	86.5	87.3
sa	86.6-75.8	90.3-78.8	87.9-78.2	91.8-81.6	91.6-81.2	92.1-82.2
G4	82.5	82.5	82.7	82.7	82.7	82.7
sa	90.6-74.5	90.6-74.5	90.7-74.6	90.7-74.6	90.7-74.6	90.7-74.6
A1	95.8	95.9	95.9	96.1	96.1	96.1
sa	99.9-87.0	100-87.1	99.9-87.4	100-87.6	100-87.6	100-87.6
A2	95.9	97.3	96.1	97.5	97.5	97.5
sa	98.6-87.8	100-89.1	98.6-88.6	100-89.9	100-89.9	100-90.0
A3	92.2	96.1	92.5	96.5	96.5	96.7
sa	95.8-84.8	100-88.4	95.8-85.7	100-89.5	100-89.3	100-89.8
A4	88.2	88.4	88.7	88.9	88.9	88.9
sa	96.1-80.2	96.4-80.5	96.3-81.0	96.5-81.2	96.5-81.2	96.5-81.3

sa: sensitivity analyses, X_{ERR} is varied plus to minus 10%

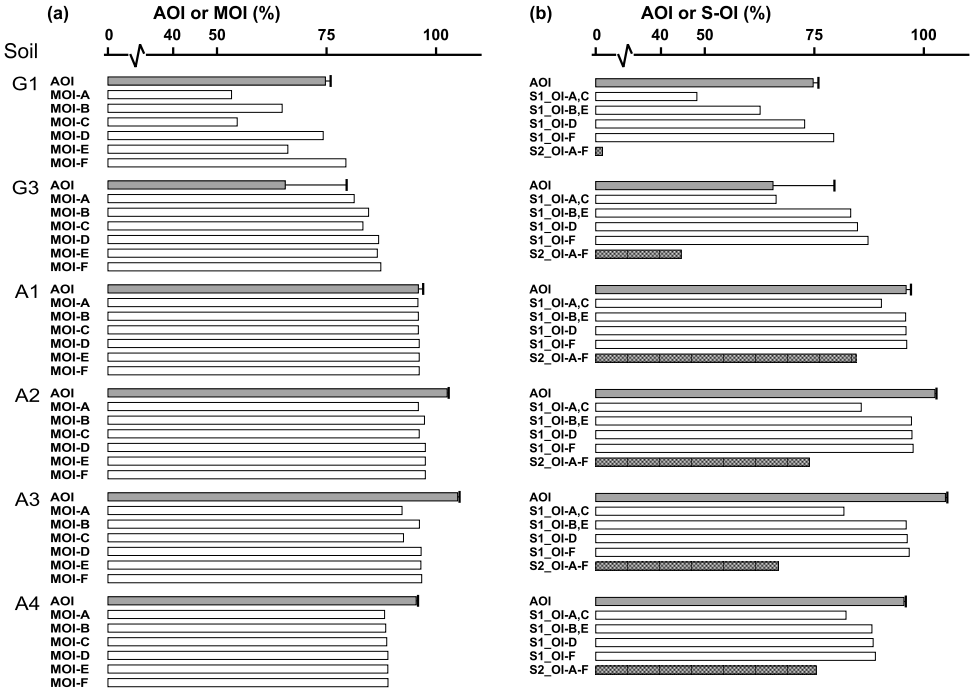

Figure 4.2: Comparison of the actual (AOI) and maximum O incorporation for all scenario evaluations (MOI) for soils G1, G3, A1, A2, A3 and A4. In these soils minimally 10% of the N_2O was NH_4^+-derived, i.e. an indirect (NCD) or direct (NN or ND) contribution of nitrifiers. Error bars indicate the SE of the AOI. **(a)** The AOI and the MOI for all scenarios. For the calculation of the MOI, the N_2O_{NCD} is maximized. **(b)** The AOI and the S1-OI and S2-OI for all scenarios. Under S1 and S2, N_2O_{ND} and N_2O_{NN} are respectively maximized. For S1, the OI under C will equal A, and E will equal B. For S2, the OI equals for all scenarios.

Discussion

Oxygen exchange during nitrifier-mediated N_2O production pathways

The measured O incorporation from H_2O into N_2O cannot for all soils be explained by O exchange during denitrification alone. The results therefore suggest that O exchange can also occur during nitrifier-mediated processes. In some of the soils, the contribution of the nitrifier pathways to total N_2O production was negligible. We therefore focus on those soils which showed a contribution of nitrifiers to N_2O production, either directly (NN and ND) and/or indirectly (NCD), of at least 10% (soils G1, G3, A1, A2, A3 and A4; Table 4.5).

Our results showed that O exchange between H_2O and intermediates of N_2O production occurred in nitrifier pathways in at least two out of the six above mentioned soils. For these soils, G1 and A3, comparison of the AOI and MOI for the different scenarios implied that O exchange must have been present in nitrifier pathways to reach the measured AOI. For the pathway of nitrifier denitrification (ND, Figure 4.1(ii)), the analyses of both soils confirmed the presence of O exchange because the AOI is in better agreement with the MOI when it is included in the scenarios (scenario B, E, and F vs. A, C, and D, respectively). We anticipated that the presence of O exchange in nitrifier denitrification would be likely since the enzymes involved in N_2O production of that pathway are similar to the denitrifier enzymes (Casciotti et al., 2001; Chain et al., 2003; Casciotti et al., 2005; Cantera et al., 2007; Garbeva et al., 2007). Moreover, several monoculture studies with denitrifiers have identified O exchange during reduction of NO_2^- (Garber et al., 1982; Aerssens et al., 1986; Shearer et al., 1988; Ye et al., 1991). Next to the ND pathway, results also supported the notion that O exchange may take place during NO_2^- conversion to NO_3^- by nitrite oxidizers, affecting the O isotopic composition of both NO_2^- and NO_3^- (Figure 4.1, iii-b). In soil G1, comparison of scenarios D with A and F with B showed that the AOI was better explained when such O exchange was assumed to be present (Figure 4.2a, Table 4.6). Oxygen exchange during NO_2^- conversion to NO_3^- without affecting the NO_2^--O (Figure 4.1, iii-a) would not provide sufficient additional O incorporation to explain the observed AOI (scenario C versus A).

Apart from the large relative contribution of FD to total N_2O production, our evaluation of O exchange during nitrifier pathways is complicated by two main (required) assumptions: i.e. the assumptions on the contribution of NCD to N_2O and on the extent of O exchange. Due to the large allocated N_2O_{NCD} and X_{ERR}, the calculated MOIs are high and vary only marginally for the different scenarios. However, for both assumptions it holds that this evaluation likely underestimates the presence of O exchange, and that O exchange in N_2O production pathways by nitrifiers in reality would be more profound, as discussed below.

A smaller percentage of NH_4^+ derived N_2O assigned to NCD would leave a larger contribution to NN plus ND. The pathways of NN and ND allow less O incorporation from H_2O through reaction stoichiometry, so the maximization of NCD implies that the MOI we calculate may overestimate the stoichiometric OI.

It would then jointly underestimate the OI through O exchange, i.e. a larger part of the AOI would in fact be due to O exchange. When ND or NN were maximized (Figure 4.2b) at the expense of NCD (S1 and S2 respectively), analyses of soil G1 and A3 further support the presence of O exchange during nitrifier pathways as already confirmed with the MOI. In three of the other four soils that showed a minimal nitrifier contribution of 10% (A1, A2 and A4), the majority of the $N_2O_{(NH4)}$ could theoretically be ascribed to NCD. Under S1 and S2, all three showed that additional O exchange by nitrifiers would be required to explain the AOI when ND or NN were maximized instead of NCD. Of all soils, the effect of maximizing the NCD contribution was most prominent for soil G3. In this single soil, the AOI did not exceed the MOI, not even when O exchange was considered to be present only during denitrification (scenario A). However, when all N_2O would have been produced through NN and FD (S2), the AOI did exceed S2-OI. Hence, these results indicate that, also in soil G3, O exchange may have occurred during nitrifier pathways in addition to O exchange during denitrification. Here it remains uncertain whether the measured AOI is partially the result of nitrifier-mediated O exchange, or solely the result of O incorporation through reaction stoichiometry plus O exchange through denitrifiers. Unfortunately, the evaluation of the results of this soil (G3) was also complicated by the relatively large variation in the measured AOI.

Our assumption on the extent of O exchange may also complicate the evaluation. We assume that wherever O exchange might occur, it takes place at the same rate as quantified for denitrification of NO_3^- to N_2O. Fortunately, the results of our sensitivity analyses showed that a variation of 10% in the X_{ERR} would not result in large changes in the MOIs. However, it is likely that the extent of exchange in separate reaction steps is smaller than X_{ERR}, as the total exchange during NO_3^- reduction to N_2O can be a sum of exchange in separate reaction steps. The ND pathway does not include the NO_3^- to NO_2^- reduction step (Figure 4.1, ii) and NO_2^- oxidation comprises only the conversion of NO_2^- to NO_3^- (Figure 4.1 iii). A lower level of exchange in separate reaction processes would result in lower MOIs. Oxygen exchange may then be required in both the ND pathway (Figure 4.1, ii) and the nitrite oxidation step (Figure 4.1 iii) to explain the observed AOI. In conclusion, if our X_{ERR} overestimates the extent of exchange per process, O exchange would in fact be more substantial for the nitrifier pathways.

Consideration of other N_2O production pathways

In our analyses we considered nitrification, denitrification, and nitrifier denitrification as major N_2O production processes. However, N_2O may also evolve from dissimilatory nitrate reduction to ammonia (DNRA) (Stevens et al., 1998), aerobic denitrification (Lloyd et al., 1987; Bell et al., 1991; Patureau et al., 2000; Bateman et al., 2005), fungal denitrification (Bollag et al., 1972; Shoun et al., 1992) and co-denitrification (Garber et al., 1982; Tanimoto et al., 1992b; Laughlin et al., 2002; Morozkina et al., 2007).

Regarding DNRA, the intermediate levels of anaerobicity made its occurrence in our experiment unlikely, and insignificant ^{15}N enrichment of the NH_4^+ after application of enriched NO_3^- showed that N_2O production through this pathway was indeed negligible in all soils. Consideration of fungal and aerobic denitrification would not change the outcome of our evaluation based on isotope tracing: their contribution is included in the fertilizer and nitrification-coupled denitrification (FD and NCD), regardless of whether the process is carried out by fungi or bacteria, or under aerobic or anaerobic conditions. However, in co-denitrification part of the denitrification-derived N_2O may have been obtained from another N source than fertilizer- or nitrification-derived NO_3^-. This would dilute the anticipated ^{15}N enrichment in TR3 and thus cause an underestimation of the extent of O exchange as quantified by the X_{ERR} (Kool et al., 2009a). If part of the N_2O was produced by co-denitrification, it would also alter the allocation of N_2O production across the different pathways, i.e. the contribution of the nitrifier pathways would be overestimated. This could complicate the identification of the presence of O exchange during production processes by nitrifiers. The relative low ^{15}N-N_2O enrichment compared to that of the applied ^{15}N-NO_3^- may be indicative of the presence of another N source. However, the same is seen for the ^{18}O enrichment, where the discrepancy is even greater as depicted by the ERR. Moreover, occurrence of co-denitrification would result in an underestimation of the O exchange as quantified by our ERR approach, while the O exchange quantified here as such is high already. We therefore consider it unlikely that co-denitrification comprised a significant contribution to N_2O production from the soils in our incubation.

Conclusion

We showed that O exchange between H_2O and intermediates of N_2O production can affect the origin of O in N_2O from both nitrifier and denitrifier pathways. For denitrification, O exchange was confirmed to be present for all soils. For nitrifier pathways, results from two of the six soils that showed a minimum nitrifier contribution to N_2O production of 10% proved that O exchange can occur during nitrifier pathways. For three out of these six, based on our sub-evaluation, we suggest that it is very likely that nitrifier production pathways were accompanied by O exchange as well. Moreover, also for the last of the six soils showing a minimum nitrifier contribution to N_2O production of 10%, our evaluation showed that O exchange may not have been limited to denitrification.

Although it may be a less widespread feature across different soils than demonstrated for denitrification, we conclude that O exchange can indeed occur during nitrifier pathways. We therefore advocate that the O isotopic signature of N_2O should be interpreted with caution when used to derive information on the origin of N_2O. Oxygen isotopic analyses of N_2O can still be a useful tool to derive information on the origin of N_2O, but based on our results we conclude that the previously proposed approach by Wrage et al. (2005) does not suffice to distinguish completely between the targeted production pathways. For both nitrifier and denitrifier pathways, future studies are needed to signify the importance of O exchange between H_2O and intermediates of N_2O production in soil. This will ultimately lead to a an improved process-based understanding of different pathways of N_2O production in terrestrial ecosystems.

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Appendices

Appendix 4-1: Partial H_2O-O incorporation of the N_2O production pathways

The calculated partial H_2O-O incorporation of the pathways $OI(N_2O_p)$s under the different scenarios A-F are listed in Table 4.5. Below we provide a detailed description of their derivation.

4-1.1: Denitrifier N_2O production (FD & NCD)

For both denitrifier pathways (FD and NCD) and under all scenarios, the N_2O produced has been subject to exchange of O between H_2O and intermediate compounds of NO_3^- reduction to N_2O, as expressed by the X_{ERR}. The fraction of the O in N_2O originating from H_2O will further depend on the O incorporated from H_2O into NO_3^- ($OI(NO3_p)$), which does differ for the pathways and scenarios. For N_2O produced through FD and NCD holds:

$$OI(N_2O_{FD}) = OI(NO3_{FD}) + X_{ERR} \cdot (1\text{-}OI(NO3_{FD})) \qquad [A,B,C,D,E,F]$$

$$OI(N_2O_{NCD}) = OI(NO3_{NCD}) + X_{ERR} \cdot (1\text{-}OI(NO3_{NCD})) \qquad [A,B,C,D,E,F]$$

In case of FD, applied NO_3^- will have no O incorporated from the enriched H_2O through reaction stoichiometry during incubation, i.e. the $OI(NO3_{FD})$ is zero. As a result:

$$OI(N_2O_{FD}) = X_{ERR} \qquad [A,B,C,D,E,F]$$

For NCD, the nitrification-derived NO_3^- will have obtained 2/3rd of the O from H_2O according to reaction stoichiometry (Figure 4.1). When O exchange occurs during nitrification of NO_2^- to NO_3^- (scenario C, D, E, F), additional O will be incorporated from H_2O into the nitrification-derived NO_3^-:

$$OI(NO3_{NCD}) = 2/3 \qquad [A,B]$$

$$OI(NO3_{NCD}) = 2/3 + X_{ERR} \cdot 1/3 \qquad [C,D,E,F]$$

In the N_2O produced from NO_3^- through NCD, the $OI(N_2O_{NCD})$ thus amounts to:

$OI(N_2O_{NCD}) = 2/3 + X_{ERR} \cdot 1/3$ [A,B]

$OI(N_2O_{NCD}) = 2/3 + X_{ERR} \cdot 1/3 + X_{ERR} \cdot (1-(2/3 + X_{ERR} \cdot 1/3))$ [C,D,E,F]

A4-1.2: Nitrifier N_2O production (ND & NN)

No oxygen will be incorporated from H_2O in the N_2O produced directly through nitrifiers as a by-product of nitrification, N_2O_{NN}, through reaction stoichiometry or through O exchange, in any of the scenarios:

$OI(N_2O_{NN}) = 0$ [A,B,C,D,E,F]

The $OI(N_2O_p)$ for production by nitrifiers through ND ($OI(N_2O_{ND})$) differs for the O exchange scenarios. When no O exchange takes place during NO_2^- reduction (A,C,D), the $OI(N_2O_{ND})$ will remain the same as the fraction of the O originating from H_2O in the preceding NO_2^- ($OI(NO2)$). Under scenario B, E, and F, the O exchange during NO_2^- reduction to N_2O adds to the $OI(N_2O_{ND})$:

$OI(N_2O_{ND}) = OI(NO2)$ [A,C,D]

$OI(N_2O_{ND}) = OI(NO2) + X_{ERR} \cdot (1-OI(NO2))$ [B,E,F]

The $OI(NO2)$ also differs for the various O exchange scenarios. The NO_2^- will have obtained 50% of its O from H_2O, following reaction stoichiometry of its production (Figure 4.1). When O exchange is assumed to affect the NO_2^- (D, F; Figure 4.1 iiib), the $OI(NO2)$ will be identical to $OI(NO3_{NCD})$:

$OI(NO2) = 1/2$ [A,B,C,E]

$OI(NO2) = 2/3 + X_{ERR} \cdot 1/3$ [D,F]

The OI for N_2O produced directly through ND ($OI(N_2O_{ND})$) thus becomes:

$OI(N_2O_{ND}) = 1/2$ [A,C]

$OI(N_2O_{ND}) = 1/2 + X_{ERR} \cdot 1/2$ [B,E]

$OI(N_2O_{ND}) = 2/3 + X_{ERR} \cdot 1/3$ [D]

$OI(N_2O_{ND}) = 2/3 + X_{ERR} \cdot 1/3 + X_{ERR} \cdot (1-(2/3 + X_{ERR} \cdot 1/3))$ [F]

In summary, the $OI(N_2O_p)$s for all pathways are calculated as follows:

$OI(N_2O_{FD}) = X_{ERR}$ [A,B,C,D,E,F]

$OI(N_2O_{NCD}) = 2/3 + X_{ERR} \cdot 1/3$ [A,B]
$OI(N_2O_{NCD}) = 2/3 + X_{ERR} \cdot 1/3 + X_{ERR} \cdot (1-(2/3 + X_{ERR} \cdot 1/3))$ [C,D,E,F]

$OI(N_2O_{ND}) = 1/2$ [A,C]
$OI(N_2O_{ND}) = 1/2 + X_{ERR} \cdot 1/2$ [B,E]
$OI(N_2O_{ND}) = 2/3 + X_{ERR} \cdot 1/3$ [D]
$OI(N_2O_{ND}) = 2/3 + X_{ERR} \cdot 1/3 + X_{ERR} \cdot (1-(2/3 + X_{ERR} \cdot 1/3))$ [F]

$OI(N_2O_{NN}) = 0$ [A,B,C,D,E,F]

Appendix 4-2: Relative contribution of the N_2O production pathways

The different pathways facilitate different amounts of H_2O-O incorporation through reaction stoichiometry. To calculate the theoretical maximum oxygen incorporation (MOI), the contribution of the pathways that facilitate highest H_2O-O incorporation through reaction stoichiometry is maximized. In line with *eq 4.6*:

$MOI = \sum N_2O_p \cdot OI(N_2O_p)$,

while maximizing the N_2O_ps associated with the largest $OI(N_2O_p)$ (*eq A4.1*)

The N_2O_ps are constrained by the variables calculated from the incubation results. This way, the stoichiometric O incorporation is maximized, which ensures we never overestimate the presence of O exchange.

Briefly, the relative contribution of fertilizer denitrification (N_2O_{FD}) follows directly from the treatments where ^{15}N enriched mineral N was applied. To maximize the stoichiometric O incorporation from H_2O (Figure 4.1), subsequently the N_2O_{NCD} and N_2O_{ND} are maximized. The N_2O_ps are presented in Table 4.5 for all soils.

A4-2.1: Denitrifier N_2O production (FD & NCD)

$N_2O_{(NO3)}$ represents the contribution of fertilizer denitrification (FD) to total N_2O production, N_2O_{FD} (%). The $N_2O_{(NH4)}$ comprises the relative contribution to total

N_2O production (%) of the nitrifier nitrification (NN; N_2O_{NN}), nitrifier denitrification (ND; N_2O_{ND}), plus nitrification-coupled denitrification (NCD; N_2O_{NCD}) pathways:

$$N_2O_{(NO3)} = N_2O_{FD} \quad (eq\ A4.2)$$

$$N_2O_{(NH4)} = N_2O_{NN} + N_2O_{ND} + N_2O_{NCD} \quad (eq\ A4.3)$$

For further distinction between the relative contribution of the NN, ND and NCD pathways, we evaluate the ^{15}N enrichment of the N_2O and NO_3^- resulting from application of ^{15}N enriched NH_4^+ (TR4). The relative contribution of these pathways to the total $N_2O_{(NH4)}$ cannot be exactly calculated, so to maximize stoichiometric H_2O-O incorporation we first derive the maximal possible contribution of NCD. As long as the ^{15}N enrichment in the total N_2O does not exceed the ^{15}N enrichment of the NO_3^- during the incubation (with TR4, application of ^{15}N enriched NH_4^+), the $N_2O_{(NH4)}$ may have exclusively originated from NCD. So all $N_2O_{(NH4)}$ is then ascribed to N_2O_{NCD}, and N_2O_{NN} and N_2O_{ND} are assumed to be zero:

If $^{15}N(N_2O_{(TR4)}) \leq {}^{15}N(NO_3^{-}{}_{(TR4)})$:

$N_2O_{FD} = N_2O_{(NO3)}$

$N_2O_{NCD} = N_2O_{(NH4)}$

$N_2O_{ND} = 0$

$N_2O_{NN} = 0$

A4-2.2: Nitrifier N_2O production (ND & NN)

When the ^{15}N-N_2O exceeded the ^{15}N enrichment in the NO_3^- in TR4 , i.e. when $^{15}N(N_2O_{(TR4)}) > {}^{15}N(NO_3^{-}{}_{(TR4)})$, not all NH_4^+-N that ended up in N_2O had gone through the nitrification-coupled denitrification. In other words, a minimal contribution to N_2O production by nitrifiers through NN or ND (N_2O_{NN} plus N_2O_{ND}; N_2O_{NN+ND}) must be adopted. The ^{15}N-N_2O and ^{15}N-NO_3^- enrichment in TR4 is then used to provide information on the ratio of N_2O production by NCD versus NN plus ND. This ratio of N_2O_{NCD} versus N_2O_{NN+ND} reflects the ratio of ^{15}N enrichments of these pools, $^{15}N(N_2O_{NCD})$ and $^{15}N(N_2O_{NN+ND})$. These in turn will

reflect the N isotopic signature of their N_2O preceding compounds NO_3^- and NH_4^+, $^{15}N(NO_3^-{}_{(TR3)})$ and $^{15}N(NH_4^+{}_{(TR4)})$, respectively:

$$\frac{N_2O_{NCD}}{N_2O_{NN+ND}} = \frac{^{15}N\left(N_2O_{NCD}\right)}{^{15}N\left(N_2O_{NN+ND}\right)} = \frac{^{15}N\left(NO_{3\,(TR4)}^-\right)}{^{15}N\left(NH_{4\,(TR4)}^+\right)} \qquad (eq\ A4.4)$$

Combined with *eq A4.3* it follows;

$$N_2O_{(NH4)} = N_2O_{NCD} + N_2O_{NCD} \cdot \left(\frac{^{15}N\left(NH_{4\,(TR4)}^+\right)}{^{15}N\left(NO_{3\,(TR4)}^-\right)} \right) \text{, i.e.}$$

$$N_2O_{(NH4)} = N_2O_{NCD} \cdot \left(1 + \frac{^{15}N\left(NH_{4\,(TR4)}^+\right)}{^{15}N\left(NO_{3\,(TR4)}^-\right)} \right) \text{, i.e.}$$

$$N_2O_{(NH4)} = N_2O_{NCD} \cdot \left(\frac{^{15}N\left(NO_{3\,(TR4)}^-\right) + {}^{15}N\left(NH_{4\,(TR4)}^+\right)}{^{15}N\left(NO_{3\,(TR4)}^-\right)} \right) \text{, i.e.}$$

$$N_2O_{NCD} = N_2O_{(NH4)} \cdot \left(\frac{^{15}N\left(NO_{3\,(TR4)}^-\right)}{^{15}N\left(NO_{3\,(TR4)}^-\right) + {}^{15}N\left(NH_{4\,(TR4)}^+\right)} \right) \qquad (eq\ A4.5)$$

To maximize stoichiometric OI, the remaining portion of $N_2O_{(NH4)}$, which constitutes N_2O_{NN} plus N_2O_{ND}, is all ascribed to ND. The pathway of NN is assumed not to contribute to any O incorporation from H_2O into N_2O, and therefore set to zero. In summary, the N_2O assigned to the different pathways amounts to:

If $^{15}N(N_2O_{(TR4)}) > {}^{15}N(NO_3^-{}_{(TR4)})$:

$N_2O_{FD} = N_2O_{(NO3)}$

$N_2O_{NCD} = N_2O_{(NH4)} \cdot {}^{15}N(NO_3^-TR4) / ({}^{15}N(NO_3^-TR4) + {}^{15}N(NH_4^+{}_{(TR4)}))$ (=*eq A4.5*)

$N_2O_{ND} = N_2O_{(NH4)} - N_2O_{NCD}$

$N_2O_{NN} = 0$

A4-2.3: Sub-evaluation: maximizing direct nitrifier N_2O production (NN & ND)

Our evaluation of the MOI requires the maximization of the contribution of NCD. In reality its contribution may however be smaller. We therefore derive additional sets of OIs for two cases, S1 and S2, where the NH_4^+ derived N_2O is assigned to NCD, ND and NN differently. All $N_2O_{(NH4)}$ is ascribed to either ND (S1) or to NN (S2), i.e. the N_2O_{NCD} is zero:

S1-OI: $N_2O_{FD} = N_2O_{(NO3)}$

$N_2O_{NCD} = 0$

$N_2O_{ND} = N_2O_{(NH4)}$

$N_2O_{NN} = 0$

S2-OI: $N_2O_{FD} = N_2O_{(NO3)}$

$N_2O_{NCD} = 0$

$N_2O_{ND} = 0$

$N_2O_{NN} = N_2O_{(NH4)}$

Note that as the N_2O_{NCD} is zero, the OI resulting from these sub-evaluations under C will equal A, and E will equal B. Under S2 the N_2O_{ND} is zero as well, and as the $OI(N_2O_{NN})$ is zero under all scenarios, the total S2-OI will only depend on the FD contribution and its partial OI, and thus be the same for scenario A to F.

Chapter 5

Nitrifier denitrification can be a source of N_2O from soil: a revised approach to the dual isotope labeling method

Abstract Nitrifier denitrification, i.e. nitrite reduction by ammonia oxidizers, is one of the biochemical pathways of nitrous oxide (N_2O) production. It is increasingly suggested that this pathway may contribute substantially to N_2O production in soil, the major source of this greenhouse gas. However, although monoculture studies recognize its potential, methodological drawbacks prohibit conclusive proof that nitrifier denitrification occurs in actual soils. Here we suggest and apply a new isotopic approach to identify its presence in soil. In incubation experiments with twelve soils, N_2O production was studied using oxygen (O) and nitrogen (N) isotope tracing, accounting for O exchange. Microbial biomass C and N and phospholipid fatty acid (PLFA) patterns were analyzed to explain potential differences in N_2O production pathways. We found that in at least five of the soils, nitrifier denitrification must have contributed to N_2O production. Moreover, it may even have been responsible for all NH_4^+ derived N_2O in most soils. In contrast, N_2O as a by-product of ammonia oxidation contributed very little to total production. Microbial biomass C and N and PLFA-distinguished microbial community composition were not indicative of differences in N_2O production pathways. Overall, we show that combined O and N isotope tracing may still provide a powerful tool to understand N_2O production pathways, provided that O exchange is accounted for. We conclude that nitrifier denitrification can indeed occur in soils, and may in fact be responsible for the majority of total nitrifier-induced N_2O production.

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Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas and contributes to the breakdown of stratospheric ozone (Crutzen, 1981). Globally, soils constitute the major source of N_2O to the atmosphere (IPCC, 2007). Rising atmospheric N_2O concentrations over the last decades have led to increased interest in understanding the production pathways of N_2O, in order to enable development of adequate mitigation strategies.

Traditionally, autotrophic nitrification and heterotrophic denitrification have been considered to be the major N_2O forming processes. However, it has long been acknowledged that these are not the sole production pathways of N_2O. Nitrifier denitrification (denitrification by autotrophic nitrifiers) (Hooper, 1968; Ritchie et al., 1972), heterotrophic nitrification (Verstraete et al., 1973; Papen et al., 1989; Laughlin et al., 2008) and co-denitrification (Shoun et al., 1991; Tanimoto et al., 1992b; Laughlin et al., 2002) by both fungi and bacteria, as well as dissimilatory nitrate reduction to ammonia (DNRA) (Caskey et al., 1979; Smith et al., 1981; Bleakley et al., 1982) may all produce N_2O as (by-) product. For most of these processes the relative significance for N_2O production was long thought to be minor in soils compared to nitrification (NN) and denitrification (from fertilizer, FD, or coupled with nitrification, NCD) (Figure 5.1). However, for nitrifier denitrification (ND) it is increasingly suggested that it may constitute a considerable contribution to N_2O production in soil (Webster et al., 1996; Wrage et al., 2004a; Ma et al., 2007; Sánchez-Martín et al., 2008).

In pure cultures the existence of nitrifier denitrification has long been established (Hooper, 1968; Ritchie et al., 1972). Several ammonia-oxidizing bacteria (AOB) have since then been identified to be able to denitrify nitrite (NO_2^-) to N_2O (Poth et al., 1985; Remde et al., 1990; Zart et al., 1998; Colliver et al., 2000; Schmidt et al., 2004b; Shaw et al., 2006). *Nitrosomonas europaea* has been studied most extensively, but is less representative of the microbial community commonly found in soils (Kowalchuk et al., 2001; Wrage et al., 2001; Arp et al., 2003; Shaw et al., 2006). The AOB most commonly found in soils are *Nitrosospira* spp. (Stephen et al., 1996; Stephen et al., 1998; Kowalchuk et al., 2001; Smith et al., 2001). Wrage et al. (2004b) first suggested that N_2O production by a representative of this genus (*Nitrosospira briensis)* occurred partly through ND.

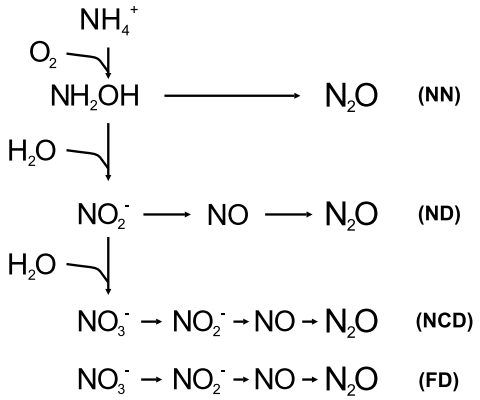

Figure 5.1. Major pathways of N_2O formation as considered in this study; nitrification (NN), nitrifier denitrification (ND), nitrification-coupled denitrification (NCD), and fertilizer (applied NO_3^-) denitrification (FD).

Shaw *et al.* (2006) found that all their seven *Nitrosospira* spp. strains tested could indeed produce N_2O through ND in pure cultures, and suggested that the ability to denitrify may be a common trait among AOB. Moreover, the recently presented complete genome sequence of *Nitrosospira multiformis* revealed that, similar to *Nitrosomonas europaea*, it contains orthologs to copper-containing nitrite-reductase (*nirK*) and nitric oxide reductase (*norCBQD*), and no coding sequence with similarity to (known) nitrate or nitrous oxide reductases (Norton et al., 2008). However, Dundee and Hopkins (Dundee et al., 2001) suggested that observed differences in O_2 sensitivities with respect to N_2O production between *Nitrosomonas europaea* and *Nitrosolobus multiformis* (currently classified as *Nitrosospira* spp. (Head et al., 1993)) implied differences in their ability to produce N_2O through nitrifier denitrification (Dundee et al., 2001). Therefore, even if the ability for ND is a common trait among nitrifiers in pure cultures, the actual occurrence in soil and level of significance remains unclear.

Although soil-based studies increasingly propose that ND may be contributing to N_2O emission from soils (Webster et al., 1996; Hütsch et al., 1999; Wrage et al., 2004a; McLain et al., 2005; Ma et al., 2007; Venterea, 2007; Sánchez-Martín et al., 2008), conclusive proof of its presence in soil remains elusive due to the lack of reliable analytical methodology. Earlier approaches were shown to entail various important drawbacks. The use of oxygen suppression and

acetylene inhibition (Yoshinari et al., 1977; Robertson et al., 1987; Klemedtsson et al., 1988; Webster et al., 1996) was shown to be unreliable (Tilsner et al., 2003; Beaumont et al., 2004b; Beaumont et al., 2004a; Wrage et al., 2004a; Wrage et al., 2004b; Shaw et al., 2006). ^{15}N isotopic labeling techniques have been employed to differentiate and quantify N_2O production from denitrification and nitrification in soil (Stevens et al., 1997; Baggs et al., 2003; Tilsner et al., 2003; Bateman et al., 2005) but it does not distinguish the N_2O that results from nitrite reduction (i.e. nitrifier denitrification; ND) from the N_2O generated as by-product from ammonia oxidation (i.e. nitrifier nitrification; NN) (Wrage et al., 2005). To enable this further distinction a dual-isotope approach was proposed (Wrage et al., 2005) that combined ^{15}N labeling with the use of ^{18}O labeled water. However, it was recognized that potential O exchange between H_2O and intermediate compounds of N_2O production complicates data interpretation for this method (Wrage et al., 2005; Kool et al., 2007; Kool et al., 2009b; Kool et al., 2009a). Introducing an additional treatment with ^{18}O labeled NO_3^-, Kool et al. (2009a) were able to quantify O exchange during denitrification. We here propose that these insights allow to partially account for O exchange and with a revised approach will allow to confirm whether ND occurs in soils, and to quantify margins of its relative contribution.

The aim of this study was thus to evaluate the N and O isotopic data from soil incubations with the revised dual isotope approach to assess whether N_2O production through ND may be conclusively proven. Further, we aim to investigate whether these results may reflect differences in soil characteristics and/or in the soil microbial community.

Methods

Soil sampling

Soil samples were collected from 12 sites across Europe, encompassing forest (F), grassland (G), and arable (A) fields (Table 5.1). For the soil incubation (isotope tracing) experiment, samples (0-10 cm) were dried at 40°C, sieved (2mm) and stored at 4°C until further use. Analyses for microbial biomass and community composition analyses were carried out on fresh soil samples (0-5cm). All soils were sampled simultaneously for the isotope tracing experiment and the

Table 5.1. Soil properties, microbial biomass N and C, and microbial biomass PLFA of all soils (except G3). The soils originated from forest (F), grassland (G), and arable (A) sites across Europe.

Soil	pH (H_2O)	Corg mgC g^{-1}dm	C/N ratio	Microbial Biomass N and C µgN g^{-1}dm	µgC g^{-1}dm	Total PLFA Biomass[b] nmol g^{-1}dm	Bacterial Biomass[b] nmol g^{-1}dm	Fungal Biomass[b] nmol g^{-1}dm	Actinomycete Biomass[b] nmol g^{-1}dm	VAM Biomass[b] nmol g^{-1}dm
F1	3.3[a]	101.7	23.3	20.7	210.9	136.2 (6.4)	123.8 (6.0)	8.1 (0.6)	3.3 (0.1)	4.3 (0.4)
F2	3.6	65.3	31.7	27.3	316.9	151.3 (15.1)	125.9 (13.2)	16.8 (2.6)	5.3 (0.4)	8.6 (0.9)
F3	4.2	72.3	17	21.7	283.6	110.0 (11.6)	102.8 (10.6)	4.6 (0.7)	4.1 (0.5)	2.6 (0.5)
F4	3.8	156.0	25	36.0	203.1	118.1 (15.0)	109.0 (14.3)	5.0 (1.1)	2.7 (0.4)	4.1 (0.4)
G1	7.8	86.0	10.3	218.3	672.2	171.2 (33.5)	147.4 (29.6)	13.7 (2.2)	9.2 (1.6)	10.2 (1.8)
G2	6.0	42.0	10.3	73.4	330.3	127.5 (7.4)	112.3 (6.2)	6.4 (0.4)	9.8 (0.4)	8.8 (1.5)
G3	6.2									
G4	5.9	52.3	13	141.9	823.4	310.4 (36.2)	281.1 (34.5)	8.6 (0.6)	18.3 (3.0)	20.7 (1.6)
A1	7.1	25.3	11	21.9	102.4	20.4 (2.0)	17.5 (1.7)	2.2 (0.3)	2.5 (0.2)	0.6 (0.1)
A2	7.2	19.7	11	70.1	193.0	45.7 (1.5)	39.6 (1.0)	3.2 (0.6)	2.5 (0.1)	2.9 (0.1)
A3	7.5	12.0	7.3	29.4	131.7	44.2 (2.9)	38.5 (2.7)	3.1 (0.8)	1.8 (0.2)	2.6 (0.2)
A4	7.1	30.3	10	14.4	127.6	64.6 (2.9)	59.4 (2.9)	2.3 (0.3)	2.1 (0.1)	2.9 (0.1)

[a] pH measured in $CaCl_2$

[b] values in brackets denote the standard error of the mean

microbial analysis, except for soil G4. Due to logistic complications, soil G3 was excluded from the soil microbial analyses.

Soil incubation experiment

Details of the incubation experiment were described by Kool et al. (2009a). In brief, for each treatment five replicate samples (75 g soil) were pre-incubated at 16°C and 40% water holding capacity (WHC) a week prior to the incubation. At the start of the incubation, all samples received equal amounts of mineral N (50 mg NH_4^+-N kg^{-1} and 50 mg NO_3^--N kg^{-1} soil). They were incubated at 80% WHC by adding appropriate amounts of H_2O. The samples were treated with one of four combinations of ^{18}O and ^{15}N labeled compounds; ^{18}O enriched H_2O at 1.0 atom% excess (TR1), ^{18}O enriched NO_3^- at 1.0 atom% excess (TR2), ^{15}N enriched NO_3^- at 40.0 atom% excess (TR3), and ^{15}N enriched NH_4^+ at 40.0 atom% excess (TR4). The experiment was set up as a completely randomized design. The sample jars were closed by lids equipped with rubber septa. At the end of the 28 h incubation period, gas and soil samples were taken. Gas samples were extracted from the headspace of the jars and transferred to 12ml exetainer vials. The N_2O concentration and its isotopic signature were measured at the UC Davis Stable Isotope Facility, using a Sercon Cryoprep trace gas concentration system interfaced to a Sercon 20/20 isotope ratio mass spectrometer (Sercon Ltd., Crewe, Cheshire, UK).

Soil samples were taken after gas sampling. The exact soil moisture content was determined from one set of sub-samples, other sub-samples of approximately 20 g moist soil were taken for analyses of mineral N (NH_4^+-N and NO_3^--N) after extraction with 1M KCl (50 ml per 20g soil) followed by segmented flow analysis (Skalar Analytical, Breda, The Netherlands) (Kool et al., 2006). The ^{15}N enrichments of the mineral N were derived using a microdiffusion method as described in Kool et al. (2009b).

Soil microbial analyses

Four replicate samples from each site were analyzed for several microbial parameters. Microbial biomass N was determined as ninhydrin-reactive N by chloroform fumigation-extraction and calculated as ninhydrin-reactive N times

3.1 (Hackl et al., 2000). Microbial biomass C was determined by chloroform fumigation followed by DOC analysis of extracts by dry combustion (Schinner et al., 1995). Phospholipid fatty acid (PLFA) analyses were carried out as described by Hackl et al. (2005) to profile the microbial community composition. The total amount of PLFAs was taken to represent total microbial biomass. Specific groups of PLFAs were considered as indicators of bacterial (i14:0, a15:0, i15:0, i16:0, i17:0, a17;0, 10Me16:0, 10Me17:0, cy17:0, cy18:0, cy19:0, 16:1(9), 18:1(13), 18:1(11)), fungal (18:2(9,12)), actinomycete (10Me18:0) and vesicular arbuscular mycorrhizal (VAM) (16:1(11)) biomass (nmol g^{-1} soil (dry matter)).

Data analysis: N_2O production

Total N_2O production, as well as isotopic enrichment of the N_2O and soil mineral N pools from our soil incubation experiment have been reported in Kool et al. (2009b). From the ^{15}N-tracing data we obtained the proportions of NH_4^+ and NO_3^- derived N_2O ($N_2O_{(NH4)}$ and $N_2O_{(NO3)}$, respectively), and the relative pathway contribution of fertilizer denitrification (FD) and the (theoretical) maximum fraction of nitrification-coupled denitrification (NCD) (N_2O_{FD} and $N_2O_{NCD\text{-}max}$, respectively). In appendix 5-1 (equations *eq A5.1* through *eq A5.7*), we added a short description of the derivation of these parameters.

From the measured N_2O production and the $N_2O_{(NH4)}$ and $N_2O_{(NO3)}$ proportions (%), we calculated the absolute NH_4^+ and NO_3^- derived N_2O production over the incubation period as well:

$$[N_2O_{(NH4)}] = [N_2O] \cdot \frac{N_2O_{(NH4)}}{100} \quad \text{, and} \qquad (eq\ 5.1)$$

$$[N_2O_{(NO3)}] = [N_2O] \cdot \frac{N_2O_{(NO3)}}{100} \qquad (eq\ 5.2)$$

where $[N_2O]$, $[N_2O_{(NH4)}]$ and $[N_2O_{(NO3)}]$ represent the absolute N_2O production in total and that derived from NH_4^+-N and NO_3^--N, respectively, in µg N_2O-N kg^{-1} soil. In addition we calculated the production of N_2O-N as parts per mil (‰) of the amount of applied N, i.e. emission rate (total N, NH_4^+-N, or NO_3^--N).

Data analysis: evaluation of the nitrifier contribution to N_2O

Wrage et al. (2005) suggested that the targeted N_2O production pathways could be distinguished by analyzing both ^{18}O incorporation from ^{18}O enriched H_2O as well as ^{15}N incorporation from ^{15}N enriched NH_4^+ and NO_3^- into N_2O. However, the occurrence of O exchange complicates data interpretation in this original approach (Kool et al., 2007; Kool et al., 2009b; Kool et al., 2009a). Our additional treatment with ^{18}O labeled NO_3^- made it possible to partly quantify O exchange. Here we propose a revised approach to the dual isotope method of Wrage et al. (2005) including the use of ^{18}O labeled NO_3^- to evaluate the presence and potential significance of the nitrifier denitrification (ND) pathway to total N_2O production.

To explore the potential presence and significance of nitrifier denitrification in our incubation experiment, we used the calculated actual O incorporation from H_2O into N_2O (AOI) and the O exchange during denitrification of NO_3^- to N_2O (X_{ERR}) derived from ^{18}O and ^{15}N labeling in addition to the above mentioned data derived from ^{15}N tracing ($N_2O_{(NH4)}$, $N_2O_{(NO3)}$, N_2O_{FD} and $N_2O_{NCD\text{-}max}$). These calculations were described before in detail (Kool et al., 2009b; Kool et al., 2009a). A summary of the AOI and X_{ERR} derivation is provided in appendix 5-1 (*eq A5.8* through *eq A5.10*) as well.

As we aimed to discern the potential extent of the nitrifier denitrification pathway, we confined our further data analysis to those soils where the combined nitrifier pathways (NN, ND, NCD) (total $N_2O_{(NH4)}$) accounted for at least 10% of total N_2O production, or where absolute amounts of NH_4^+ derived N_2O revealed a relevant nitrifier contribution (N_2O-N>0.01% of applied NH_4^+-N).

Our evaluation is based on the O incorporation from H_2O into N_2O. We describe this approach and the calculations in detail in appendix 5-2. By calculating theoretical amounts of O incorporation (TOI) and comparing these with the measured actual O incorporation (AOI), we determined what the minimum and maximum contributions of the pathways could have been in order to agree with the observed AOI (appendix 5-2). When the relative contribution to N_2O production (N_2O_p) and the O incorporation from H_2O into N_2O during production ($OI(N_2O_p)$) was known for each pathway p, we could calculate the ^{18}O incorporation from ^{18}O-H_2O into N_2O that should be measured (Kool et al., 2009b):

$$OI(N_2O) = \sum N_2O_p \cdot OI(N_2O_p)$$
$$= N_2O_{FD} \cdot OI(N_2O_{FD}) + N_2O_{NCD} \cdot OI(N_2O_{NCD})$$
$$+ N_2O_{ND} \cdot OI(N_2O_{ND}) + N_2O_{NN} \cdot OI(N_2O_{NN}) \quad (eq\ 5.3)$$

Following this notion, we determined what TOI would be expected under certain assumptions on the contributions of the pathways to N_2O and the occurrence of O exchange (appendix 5-2, *eq A5.11*, *eq A5.13*, *eq A5.16* for TOI_1, TOI_2 and TOI_3). We then determined whether these assumptions on the pathway contributions could hold, or whether they were violated and should be rejected based on the measured AOI. First we assessed whether the $N_2O_{(NH4)}$ must have been at least partially derived through nitrifier denitrification (ND), i.e. whether N_2O_{ND}>0, by evaluating a TOI under the assumption that N_2O_{ND} is zero (TOI_1, *eq A5.11* and *eqA5.12*). Next, we assessed whether ND might have accounted for all $N_2O_{(NH4)}$ (TOI_2, *eq A5.13*, *eq A5.14*, *eq A5.15*). Concurrently, we considered the potential contributions of the pathways nitrifier nitrification (NN) and nitrification-coupled denitrification (NCD) to the $N_2O_{(NH4)}$, and to total N_2O production in general. The pathways evaluated to have had a minimum contribution (i.e. N_2O_p> 0) are further quantified (*eq A5.17* through *eq A5.20*).

Statistical analyses: data accuracy

The uncertainty of the average values over the replicates was quantified with the standard error:

$$se(\bar{x}) = \sqrt{\frac{s^2(x)}{n_x}}$$

with $s^2(x)$ the variance of the individual measurements, and n the number of replicates.

Parameters $N_2O_{(NH4)}$, $N_2O_{(NO3)}$, and X_{ERR} are defined as ratios (appendix 5-1):

$$N_2O_{(NH4)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR4)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \quad (eq\ 5.4)$$

$$N_2O_{(NO3)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR3)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \quad (eq\ 5.5)$$

$X_{ERR} = 100 - ERR$, *with*

$$ERR = 100 \cdot \frac{^{18}O\left(N_2O_{(TR2)}\right)}{^{15}N\left(N_2O_{(TR3)}\right)} \cdot \frac{^{15}N\left(NO_3^-{}_{(TR3)}\right)}{^{18}O\left(NO_3^-{}_{(TR2)}\right)} \quad (eq\ 5.6)$$

Both for the numerator and denominator of these ratios we have replicate measurements. The $N_2O_{(NH4)}$, $N_2O_{(NO3)}$, and X_{ERR} were estimated by the ratios of the averages over the replicates. The variance of these estimated ratios are approximated by a first-order Taylor linearization (Kendall et al., 1977). For instance, if we denote $^{15}N(N_2O_{(TR4)})$ by $\bar{x}$ and $^{15}N(N_2O_{(TR3)})$ by $\bar{y}$, and the sum of $\bar{x}$ and $\bar{y}$ by $\bar{z}$, then the variance of $N_2O_{(NH4)}$ can be approximated by:

$$v\left(\frac{\bar{x}}{\bar{z}}\right) = 100^2\left(\frac{\mu(\bar{x})}{\mu(\bar{z})}\right)^2\left(\frac{v(\bar{x})}{\mu(\bar{x})} + \frac{v(\bar{z})}{\mu(\bar{z})} - \frac{2\,cov(\bar{x},\bar{z})}{\mu(\bar{x})\mu(\bar{z})}\right) \quad (eq\ 5.7)$$

Similarly, the variance of $N_2O_{(NO3)}$ can be approximated by substituting $\bar{y}$ for $\bar{x}$ in the above equation. By experimental design, $\bar{x}$ and $\bar{y}$ are independent, however the sample means $\bar{x}$ and $\bar{z}$ are logically dependent and $\bar{y}$ and $\bar{z}$ likewise. The covariance of $\bar{x}$ and $\bar{z}$, or $\bar{y}$ and $\bar{z}$, with $\bar{z} = \bar{x} + \bar{y}$ and $\bar{x}$ and $\bar{y}$ independent, equals $v(\bar{x})$ or $v(\bar{y})$ respectively. For the approximation of the variance of X_{ERR}, we define $^{18}O(N_2O_{(TR2)})$ as $\bar{x}$ and $^{15}N(N_2O_{(TR3)})$ as $\bar{z}$. Here, $\bar{x}$ and $\bar{z}$ are independent so their covariance is zero.

For an estimate of these approximated variances the true variances $v(\bar{x})$ and $v(\bar{z})$, and the squared expectations $\mu^2(\bar{x})$ and $\mu^2(\bar{z})$ and the product $\mu(\bar{x})\mu(\bar{z})$, are replaced by their unbiased estimators:

$$\hat{v}(\bar{x}) = \frac{s^2(x)}{n(x)}, \quad \hat{v}(\bar{z}) = \frac{s^2(z)}{n(z)}, \quad \hat{\mu}^2(\bar{z}) = (\bar{z})^2 - \frac{s^2(z)}{n(z)} \quad \text{and} \quad \hat{\mu}(\bar{x})\hat{\mu}(\bar{z}) = \bar{x} \cdot \bar{z} - cov(\bar{x},\bar{z}).$$

Statistical analyses: linear regression

Relations between the soil parameters (including microbial parameters) and N_2O emissions were evaluated by linear regression analysis in GenStat eleventh edition (VSN international Ltd.). Analyses were carried out for total N_2O production, both the relative and absolute contributions of NH_4^+ and NO_3^- derived N_2O, the potential maximum contribution of the NCD and minimum of

the ND pathway. To avoid pseudo-replication the averages were taken as response values. For the absolute productions averages were log-transformed (natural logs), and the relative contributions were logit transformed. As we have only sparse data, we decided to fit simple linear models only, i.e. models with one predictor. Data from soils F3 and F4 were not included in the regression analyses because they exhibited very marginal total N_2O production which would be likely to bias the results.

Results

Basic soil characteristics (pH, organic C content and C:N ratio), data on microbial biomass N and C, and the microbial community compositions from the PLFA analyses are reported in Table 5.1. In general, the forest (F) soils had low pH, contained highest organic C contents and had higher C:N ratios than the

Table 5.2: Production of N_2O during incubation. Absolute production (µg N_2O-N kg^{-1} soil) and relative production as ‰ of applied N (emission rates) for total and NH_4^+ and NO_3^- derived N_2O, and the relative contribution of NH_4^+-N and NO_3^--N to total N_2O (%).

	Total N_2O production		NO_3^- derived N_2O			NH_4^+ derived N_2O		
Soil	*[N_2O]* [a] µg N kg^{-1}soil	emission rate (‰)	*$N_2O_{(NO3)}$* [b] % of total N_2O	*[$N_2O_{(NO3)}$]* µg N kg^{-1}	emission rate (‰)	*$N_2O_{(NH4)}$* [b] % of total N_2O	*[$N_2O_{(NH4)}$]* µg N kg^{-1}	emission rate (‰)
F1	1.7 (0.3)	0.02	98.02 (0.48)	1.7	0.03	1.98 (0.47)	0.0	0.00
F2	21.6 (2.5)	0.22	99.95 (0.02)	21.6	0.43	0.05 (0.02)	0.0	0.00
F3	0.5 (0.0)	0.00	96.04 (1.24)	0.4	0.01	3.96 (1.23)	0.0	0.00
F4	0.3 (0.0)	0.00	96.15 (1.74)	0.3	0.01	3.85 (1.67)	0.0	0.00
G1	26.2 (9.8)	0.26	10.16 (0.95)	2.7	0.05	89.84 (0.95)	23.5	0.47
G2	1031.0 (74.2)	10.31	97.44 (0.26)	1004.6	20.09	2.56 (0.26)	26.4	0.53
G3	46.2 (13.7)	0.46	56.71 (8.50)	26.2	0.52	43.29 (8.82)	20.0	0.40
G4	924.3 (122.9)	9.24	97.50 (0.97)	901.2	18.02	2.50 (0.98)	23.1	0.46
A1	239.1 (20.6)	2.39	88.43 (0.48)	211.4	4.23	11.57 (0.48)	27.7	0.55
A2	219.5 (27.3)	2.19	76.33 (0.28)	167.5	3.35	23.67 (0.28)	51.9	1.04
A3	95.4 (10.8)	0.95	70.14 (0.60)	66.9	1.34	29.86 (0.60)	28.5	0.57
A4	146.3 (14.3)	1.46	86.46 (0.19)	126.5	2.53	13.54 (0.19)	19.8	0.40

[a] values in brackets denote the standard error of the mean

[b] values in brackets denote the estimated standard error

grassland (G) and agricultural (A) soils. The organic C content was higher in the G soils than in the A soils, but their C:N ratios were comparable. Total microbial biomass N and C were lower in the agricultural (A) soils than in the forest (F) and grassland (G) soils. This was also the case for the PLFA biomass, both for total biomass and for the specified functional groups.

The data on N_2O production are listed in Table 5.2. Absolute production (µg N_2O-N kg^{-1} soil) and relative production as ‰ of applied N (emission rates) are given for total and NH_4^+ and NO_3^- derived N_2O, as well as the relative contribution of NH_4^+-N and NO_3^--N to total N_2O (%). These data were partly presented before (Kool et al., 2009b; Kool et al., 2009a). The estimated standard error of $N_2O_{(NH4)}$ and $N_2O_{(NO3)}$ was small for most soils, except for F3 and F4 which showed very little N_2O production. The contribution of NO_3^- derived N_2O shows a wide range across soils, in both absolute terms and as % of total N_2O. The relative contribution of NH_4^+ derived N_2O as % of total N_2O varies considerably as well. However, in absolute amounts and emission rate (‰ of applied N) the NH_4^+-N contribution is notably similar across the G and A soils.

Table 5.3: Results of the TOI evaluation, including the AOI and X_{ERR} that were used for the TOI calculations (appendix 5-1 and 5-2). The TOI are theoretical amounts of O incorporation from H_2O under specific assumptions on the pathway contributions to N_2O production and O exchange. The X_{ERR} is the quantified O exchange during denitrification, the AOI is the actual O incorporation from ^{18}O-H_2O into N_2O (Kool et al., 2009b).

	X_{ERR}	AOI	TOI_1[c]	TOI_2	TOI_3	Implications $TOI_{1,2,3}$ for N_2O_p			
Soil	%[a]	%[b]				NDmin	NDmax	NNmin	NNmax
G1	32.2 (12.33)	74.6 (1.2)	18.47	48.2	79.3	>0	= $N_2O_{(NH4)}$	=0	>0
G2	90.4 (0.73)	98.2 (0.4)	-	89.4	90.6	=0	= $N_2O_{(NH4)}$	=0	=0
G3	78.7 (5.01)	65.4 (14.0)	78.91	66.3	87.3	=0	< $N_2O_{(NH4)}$	>0	>0
G4	82.2 (0.84)	89.0 (1.0)	-	81.4	82.7	=0	= $N_2O_{(NH4)}$	=0	=0
A1	95.6 (0.29)	95.9 (1.1)	95.81	90.3	96.1	>0	= $N_2O_{(NH4)}$	=0	>0
A2	96.8 (0.17)	102.5 (0.3)	94.64	85.7	97.5	>0	= $N_2O_{(NH4)}$	=0	=0
A3	95.3 (0.22)	104.9 (0.4)	88.32	81.8	96.7	>0	= $N_2O_{(NH4)}$	=0	=0
A4	87.3 (0.45)	95.4 (0.4)	88.37	82.2	88.9	>0	= $N_2O_{(NH4)}$	=0	=0

[a] values in brackets denote the estimated standard error

[b] values in brackets denote the standard error of the mean

[c] only relevant when $N_2O_{NCD\text{-}max} < N_2O_{(NH4)}$

The $[N_2O_{(NH4)}]$ was in the range of 20 to 30 µgN_2O-N kg^{-1} soil for most G and A soils, with A2 as an upper outlier (51.9 µg N_2O-N kg^{-1} soil). In the F soils on the contrary, the NH_4^+-N contribution to N_2O was negligible.

Table 5.3 provides the calculated TOIs and the result of their assessment regarding the potential contributions of the nitrifier pathways to N_2O production. The (previously derived) X_{ERR} and AOI used for this evaluation are included. The further defined margins of the relative contributions of all pathways are listed in Table 5.4 as percentage of total production (Table 5.4a) and as percentage of NH_4^+ derived N_2O (Table 5.4b). Figure 5.2 presents the identified minimum and maximum contribution of nitrifier denitrification (ND). The results identified that nitrifier denitrification had contributed to at least some of the N_2O production in most soils (Table 5.3, Table 5.4, Figure 5.2). In all soils except G3, ND may even have been responsible for up to 100% of the $N_2O_{(NH4)}$, constituting up to 89.8% (G1) of the total N_2O production (Table 5.4, Figure 5.2). $N_2O_{(NH4)}$ could also have been derived through nitrification-coupled denitrification (NCD), but the ^{15}N tracing revealed that this contribution was constrained to less than all $N_2O_{(NH4)}$ for most soils except G2 and G4 ($N_2O_{NCD\text{-}max}$, Table 5.4). For most other soils, part of

Table 5.4: The minimum and maximum potential contributions of the pathways to N_2O production (a) as % of total N_2O, and (b) as % of NH_4^+ derived N_2O. The contribution of fertilizer denitrification (N_2O_{FD}) and the theoretical maximum contribution of nitrification-coupled denitrification ($N_2O_{NCD\text{-}max}$) were derived previously (Kool et al., 2009b); the contributions of nitrifier denitrification ($N_2O_{ND\text{-}min}$ and $N_2O_{ND\text{-}max}$) and nitrifier nitrification ($N_2O_{NN\text{-}min}$ and $N_2O_{NN\text{-}max}$) were derived as described in appendix 5-2.

a	**N_2O_p as % of total N_2O**					
Soil	FD	NCDmax	NDmin	NDmax	NNmin	NNmax
G1	10.2	18.0	66.3	89.8	0.0	5.6
G2	97.44	2.56	0.0	2.56	0.0	0.0
G3	56.7	34.8	0.0	41.6	1.7	8.5
G4	97.50	2.50	0.0	2.50	0.0	0.0
A1	88.4	11.3	0.1	11.6	0.0	0.2
A2	76.3	20.8	2.9	23.7	0.0	0.0
A3	70.1	21.5	8.3	29.9	0.0	0.0
A4	86.5	13.0	0.6	13.5	0.0	0.0

b	**N_2O_p as % of NH_4^+ derived N_2O**				
Soil	NCDmax	NDmin	NDmax	NNmin	NNmax
G1	20.0	73.8	100.0	0.0	6.2
G2	100.0	0.0	100.0	0.0	0.0
G3	80.4	0.0	96.1	3.9	19.6
G4	100.0	0.0	100.0	0.0	0.0
A1	97.6	0.5	100.0	0.0	2.0
A2	87.8	12.2	100.0	0.0	0.0
A3	72.1	27.9	100.0	0.0	0.0
A4	95.8	4.2	100.0	0.0	0.0

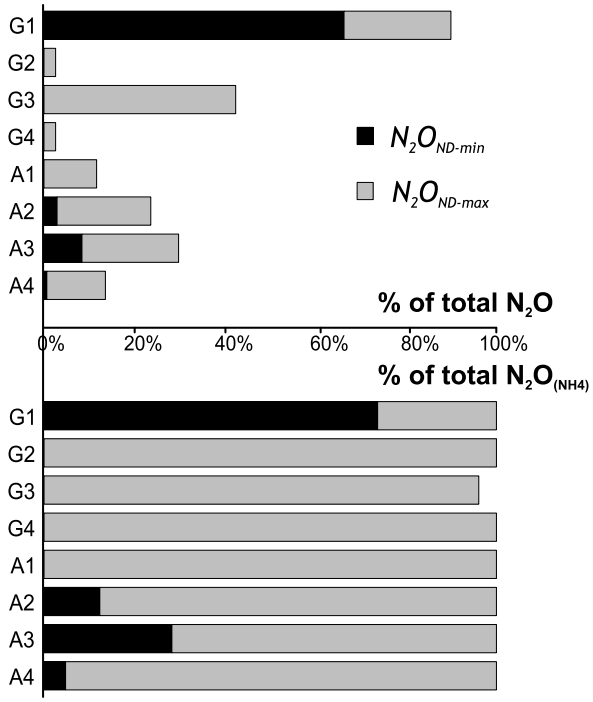

Figure 5.2: Minimum and maximum potential contribution of nitrifier denitrification (ND) to N_2O production, as % of total and NH_4^+ derived N_2O (N_2O_{ND-min} and N_2O_{ND-max}). This was evaluated for all soils where the combined nitrifier pathways, i.e. total $N_2O_{(NH4)}$ (N_2O_{NN} plus N_2O_{ND} plus N_2O_{NCD}), accounted for at least 10% of total N_2O production, or where absolute amounts of NH_4^+ derived N_2O revealed a relevant nitrifier contribution (emission rate >0.1‰ of applied NH_4^+-N).

$N_2O_{(NH4)}$ must have been produced through nitrifier denitrification (ND): the minimum contribution of ND was zero or negligible for G3 and A1, but comprised at least 73.8, 12.2, 27.9, and 4.2% of nitrifier-N_2O ($N_2O_{(NH4)}$) in soils G1, A2, A3 and A4 respectively (Table 5.4b, Figure 5.2).

For five of the eight soils, the nitrifier nitrification (NN) contribution was conclusively shown to be zero (G2, G4, A2, A3, and A4, Table 5.4a). There was no minimal contribution of NN confirmed for any soil except G3 (Table 5.3), and the maximum potential contribution was less than 10% of total N_2O for all soils (Table 5.4a). The highest N_2O_{NN-max} was found for G3, at 8.5% of total N_2O production (Table 5.4a). It must be noted that the data of this single soil (G3),

which was also the only soil where $N_2O_{ND\text{-}max}$ was not equal to $N_2O_{(NH4)}$ and $N_2O_{ND\text{-}min}$ could have been zero, displayed a relatively large variation in the primary data (Table 5.2).

For G2 and G4, the applicability of the TOI approach was limited because the TOI_1 is not relevant in those cases where $N_2O_{NCD\text{-}max}$ could have comprised the total $N_2O_{(NH4)}$ ($N_2O_{NCD\text{-}max}$ is not constrained to less than $N_2O_{(NH4)}$ by the ^{15}N tracing). For these soils therefore both N_2O_{NCD} and N_2O_{ND} could range from zero as minimum up to the total $N_2O_{(NH4)}$ as maximum.

Regression analysis showed that none of the microbial parameters was a significant predictor for any of the N_2O variables, with the single exception of $N_2O_{NCD\text{-}max}$ which seemed to be negatively related to actinomycete PLFA biomass (Table 5.5). Of the non-microbial parameters, pH was a significant predictor of the relative contributions of NO_3^--N and NH_4^+-N, and of theoretical maximum nitrification-coupled denitrification and nitrifier denitrification contributions ($N_2O_{NCD\text{-}max}$ and $N_2O_{ND\text{-}max}$) to total N_2O production. The variables of nitrifier-induced production ($N_2O_{(NH4)}$, $N_2O_{NCD\text{-}max}$ and $N_2O_{ND\text{-}max}$) were all positively related to pH, the $N_2O_{(NO3)}$ intrinsically decreased with pH (as it is inversely related to $N_2O_{(NH4)}$) (Table 5.5). The organic C content demonstrated a negative effect on absolute total and NO_3^- derived N_2O (Table 5.5). Absolute NH_4^+ derived N_2O showed a negative relation with C:N ratio. The relative $N_2O_{(NO3)}$ and $N_2O_{(NH4)}$ were respectively positively and negatively related to C:N ratio (Table 5.5).

Discussion

The results from our study provide the best evidence so far that nitrifier denitrification can indeed occur in soils, and can do so at substantial rates (Table 5.4, Figure 5.2). Eight of the twelve incubated soils showed a considerable contribution of nitrifier-induced (NH_4^+ derived) N_2O production. We found that nitrifier denitrification must have contributed to N_2O production in at least five of these eight soils in order to explain the observed O incorporation from H_2O into the produced N_2O (Table 5.4, Figure 5.2). In all eight soils, nitrifier denitrification may even have been responsible for virtually all NH_4^+ derived N_2O. In contrast, N_2O production as by-product from nitrification had hardly occurred, if at all (Table 5.4).

Table 5.5: Results of the linear regression analyses between dependent variables on N_2O production and predictor variables on soil properties and soil microbial parameters. Predictor variables are considered significant when $P < 0.05$; *ns* = non-significant.

Response variable	Total N_2O		NO_3^- derived N_2O				NH_4^+ derived N_2O				NCDmax[a]		NDmax[a]	
	µg N kg^{-1}soil		% of total N_2O		µg N kg^{-1}soil		% of total N_2O		µg N kg^{-1}soil		% of total N_2O		% of total N_2O	
Predictor	P	R_{adj}^2	P	R_{adj}^2	P	R_{adj}^2	P	R_{adj}^2	P	R_{adj}^2	P	R_{adj}^2	P	R_{adj}^2
pH	*ns*		0.005	0.66	*ns*		0.005	0.66	0.000	0.83	0.001	0.89	0.006	0.77
Corg	0.037	0.41	*ns*		0.028	0.45	*ns*		*ns*		*ns*		*ns*	
C/N	*ns*		0.012	0.56	*ns*		0.012	0.56	0.000	0.92	*ns*		*ns*	
Microbial Biomass N	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
Microbial Biomass C	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
Total PLFA Biomass	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
Bacterial Biomass	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
Fungal Biomass	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
Actinomycete Biomass	*ns*		*ns*		*ns*		*ns*		*ns*		0.040	0.52	*ns*	
VAM Biomass	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	

Model fit[b]

Predictor	*Corg*	*pH*	*Corg*	*pH*	*pH*	*pH*	*pH*
Constant	6.76 (1.00)	10.82 (2.13)	6.79 (1.09)	-10.82 (2.13)	-9.30 (1.83)	-11.63 (1.31)	-18.69 (3.79)
Parameter	-0.05 (0.02)	-1.35 (0.34)	-0.05 (0.02)	1.35 (0.34)	1.78 (0.29)	1.36 (0.19)	2.47 (0.54)
Predictor		*C/N*		*C/N*	*C/N*	*Actin Bm*	
Constant		-1.26 (1.27)		1.26 (1.27)	7.25 (0.66)	-1.35 (0.41)	
Parameter		0.27 (0.08)		-0.27 (0.08)	-0.39 (0.04)	-0.13 (0.05)	

[a] only includes G and A soils

[b] for significant predictors only; model parameters (and standard errors (se)) for the ln-transformed data

In most soils, the dominant pathway of N_2O production was fertilizer denitrification (FD) (Table 5.4). The high moisture level was very likely the major determinant for this. Relative to FD, nitrifier denitrification (ND) and nitrification-coupled denitrification (NCD) may likely be suppressed because the required first step of ammonium oxidation is limited by more anaerobic conditions. At low O_2 levels, ND in turn is thought to be favored relative to nitrifier nitrification (NN) (Wrage et al., 2001), which may explain why we observed very little N_2O production through the latter pathway (Table 5.4). However, although the high moisture levels were expected to be sub-optimal for NN relative to the other pathways, ammonium oxidation *did* occur. It therefore remains remarkable that N_2O production as by-product from nitrification (NN) appears to be completely absent.

Nitrifier-induced N_2O production, i.e. total NH_4^+ derived N_2O, was evident in all grassland (G) and arable (A) soils (Table 5.2). In contrast, all forest (F) soils exhibited very little N_2O derived from NH_4^+. Remarkably, across the G and A soils the absolute amounts of NH_4^+ derived N_2O production were very similar, whereas fertilizer denitrification varied considerably. This may again suggest that nitrification was limited under the experimental conditions, so that differences in the potential for nitrifier N_2O production pathways across soils are less expressed.

Next to the moisture conditions, the presence and significance of N_2O production pathways may likely be related with soil properties such as pH, C content and microbial community composition (Knowles, 1982; Bock et al., 1986; Haynes et al., 1986; Paul et al., 1996).

The pH was a significant predictor of the relative contributions to N_2O production. At higher pH, the relative proportion of N_2O derived from NH_4^+ increased at the cost of NO_3^- derived N_2O. In general, low soil pH may have inhibited the nitrifier pathways altogether (NH_4^+-N derived N_2O) in the F soils, which limited the evaluation of the relation between soil pH and the different nitrifier-induced N_2O production pathways to the G and A soils. However, the potential (theoretical maximum) contribution of NCD and ND to N_2O were positively related with soil pH as well. As nitrite oxidation seems more sensitive to low pH than ammonia oxidation (Anthonisen et al., 1976) and accumulating NO_2^- levels can become toxic to ammonia oxidizers, we speculate that lower pH

might favor nitrifier denitrification (ND) over nitrification-coupled denitrification (NCD). Based on culture studies it is indeed thought that in general nitrification is favored at pH of approximately 6.5 and higher (Bock et al., 1986; Haynes, 1986; Stephen et al., 1998), though nevertheless nitrification is demonstrated in a wide variety of acid soils (Rosswall, 1982; Haynes, 1986; De Boer et al., 2001). In pure cultures *N. europaea* remains the most studied AOB, although *Nitrosospira spp*. are more common in soil (Stephen et al., 1998; Kowalchuk et al., 2001; Smith et al., 2001). Different clusters of the latter were demonstrated to favor different pH conditions, ranging from 4.2 to 7 (Stephen et al., 1996; Stephen et al., 1998). Heterotrophic nitrification and/or the presence of micro-sites for autotrophic nitrification have been thought to explain the occurrence of nitrification in acid soils (De Boer et al., 1991; Paul et al., 1996), but De Boer et al. (1991) also suggested that aggregated autotrophic bacteria may actually dominate nitrification at low soil pH. Overall, pH is clearly a driving factor in N_2O production in total, and may likely affect the relative significance of the contributions of the different pathways.

Soil organic C content is also considered to be a determining factor in N_2O production (Firestone, 1982; Haynes et al., 1986; Weier et al., 1993; Sánchez-Martín et al., 2008). Especially production through denitrification would be enhanced at higher C content, by providing an energy source for this heterotrophic process. However, absolute total and NO_3^- derived N_2O showed to be negatively related to organic C content in our study. This could be explained by the suggestion that increased C availability leads to more complete denitrification with N_2 rather than N_2O as the end-product (Weier et al., 1993; Mathieu et al., 2006; Miller et al., 2009). The relative contributions of mineral N pools to total N_2O emission, however, seemed to be significantly affected by C:N ratios by promoting the NO_3^- derived N_2O at the cost of $N_2O_{(NH4)}$ at higher ratios.

The microbial community analyses revealed considerable variation across the soils. Drying and sieving of the soil prior to incubation will have affected the microbial population, so data should be compared only in a semi-quantitative way. Although these microbial analyses are not directly indicative of the processes undertaken by the microbial community, comparative differences in microbial biomass N, C, and PLFA could be reflected in the N_2O production as studies have suggested that microbial community may influence its production

and the N_2O:N_2 ratio (Holtan-Hartwig et al., 2000; Dembreville et al., 2006). However, except for a relation between actinomycete biomass and $N_2O_{NCD\text{-}max}$, neither total N_2O production nor the relative contributions of the distinguished pathways were shown to be related to the microbial community composition (Table 5.5). Many actinomycetes are indeed able to denitrify (Shoun et al., 1998), but Miller et al. (2008; 2009) also found that there was no significant relation between the abundance of denitrifiers and N_2O emissions in their soil incubation studies. The here observed positive effect of actinomycete PLFA biomass on the $N_2O_{NCD\text{-}max}$ might also be explained by their saprotrophic nature. Their ability to degrade more resistant organic substances (Paul et al., 1996) may increase C availability to other organisms in the soil. This may favor denitrification relative to nitrification, and thereby positively affect the relative contribution of NCD. However, we might then expect to see this effect on the contribution of FD as well, but both absolute and relative NO_3^- derived N_2O did not show a significant relation with actinomycete biomass. Overall, differences in microbial community composition as distinguished by PLFA analyses are not necessarily reflected in N_2O production pathways, as similar microbial functions are spread across different functional groups and vice versa diverse functions can be undertaken by the same group. We suggest that future research would mainly benefit from combining isotope tracing with molecular techniques involving the analyses of functional genes rather than community composition.

This study comprised quite a wide range of soil types and land uses, including temperate, continental and Mediterranean climate regions. Differences in climatic origin of the soil may be of interest in examining the pathways of N_2O production. For example, Crenshaw et al. (2008) concluded that fungi may be the main producers of N_2O in semi-arid soil (2008). In the semi-arid soil studied by Sánchez-Martín et al. (2008), nitrifier denitrification was thought to be the main source of N_2O in contrast to a temperate soil where (nitrification-coupled) denitrification was dominant. Ma et al. (2007) found that in Arctic soil the role of denitrifiers was minor and suggest that nitrifier denitrification was in fact the dominant pathway of N_2O production. Although (semi-) arid and Arctic regions may not be a major contributor to total atmospheric N_2O inputs, extending further investigations to such regions could advance our understanding of the intricate complexity of N_2O formation in (agro-) ecosystems.

Conclusions

This study aimed to assess whether N_2O production through nitrifier denitrification in soil may be conclusively proven. Evaluation of the data from our isotopic labeling experiment revealed that O isotopic enrichment of the produced N_2O could not be explained without assuming the presence of nitrifier denitrification (ND) in some of our soils. We thus conclude that ND can indeed take place and constitute an important contribution to total N_2O production in actual soils. Further research remains needed to study how different soil types and variable conditions like moisture and fertilizer treatments affect the (relative and absolute) N_2O production through the distinctive pathways.

Acknowledgements

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Appendix 5-1: Summary of the derivations of $N_2O_{(NH4)}$, $N_2O_{(NO3)}$, N_2O_{FD}, $N_2O_{NCD\text{-}max}$, AOI and X_{ERR}

These calculations and the reasoning behind them are explained in detail in Kool et al. (2009b; 2009a). In short, the proportions of total N_2O derived from NH_4^+ and NO_3^-, $N_2O_{(NH4)}$ and $N_2O_{(NO3)}$ respectively, were calculated from the ^{15}N-N_2O enrichment data. The relative contribution of FD to N_2O production (N_2O_{FD}) was then defined as $N_2O_{(NO3)}$. The $N_2O_{(NH4)}$ comprised the sum of the relative contribution of nitrification (NN; N_2O_{NN}), nitrifier denitrification (ND; N_2O_{ND}), and nitrification-coupled denitrification (NCD; N_2O_{NCD}) pathways together:

$$N_2O_{(NH4)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR4)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \quad (eq\ A5.1)$$

$$N_2O_{(NO3)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR3)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \quad (eq\ A5.2)$$

$$N_2O_{(NO3)} = N_2O_{FD} \quad (eq\ A5.3)$$

$$N_2O_{(NH4)} = N_2O_{NN} + N_2O_{ND} + N_2O_{NCD} \quad (eq\ A5.4)$$

Next, the maximum possible contribution of NCD, $N_2O_{NCD\text{-}max}$, was derived based on the ^{15}N enrichment of the N_2O and NO_3^- resulting from application of ^{15}N enriched NH_4^+ (TR4). Nitrate is assumed to be an obligatory intermediate for NCD, i.e. the use of nitrite by heterotrophic denitrifiers is assumed to be minimal (as NO_3^- was abundant and is energetically more profitable to use). Therefore, where the ^{15}N enrichment in the total N_2O did not exceed the ^{15}N enrichment of the NO_3^- (from TR4), the $N_2O_{(NH4)}$ could have exclusively originated from NCD. The $N_2O_{NCD\text{-}max}$ then equaled the total $N_2O_{(NH4)}$. When ^{15}N-N_2O exceeded the ^{15}N enrichment in the NO_3^- in TR4, the $N_2O_{NCD\text{-}max}$ comprised a fraction of the $N_2O_{(NH4)}$ that could be derived from the ^{15}N enrichment data (from TR4)(Kool et al., 2009b):

$$If\ ^{15}N(N_2O_{(TR4)}) \leq {}^{15}N(NO_3^-{}_{(TR4)})\ ,\ then\ N_2O_{NCD\text{-}max} = N_2O_{(NH4)} \quad (eq\ A5.5)$$

$$If\ ^{15}N(N_2O_{(TR4)}) > {}^{15}N(NO_3^-{}_{(TR4)}),\ then\ N_2O_{NCD\text{-}max} < N_2O_{(NH4)} \quad (eq\ A5.6)$$

$$N_2O_{NCD\text{-}max} = N_2O_{(NH4)} \cdot \left(\frac{^{15}N\left(NO_3^-{}_{(TR4)}\right)}{^{15}N\left(NO_3^-{}_{(TR4)}\right) + {}^{15}N\left(NH_4^+{}_{(TR4)}\right)} \right) \quad (eq\ A5.7)$$

From the application of ^{18}O enriched H_2O (TR1), the actual O incorporation from H_2O into N_2O (AOI, %) was determined. It is calculated from the measured ^{18}O enrichment of N_2O relative to the applied enrichment of the ^{18}O-H_2O:

$$AOI = 100 \cdot \frac{^{18}O\left(N_2O_{(TR1)}\right)}{^{18}O\left(H_2O_{(TR1)}\right)} \qquad (eq\ A5.8)$$

The O exchange during denitrification, X_{ERR}, was quantified from the ^{18}O:^{15}N enrichment ratio of the N_2O relative to that of the applied NO_3^- (TR2 and TR3). Without O exchange, the retention of this enrichment ratio from NO_3^- into N_2O (ERR) would be 100%. The exchange was thus quantified as the loss in this enrichment ratio, i.e. 100% minus the ERR:

$$X_{ERR} = 100 - ERR \qquad (eq\ A5.9)$$

$$ERR = 100 \cdot \frac{^{18}O\left(N_2O_{(TR2)}\right)}{^{15}N\left(N_2O_{(TR3)}\right)} \cdot \frac{^{15}N\left(NO_{3\,(TR3)}^-\right)}{^{18}O\left(NO_{3\,(TR2)}^-\right)} \qquad (eq\ A5.10)$$

Appendix 5-2: Evaluation of nitrifier denitrification

Identification of the presence of ND

With the use of the previously derived data from ^{18}O and ^{15}N tracing, we further evaluated the N_2O ^{18}O enrichment data in order to assess the potential contribution of the nitrifier pathways to N_2O production. Basic assumptions on the O incorporation into N_2O through the different pathways were that (i) N_2O resulting from NN would not have any O incorporated from H_2O (only from O_2), (ii) the N_2O resulting from ND and NCD would obtain respectively 1/2nd and 2/3rd of the O from H_2O through reaction stoichiometry, and (iii) the OI for N_2O resulting from ND, NCD, and FD could be increased as an effect of O exchange, at the level of X_{ERR} for all these pathways. For details on the derivation of the various OIs for the pathways under different assumptions regarding O exchange, we refer to Kool *et al.* (2009b).

We first identified whether ND must have contributed at least some part to total N_2O production. For this purpose we assumed its contribution to be zero, N_2O_{ND} = 0, and subsequently evaluate whether this assumption could hold or

must be rejected in order to concur with the measured isotopic enrichments. We calculated the theoretical oxygen incorporation from H_2O (TOI) under these assumptions, TOI_1, by adopting the $N_2O_{NCD\text{-}max}$ and ascribing the remaining $N_2O_{(NH4)}$ to NN (N_2O_{NN}). By using $N_2O_{NCD\text{-}max}$ and in addition assuming that NCD is affected by O exchange, the TOI was maximized. This TOI_1 thus amounted to:

$$TOI_1 = N_2O_{(NO3)} \cdot X_{ERR} + N_2O_{NCD\text{-}max} \cdot (2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2) \quad (eq\ A5.11)$$

Parameter settings for TOI_1:

$N_2O_{FD} = N_2O_{(NO3)}$

$N_2O_{NCD} = N_2O_{NCD\text{-max}}$

$N_2O_{ND} = 0$

$N_2O_{NN} = N_2O_{(NH4)} - N_2O_{NCD\text{-max}}$

&

$OI(N_2O_{FD}) = X_{ERR}$

$OI(N_2O_{NCD}) = 2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2$

$OI(N_2O_{ND}) = n.a.$

$OI(N_2O_{NN}) = 0$

When the AOI would be similar or lower than TOI_1, this would imply that no ND was needed to explain the ^{18}O incorporation from H_2O into the produced N_2O (the AOI). However, when the AOI exceeded the TOI, part of the N_2O must have been produced through ND instead of NN to allow for 'additional' O incorporation to explain the AOI. This would identify a minimum value for N_2O_{ND}, $N_2O_{ND\text{-}min}$, and would simultaneously imply a maximum for the potential contribution of NN, $N_2O_{NN\text{-}max}$:

If $AOI > TOI_1$,

$$then\ N_2O_{ND\text{-}min} > 0\ \&\ N_2O_{NN\text{-}max} < N_2O_{(NH4)} - N_2O_{NCD\text{-}max} \quad (eq\ A5.12)$$

Please note that TOI_1 would only be relevant when $N_2O_{NCD\text{-}max}$ was constrained to less than $N_2O_{(NH4)}$ by the ^{15}N tracing data. (We further quantified the $N_2O_{ND\text{-}min}$ and $N_2O_{NN\text{-}max}$ where relevant, as described below).

Next, we assessed the potential significance of the ND contribution. This was

done by conversely ascribing the $N_2O_{(NH4)}$ completely to ND, i.e. $N_2O_{ND} = N_2O_{(NH4)}$, to evaluate whether we had to identify a maximum to its contribution ($N_2O_{ND\text{-}max}$). For this TOI_2 we minimized the theoretical O incorporation by assuming that O exchange did *not* take place under ND:

$$TOI_2 = N_2O_{(NO3)} \cdot X_{ERR} + N_2O_{(NH4)} \cdot 0.5 \qquad (eq\ A5.13)$$

Parameter settings for TOI_2:

$N_2O_{FD} = N_2O_{(NO3)}$

$N_2O_{NCD} = 0$

$N_2O_{ND} = N_2O_{(NH4)}$

$N_2O_{NN} = 0$

&

$OI(N_2O_{FD}) = X_{ERR}$

$OI(N_2O_{NCD}) = n.a.$

$OI(N_2O_{ND}) = 0.5$

$OI(N_2O_{NN}) = n.a.$

When the AOI would be smaller than TOI_2, this would mean that not all of the $N_2O_{(NH4)}$ could have originated from ND. It would simultaneously imply that part of the $N_2O_{(NH4)}$ would need to be ascribed to NN (to obtain a lower TOI that better explains the AOI):

$$If\ AOI < TOI_2,$$

$$then\ N_2O_{ND\text{-}max} < N_2O_{(NH4)}\ \&\ N_2O_{NN\text{-}min} > 0 \qquad (eq\ A5.14)$$

(Quantification of the minimum NN contribution, $N_2O_{NN\text{-}min}$, follows below).

Conversely, when the AOI was not smaller than TOI_2, all $N_2O_{(NH4)}$ could indeed have been derived through ND, without proof for any minimum contribution to N_2O production through NN:

$$If\ AOI \geq TOI_2,$$

$$then\ N_2O_{ND\text{-}max} = N_2O_{(NH4)}\ \&\ N_2O_{NN\text{-}min} = 0 \qquad (eq\ A5.15)$$

We also consider whether $N_2O_{ND\text{-}max}$ should be limited (i.e. less than $N_2O_{(NH4)}$) because part of the $N_2O_{(NH4)}$ would need to be assigned to NCD instead. Another TOI could be derived, for which all $N_2O_{(NH4)}$ is again ascribed to ND but where O exchange is assumed to affect the $OI(N_2O_{ND})$ as well. However, when O exchange is maximized (by being present during ND as well as during NO_2^- oxidation to NO_3^-) the $OI(N_2O_{ND})$ in fact equals the $OI(N_2O_{NCD})$. In other words, assigning part of the $N_2O_{(NH4)}$ to NCD would not further improve the TOI estimation of the AOI. Therefore, the conclusion of *eq A5.15* remains.

However, it remained valuable to compare such a TOI that maximizes O exchange, TOI_3, with our AOI as well:

$$TOI_3 = N_2O_{(NO3)} \cdot X_{ERR} + N_2O_{(NH4)} \cdot (2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2) \qquad (eq\ A5.16)$$

Parameter settings for TOI_3*:*

$N_2O_{FD} = N_2O_{(NO3)}$

$N_2O_{NCD} + N_2O_{ND} = N_2O_{(NH4)}$

$N_2O_{NN} = 0$

&

$OI(N_2O_{FD}) = X_{ERR}$

$OI(N_2O_{NCD}) = OI(N_2O_{ND}) = 2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2$

$OI(N_2O_{NN}) = n.a.$

As this TOI_3 is the 'ultimate' maximum TOI we could compute based on our data, it should not severely underestimate our AOI. When it closely estimates the AOI, we in fact recognize that the contribution of NN to N_2O production must have been negligible, i.e. $N_2O_{NN\text{-}max}$ should be zero.

Quantification of the $N_2O_{NN\text{-}max}$, $N_2O_{ND\text{-}min}$, and $N_2O_{NN\text{-}min}$

As described above (*eq A5.12*), when AOI > TOI_1 we identified a minimum contribution of ND, i.e. $N_2O_{ND\text{-}min} > 0$. Also, the contribution of NN must have been less than assumed under TOI_1, i.e. $N_2O_{NN\text{-}max} < N_2O_{(NH4)} - N_2O_{NCD\text{-}max}$. From the evaluation of TOI_2 we may have confirmed a minimum contribution of NN to N_2O production, i.e. $N_2O_{NN\text{-}min} > 0$ (*eq A5.14*). We now aimed to further quantify these $N_2O_{ND\text{-}min}$, $N_2O_{NN\text{-}max}$ and $N_2O_{NN\text{-}min}$.

First, evaluation of TOI_2 and TOI_3 could have determined that $N_2O_{NN\text{-}max}$ was zero. In that case, all $N_2O_{(NH4)}$ was derived through N_2O_{NCD} and N_2O_{ND}. The minimum N_2O_{ND} is then derived using the $N_2O_{NCD\text{-}max}$:

If $N_2O_{NN\text{-}max} = 0$,

then $N_2O_{ND\text{-}min} = N_2O_{(NH4)} - N_2O_{NCD\text{-}max}$,

i.e. $N_2O_{ND\text{-}min} = 100 - N_2O_{FD} - N_2O_{NCD\text{-}max}$ (*eq A5.17*)

When our data indicated that there may have been N_2O production through NN, i.e. $N_2O_{NN\text{-}max} > 0$, we quantified the $N_2O_{NN\text{-}max}$ by determining what minimal contributions of the other pathways together would be required to explain the AOI. The N_2O_{NN} itself does not contribute to the AOI ($OI(N_2O_{NN}) \equiv 0$). We minimized the N_2O_{ND} and N_2O_{NCD} that would be needed to achieve the AOI by assuming that both are affected by O exchange (i.e. maximizing the $OI(N_2O_{ND})$ and the $OI(N_2O_{NCD})$). As noted above, the OI for those pathways would be equal, so it does not matter (for the OI) whether the N_2O has been produced through ND or NCD. The combined contribution of ND and NCD to N_2O production is defined as $N_2O_{(NCD+ND)\text{-}min}$, and their OI denoted as $OI(N_2O_{NCD+ND})$. We then calculated the $N_2O_{NN\text{-}max}$ as follows:

$N_2O_{FD} + N_2O_{(NCD+ND)\text{-}min} + N_2O_{NN\text{-}max} = 100$

$AOI = N_2O_{FD} \cdot X_{ERR} + N_2O_{(NCD+ND)\text{-}min} \cdot OI(N_2O_{NCD+ND})$, *i.e.*

$N_2O_{(NCD+ND)\text{-}min} \cdot OI(N_2O_{NCD+ND}) = AOI - N_2O_{FD} \cdot X_{ERR}$, *i.e.*

$N_2O_{(NCD+ND)\text{-}min} = (AOI - N_2O_{FD} \cdot X_{ERR}) / OI(N_2O_{NCD+ND})$, *where*

$OI(N_2O_{NCD+ND}) = 2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2$

$N_2O_{NN\text{-}max} = 100 - N_2O_{FD} - N_2O_{(NCD+ND)\text{-}min}$ (*eq A5.18*)

The $N_2O_{ND\text{-}min}$ would then amount to $N_2O_{(NCD+ND)\text{-}min}$ minus the $N_2O_{NCD\text{-}max}$:

$N_2O_{ND\text{-}min} = N_2O_{(NCD+ND)\text{-}min} - N_2O_{NCD\text{-}max}$ (*eq A5.19*)

From the evaluation of TOI_2 we might have concluded that NN had a minimum contribution to N_2O, i.e. $N_2O_{NN\text{-}min} > 0$, if the $AOI < TOI_2$ (*eq A5.14*). To quantify the $N_2O_{NN\text{-}min}$, we evaluated a theoretical scenario where O incorporation

is minimized. The N_2O_{NCD} was thus set to zero and ND was not subject to O exchange, similar to the approach for TOI_2. However, as TOI_2 indicates (*eq A5.14*), part of the $N_2O_{(NH4)}$ needs to be assigned to NN instead of ND:

$$N_2O_{(NH4)} = N_2O_{ND\text{-}max} + N_2O_{NN\text{-}min}$$

$$AOI = N_2O_{FD} \cdot X_{ERR} + N_2O_{ND\text{-}max} \cdot OI(N_2O_{ND}), \text{ i.e.}$$

$$N_2O_{ND\text{-}max} \cdot OI(N_2O_{ND}) = AOI - N_2O_{FD} \cdot X_{ERR}, \text{ i.e.}$$

$$N_2O_{ND\text{-}max} = (AOI - N_2O_{FD} \cdot X_{ERR}) / OI(N_2O_{ND}), \text{ where}$$

$$OI(N_2O_{ND}) = 0.5$$

$$N_2O_{NN\text{-}min} = N_2O_{(NH4)} - N_2O_{ND\text{-}max} \qquad (eq\ A5.20)$$

Chapter 6

Nitrifier denitrification as a distinct and significant source of N_2O from soil

Abstract As soils comprise the premier source of the greenhouse gas nitrous oxide (N_2O), it is essential to understand its key N_2O production pathways. The potential of nitrifier denitrification as production pathway of N_2O has been well established in pure culture studies, but proof of its occurrence in terrestrial ecosystems has remained elusive. Only recently empirical research has confirmed that nitrifier denitrification can produce N_2O in soil, but its relative significance was minor as experimental moisture conditions favored nitrate driven denitrification. Here we assess the relative importance of nitrifier denitrification under a range of moisture regimes, including conditions less optimal for denitrification. Using a novel multi-isotope tracing approach we show that nitrifier denitrification can be a major contributor to total N_2O emission from soil. The role of nitrifier denitrification can be equally significant as that of N_2O produced as by-product of ammonia oxidation. With respect to total denitrifying activity, nitrifier denitrification dominated N_2O production under conditions sub-optimal for heterotrophic denitrification. We conclude that nitrifier denitrification is distinct from conventional nitrification and denitrification and affected idiosyncratically by environmental conditions. Accordingly, nitrifier denitrification should be routinely addressed as one of the major sources of N_2O from soil.

Introduction

Nitrous oxide has become the third most important anthropogenic greenhouse gas (IPCC, 2007), and is today's single most important ozone-depleting emission (Ravishankara et al., 2009). When aiming to mitigate N_2O emissions, accurate understanding of the biochemical processes responsible for N_2O production is crucial (Baggs, 2008). Although a wide range of processes has the potential to produce N_2O, its production in soil is generally primarily attributed to nitrification and denitrification. Semantics may confuse this apparently simple paradigm, since various nitrifiers are able to denitrify as well. This nitrifier denitrification (ND) by ammonia oxidizing bacteria (AOB) has long been acknowledged in pure cultures (Hooper, 1968; Ritchie et al., 1972), and it has been suggested that ND could be a universal trait in beta-proteobacterial ammonium oxidizers, which are thought to be the dominant ammonium oxidizing bacteria in soil (Shaw et al., 2006). As it is well established that nitrifying micro-organisms contribute significantly to N_2O emission from soils (Bremner, 1997), and as soils are the major source of N_2O to the atmosphere (IPCC, 2007), insight in the potential of ND in soils is of global environmental interest. An increasing number of studies suggests that ND may contribute significantly to N_2O production in soil (Webster et al., 1996; McLain et al., 2005; Wrage et al., 2005; Venterea, 2007), but definite proof has remained elusive due to methodological constraints (Wrage et al., 2001; Wrage et al., 2005; Kool et al., 2007). Only recently a novel multi-isotope tracing approach was presented (Kool et al., 2010) that accounts for the potential exchange of oxygen (O) between H_2O and intermediate compounds of N_2O production (Kool et al., 2007; Kool et al., 2009b). This enabled further discrimination of nitrifier denitrification (ND) as an N_2O production pathway that is distinct from conventional nitrification (NN) and denitrification (FD and NCD, denitrification of applied -fertilizer- NO_3^- and nitrification-coupled denitrification, respectively) (Figure 6.1). It provided best proof to date in soil-based experiments that ND can indeed produce N_2O in soil. However, the relative contribution of ND to total N_2O production was minor in this set-up, as production was dominated by FD (Kool et al., 2010). This may be explained by experimental conditions, which at 80% water holding capacity (WHC) were optimal for denitrification. To study the significance of ND under conditions less

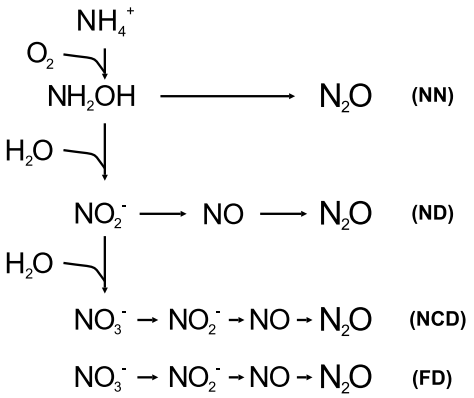

Figure 6.1. Depiction of the major pathways of N_2O formation. We distinguish N_2O production from nitrifiers (ammonia oxidizers) through nitrification (NN) and nitrifier denitrification (ND), and from denitrifiers through reduction of NO_3^- produced from nitrification, i.e. nitrification-coupled denitrification (NCD), and reduction of applied NO_3^-, i.e. fertilizer denitrification (FD).

optimal for denitrification and more representative soil conditions we here imposed a variety in moisture conditions. N_2O production was studied from soil incubated at 50, 70, and 90% WHC using the novel multi-isotope tracing approach (Kool et al., 2010).

Methods

Replicate samples of a poor sandy soil (pH 5.4) were incubated in glass jars for 28h after application of 50 mg NH_4^+-N kg^{-1} and 50 mg NO_3^--N kg^{-1} soil, with treatment-specific isotopically enriched compounds: ^{18}O labeled H_2O or NO_3^-, or ^{15}N labeled NO_3^- or NH_4^+ (TR1, TR2, TR3 and TR4 respectively). Three moisture treatments were imposed, i.e. 50, 70, and 90% WHC. Lids were kept closed (airtight) during the incubation period. Analyses on a random selection of gas samples confirmed that O_2 concentrations in the headspace had not notably declined during the incubation.

At the end of the incubation, N_2O production, soil mineral N content, and their relevant O and N isotopic signatures were determined (Kool et al., 2009a). From the ^{15}N enrichment data the relative contributions of NH_4^+-N and NO_3^--N to total N_2O production were derived (Kool et al., 2009b). Analyses of the ^{15}N-NH_4^+

in the $^{15}N\text{-}NO_3^-$ labeling treatment confirmed that the contribution of DNRA as potential N_2O producing pathway was negligible. Oxygen exchange during production of N_2O from NO_3^- reduction (X_{ERR}) was determined by the ERR method (Kool et al., 2009a) (using the data from the ^{18}O- and $^{15}N\text{-}NO_3^-$ labeling treatments) and taken into account with further data evaluation. The potential minimum and maximum contribution of the different pathways to total N_2O production was calculated following the combined O and N isotope tracing approach presented by Kool et al. (2010). A summary of the main calculations of this approach is provided as supplementary information. The main assumptions underlying the approach are that (i) N_2O produced as byproduct of ammonia oxidation (NN) obtains all O from O_2 (no O incorporation from H_2O), (ii) O incorporation from H_2O into N_2O from FD, ND and NCD is respectively zero, 1/2nd and 2/3rd through reaction stoichiometry, and can be increased as an effect of O exchange at the level of X_{ERR} for all these pathways, and that (iii) nitrate (not only nitrite) is an obligatory intermediate for nitrification-coupled denitrification (NCD). Table 6.1 presents total N_2O production, relative contributions of NH_4^+-N ($N_2O_{(NH4)}$) and NO_3^--N ($N_2O_{(NO3)}$) to total N_2O production and several intermediate parameters of the data evaluation.

All treatments were replicated five times, which provided for the standard errors of the means of isotope enrichment data. The variables X_{ERR}, $N_2O_{(NH4)}$, and $N_2O_{(NO3)}$ are defined as ratios of averages of the replicates, for which standard errors were approximated by a first-order Taylor linearization (Kool et al., 2010). As X_{ERR} is a key parameter in the analyses to derive the relative pathway contributions, we carried out a sensitivity analysis of this parameter. A full data evaluation was additionally carried out using the X_{ERR} plus or minus its standard error in the calculations. Oxygen exchange was set to zero when X_{ERR} was calculated to be negative in the evaluation.

A summary of the data calculations is given in appendix 6. More specifics about the incubation set-up, analyses, and data calculations can be found in previous work (Kool et al., 2009b; Kool et al., 2009a; Kool et al., 2010).

Results and Discussion

Table 6.1 lists the total N_2O production over the incubation and intermediate

parameters of the data calculations. The therewith calculated relative pathway contributions to N_2O production are presented in Table 6.2 and Figure 6.2.

Our results show that ND can be the prime contributor to total N_2O production from soil (Figure 6.2). Nitrifier denitrification contributed more to N_2O production than total conventional denitrification of NO_3^- (FD plus NCD) at both 50 and 70% WHC. In the nearly water saturated soil (90% WHC), N_2O production was, as expected, dominated by conventional denitrification of NO_3^-. In all moisture treatments ND constituted a major proportion of the NH_4^+ derived N_2O, ranging from minima of 30-50% to maxima of 60-100%. N_2O production as a by-product of ammonia oxidation, i.e. NN, comprised maximally 44-64% of NH_4^+ derived N_2O. Nitrifier denitrification should therefore be considered as an equally important pathway of N_2O production as NN and conventional denitrification (FD plus NCD).

In our incubation experiments, soils were amended with both NH_4^+ and NO_3^- to realize the required isotopic enrichment. As nitrification in general proceeds slower than (heterotrophic) denitrification, it would be expected that the relative contribution of FD in these incubations is larger than it would be in the field under non-nitrate fertilized conditions. Therefore in actual ecosystems where NO_3^- may be relatively more scarce, the potential contribution of ND to total N_2O is likely to be even more significant.

Although they are not known to produce N_2O, Archaea are suggested to have a potential significant role in the NH_4^+ oxidizing community in soils (Leininger et

Table 6.1: Total absolute production of N_2O over the incubation period and intermediate parameters of the calculations of the relative pathway contributions. AOI= actual oxygen incorporation (from H_2O into N_2O); TOI= Theoretical oxygen incorporation.

Moisture treatment	Total production $\mu gN_2O\text{-}N\ kg^{-1}soil$ (se)	$N_2O_{(NO3)}$ % (se) [a]	$N_2O_{(NH4)}$ % (se) [a]	AOI % (se)	TOI_1 %	TOI_2 %
50% WHC	0.78 (0.06)	20.0 (1.4)	80.0 (0.0)[b]	28.4 (1.5)	4.0	40.0
70% WHC	0.93 (0.07)	16.1 (1.9)	83.9 (0.9)	29.3 (1.1)	9.4	46.3
90% WHC	16.74 (2.15)	92.1 (0.0)[b]	7.9 (0.4)	61.5 (2.4)	57.8	61.2

[a] values between brackets denote the approximated standard error

[b] the approximated variance was negative and therefore set to zero

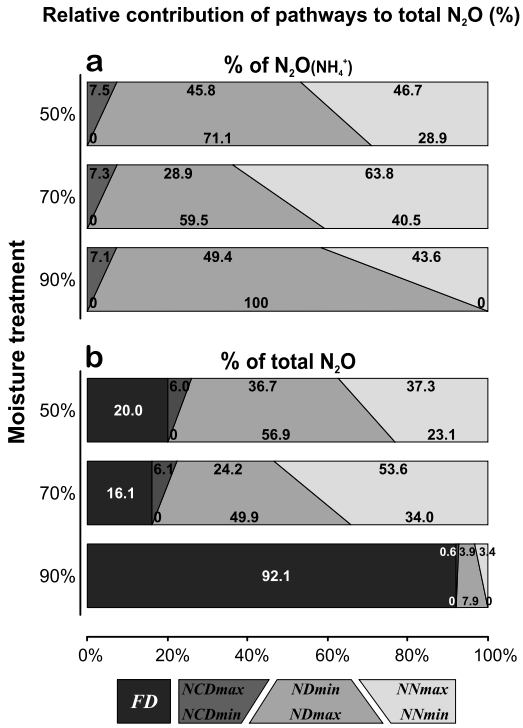

Figure 6.2: Relative contributions of the pathways to N_2O production over the incubation period at the different moisture treatments (% WHC), as percentage (%) of (a) the NH_4^+ derived N_2O, and (b) the total N_2O, with minima and maxima for the NH_4^+ derived N_2O (NCD, ND and NN). The minimum N_2O_{NCD} is zero by default of the evaluation. NCD = (N_2O from) nitrification-coupled denitrification; ND = nitrifier denitrification; NN = nitrifier nitrification (i.e. ammonia oxidation); FD = fertilizer (applied NO_3^-) denitrification.

al., 2006). Potential occurrence of archaeal ammonia oxidation however does not impair our findings on the contribution of different nitrifier pathways to N_2O emissions. On the contrary, if Archaea are in fact responsible for a significant part of the ammonia oxidation this would relegate the role of AOB in that process. This would again imply that the ammonia-derived N_2O that is interpreted as by-product from ammonia oxidation by AOB may often be overrated.

The extent of oxygen exchange may severely affect the ^{18}O signature of N_2O (Kool et al., 2009a), and is evidently an important parameter in analyses to distinguish the significance of the different pathways (Kool et al., 2010). A sensitivity analysis of this parameter confirmed the robustness and general

outcome of our results: when O exchange was varied from plus to minus its standard error the data evaluation still showed ND to be a major contributor to N_2O production in soil (Table 6.2).

Moisture conditions are a well-known driver of N_2O production (Webster et al., 1996). Our results indicate that nitrifier denitrification and 'conventional' denitrification may each respond differently to moisture conditions. The relative importance of FD was considerably less at reduced soil moisture content than at 90% WHC, while the relative contribution of ND as percentage of NH_4^+ derived N_2O did not strongly differ between moisture conditions (Figure 6.2). In terms of absolute production, both ND and FD declined with moisture content, but ND much less so than FD: about 60% versus 99% reduction respectively (Table 6.3).

The increased importance of ND relative to FD at lower moisture content is notable, as from theory one might argue that soil moisture content and related oxygen (O_2) availability control FD and ND alike. In both reduction processes the NO_3^- or NO_2^- acts as electron acceptor. Because the NO_2^- and NO reductases and genes encoding for these enzymes in AOB have been found to be similar to those in heterotrophic denitrifiers (Chain et al., 2003; Casciotti et al., 2005; Cantera et al., 2007; Garbeva et al., 2007; Norton et al., 2008), enzyme synthesis and/or activity in the two pathways might be expected to respond similarly to O_2 availability. On

Table 6.2: Relative contributions of the N_2O production pathways during the incubation period, including the results of the sensitivity analysis (SA range). For the sensitivity analyses all contributions were calculated with the X_{ERR} lowered or raised with its standard error (calculated FD and NCDmax are not affected by that). FD = (N_2O from) fertilizer denitrification (from applied NO_3^-); NCD = nitrification-coupled denitrification; ND = nitrifier denitrification; NN = nitrifier nitrification, i.e. ammonium oxidation.

Moisture treatment	% of total N_2O						% of NH_4^+ derived N_2O				
	FD	NCDmax	NDmin	NDmax	NNmin	NNmax	NCDmax	NDmin	NDmax	NNmin	NNmax
50% WHC	20.0	6.0	36.7	56.9	23.1	37.3	7.5	45.8	71.1	28.9	46.7
SA range			26.3-36.7	49.9-56.9	23.1-30.1	37.3-47.7		32.9-45.8	62.4-71.1	28.9-37.6	46.7-59.6
70% WHC	16.1	6.1	24.2	49.9	34.0	53.6	7.3	28.9	59.5	40.5	63.8
SA range			18.1-33.0	43.8-56.1	27.8-40.2	44.8-59.7		21.6-39.3	52.1-66.8	33.2-47.9	53.4-71.1
90% WHC	92.1	0.6	3.9	7.9	0.0	3.4	7.1	49.4	100.0	0.0	43.6
SA range			1.3-6.5	3.5-7.9	0.0-4.4	0.8-6.0		16.0-83.3	44.2-100	0.0-55.8	9.7-77.0

the other hand, profound (eco-)physiological differences between the microorganisms responsible for FD and ND, i.e. heterotrophic denitrifiers and autotrophic ammonia oxidizers, may conceivably induce different responses to environmental conditions. In FD, NO_3^- reduction serves respiration, but generally more energy can be gained when O_2 is used as electron acceptor. Most denitrifiers favor O_2 over NO_3^- even at quite low O_2 concentrations, thereby precluding FD activity. In ND the oxidation of ammonium is thought to provide the electron source for NO_2^- reduction (Ritchie et al., 1972; Poth et al., 1985; Bock et al., 1995). The amount of energy available from this process is thought to be similar to the amount of energy available from aerobic ammonium oxidation to nitrite (Jetten et al., 1999; Wrage et al., 2001). When NO_2^- is available from NH_4^+ oxidation, AOB could subsequently oxidize NH_4^+ with NO_2^- (ND) just as well as with O_2 (NN) to obtain a similar energy gain. Consequently, aerobic conditions would not need to inhibit ND. In a study by Ritchie and Nicholas (Ritchie et al., 1972) *Nitrosomonas europaea* indeed reduced NO_2^- to N_2O under both aerobic and anaerobic conditions. Also Shaw et al. (2006) found all AOB strains tested capable of ND under aerobic conditions. On the other hand, both the production of N_2O and the N_2O:NO_2^- ratio from nitrifiers in pure cultures have been found to decrease with increasing aerobicity (Goreau et al., 1980; Poth et al., 1985; Bock et al., 1995; Kester et al., 1997). The latter suggests that under aerobic conditions N_2O production as by-product of ammonia oxidation (NN) is more important than N_2O from nitrifier denitrification (ND). Altogether, aerobicity likely affects the occurrence of ND, but O_2 concentrations that repress heterotrophic denitrification do not necessarily constrain nitrifier denitrification to the same extent or through the same mechanisms. Despite similarities in their enzyme system, the biochemical

Table 6.3. Absolute contribution to N_2O production by the different pathways over the incubation period, calculated from the total absolute production (Table 6.1) and the evaluated relative pathway contributions (Table 6.2).

Moisture treatment	μg N_2O-N kg^{-1}					
	FD	NCDmax	NDmin	NDmax	NNmin	NNmax
50% WHC	0.16	0.05	0.29	0.45	0.18	0.29
70% WHC	0.15	0.06	0.23	0.47	0.32	0.50
90% WHC	15.43	0.09	0.65	1.32	0.00	0.57

processes of ND and FD are apparently not regulated alike.

An alternative explanation for the occurrence of ND is a response to NO_2^- toxicity (Poth et al., 1985; Stein et al., 1998; Beaumont et al., 2004b; Shaw et al., 2006). A decrease in N_2O production by *N. europaea* when co-cultured with a nitrite oxidizer (*Nitrobacter winogradskyi*) was ascribed to lower NO_2^- concentration (Kester et al., 1997). Further, the ammonium monooxygenase enzyme has been shown to be inhibited by nitrite under both aerobic and anaerobic conditions (Stein et al., 1998), and NirK has been shown to be expressed aerobically by *N. europaea* in response to increasing nitrite concentrations (Beaumont et al., 2004b). However, it could be argued that if ND serves to reduce NO_2^- toxicity it should be positively related to NO_2^- production from NH_4^+ oxidation, which conflicts with the observation that under well-aerated conditions NO_2^- reduction by AOB is limited or absent while NH_4^+ oxidation continues to produce NO_2^- (Bock et al., 1995). On the other hand expression of the genes for denitrifying enzymes (*nirK*, *norB* and *nsc*) in AOB has been found to be inhibited by NH_4^+ (Schmidt, 2009). Certain levels of NH_4^+ might thus inhibit ND, until ongoing NH_4^+ oxidation leads to increased NO_2^- and/or decreased NH_4^+ concentrations that respectively exceed and/or drop below a threshold level of NO_2^- toxicity and ND inhibition. Clearly, our study was not set-up to test hypotheses on NO_2^- toxicity or NH_4^+ inhibition as driving factors for ND. Nevertheless, the evident potential importance of this pathway should instigate future studies to further unravel the dynamics and driving factors of ND.

Conclusion

In summary, we found that the ND pathway is fundamentally distinct from other N_2O production pathways and responds idiosyncratically to environmental conditions. Our results show that ND may not only occur, but can indeed comprise a significant contribution to total N_2O production in soils, especially under conditions that are suboptimal for heterotrophic NO_3^- denitrification. The contribution of ammonia oxidation by AOB as N_2O source can be severely overrated when nitrifier denitrification is neglected. This strongly argues for nitrifier denitrification to be routinely considered as separate potential premier N_2O production pathway in biogeochemistry.

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Appendix 6: Summary of data calculations

The actual O incorporation from H_2O into N_2O (AOI) is calculated from the ^{18}O enrichment of the N_2O and H_2O in treatment TR1, $^{18}O(N_2O_{(TR1)})$ and $^{18}O(H_2O_{(TR1)})$ respectively:

$$AOI = 100 \cdot \frac{^{18}O\left(N_2O_{(TR1)}\right)}{^{18}O\left(H_2O_{(TR1)}\right)} \qquad (eq\ A6.1)$$

The oxygen exchange between H_2O and intermediates of the N_2O production pathways during reduction of NO_3^- to N_2O, X_{ERR}, is calculated from the ^{18}O and ^{15}N enrichment of the N_2O in treatment TR2 and TR3 respectively, $^{18}O(N_2O_{(TR2)})$ and $^{15}N(N_2O_{(TR3)})$, and the imposed ^{18}O and ^{15}N enrichment of NO_3^- in those treatments, $^{18}O(NO_3^-{}_{(TR2)})$ and $^{15}N(NO_3^-{}_{(TR3)})$:

$$X_{ERR} = 100 \cdot \left(1 - \frac{^{18}O\left(N_2O_{(TR2)}\right)}{^{15}N\left(N_2O_{(TR3)}\right)} \cdot \frac{^{15}N\left(NO_3^-{}_{(TR3)}\right)}{^{18}O\left(NO_3^-{}_{(TR2)}\right)}\right) \qquad (eq\ A6.2)$$

The proportions of total N_2O derived from NH_4^+ and NO_3^-, the $N_2O_{(NH4)}$ and $N_2O_{(NO3)}$, are calculated from the ^{15}N-N_2O enrichment in treatment TR3 and TR4, $^{15}N(N_2O_{(TR3)})$ and $^{15}N(N_2O_{(TR4)})$ respectively:

$$N_2O_{(NH4)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR4)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \qquad (eq\ A6.3)$$

$$N_2O_{(NO3)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR3)}\right)}{^{15}N\left(N_2O_{(TR3)}\right) + {}^{15}N\left(N_2O_{(TR4)}\right)} \qquad (eq\ A6.4)$$

The relative contribution of FD to total N_2O, N_2O_{FD}, is defined as $N_2O_{(NO3)}$:

$FD = N_2O_{(NO3)}$ ($FD = N_2O_{FD}$ in Kool et al., 2010) (*eq A6.1*)

The maximum proportion of N_2O that could have been derived from NCD, *NCDmax*, is calculated from the ^{15}N enrichment of the N_2O and NO_3^- resulting from treatment TR4, $^{15}N(N_2O_{(TR4)})$ and $^{15}N(NO_3^-{}_{(TR4)})$:

$$If\ ^{15}N(N_2O_{(TR4)}) \le {}^{15}N(NO_3^-{}_{(TR4)})\ ,\ then\ NCDmax = N_2O_{(NH4)} \qquad (eq\ A6.5)$$

$$If\ ^{15}N(N_2O_{(TR4)}) > {}^{15}N(NO_3^-{}_{(TR4)}),\ then\ NCDmax < N_2O_{(NH4)},\ \&$$

$$NCD\ max = N_2O_{(NH4)} \cdot \left(\frac{^{15}N\left(NO_3^-{}_{(TR4)}\right)}{^{15}N\left(NO_3^-{}_{(TR4)}\right) + {}^{15}N\left(NH_4^+{}_{(TR4)}\right)}\right) \qquad (eq\ A6.6)$$

The N_2O derived from NH_4^+ comprises the N_2O that is produced through NN, ND and NCD. The $N_2O_{(NH4)}$ is the sum of either (A) the maximum contribution of NN (*NNmax*), the minimum of ND (*NDmin*), and the maximum of NCD (*NCDmax)*, or (B) the minimum contribution of NN (*NNmin)*, the maximum of ND (*NDmax*), and the minimum of NCD (*NCDmin)*. A Theoretical Oxygen Incorporation from H_2O into N_2O (TOI) is calculated under (A) that maximizes the O incorporation (assuming overall presence of O exchange) while minimizing the contribution of ND (TOI_1). Under (B), the TOI_2 is calculated which maximizes the contribution of ND and encounters the minimum O incorporation, i.e. through reaction stoichiometry and O exchange during FD only. As follows:

$$N_2O_{(NH4)} = NN + ND + NCD \qquad (eq\ A6.8)$$

(i.e. $N_2O_{(NH4)} = N_2O_{NN} + N_2O_{ND} + N_2O_{NCD}$ in Kool et al., 2010)

(A): $N_2O_{(NH4)} = NNmax + NDmin + NCDmax \qquad (eq\ A6.9)$

$TOI_1 = N_2O_{(NO3)} \cdot X_{ERR} + NCDmax \cdot (2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2)$

If $AOI \leq TOI_1$,

then $NDmin = 0$ *&* $(NCD+ND)min = N_2O_{(NH4)}$

If $AOI > TOI_1$,

then $NDmin > 0$

& $(NCD+ND)min = (AOI - FD \cdot X_{ERR}) / (2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2)$

$NDmin = (NCD+ND)min - NCDmax$

$NNmax = N_2O_{(NH4)} - (NCD+ND)min$

(B): $N_2O_{(NH4)} = NNmin + NDmax \quad (NCDmin = 0) \qquad (eq\ A6.10)$

$TOI_2 = N_2O_{(NO3)} \cdot X_{ERR} + N_2O_{(NH4)} \cdot 0.5$

If $AOI \geq TOI_2$,

then $NDmax = N_2O_{(NH4)}$ *&* $NNmin = 0$

If $AOI < TOI_2$,

then $NDmax < N_2O_{(NH4)}$ *&* $NNmin > 0$

$NDmax = (AOI - FD \cdot X_{ERR}) / 0.5$

$NNmin = N_2O_{(NH4)} - NDmax$

6

Chapter 7

Oxygen exchange affects the oxygen isotopic signature of nitrate in soil

Abstract Oxygen stable isotope analyses are commonly used in nitrate (NO_3^-) source determination studies. The source and fate of NO_3^- are studied based on distinct O isotopic signatures from potential sources and production and consumption processes of nitrate. In particular, the $\delta^{18}O$ differs between sources like fertilizer, atmospheric deposition, and microbial production (nitrification), and is affected by fractionation effects during its transformation processes as well. However, O exchange between O from NO_3^- and H_2O is in those studies implicitly assumed not to affect the $\delta^{18}O$-NO_3^-. Here we show in a soil-based experiment that this assumption may not hold. In a short (24h) incubation experiment, soils were treated with ^{18}O and ^{15}N enriched NO_3^-. Production of NO_3^- during the incubation would affect both the ^{18}O and the ^{15}N enrichment. Oxygen exchange could therefore be studied by examining the change in ^{18}O relative to the ^{15}N. In two out of the three soils, we found that the imposed ^{18}O enrichment of the NO_3^- declined relatively more than the imposed ^{15}N-NO_3^- enrichment. This implies that O exchange might indeed affect the O isotopic signature of NO_3^-, which has implications for NO_3^- source determination studies. We suggest that O exchange should be considered as a defining factor of the O isotopic signature of NO_3^- when studying its origin and fate in ecosystems.

Introduction

Increasing concentrations of nitrate (NO_3^-) constitute an important environmental concern: contamination of groundwater and eutrophication of surface waters are recognized undesirable consequences of increased used of nitrogen (N) fertilizer and animal manure, atmospheric deposition and discharge of sewage waste (Howarth et al., 1996; Galloway et al., 2003). However, the significance of NO_3^- input from different sources is often not known quantitatively. Evidently, identifying the sources and evaluating the progress of the production and consumption of NO_3^- in ecosystems is of environmental interest.

Analyses of the $\delta^{15}N$ and $\delta^{18}O$ signatures of NO_3^- are commonly used to evaluate its sources and fate in ecosystems including groundwater, drainage water, and river catchments (e.g.Amberger et al., 1987; Durka et al., 1994; Burns et al., 2002; Wankel et al., 2006; Kendall et al., 2007; Burns et al., 2009). Different sources and processes are assumed to impose distinct isotopic signatures on the NO_3^- in these systems (Figure 7.1): atmospheric deposition and synthetic fertilizers are relatively highly enriched in ^{18}O; organic fertilizer (manure, slurry) has relatively high $\delta^{15}N$ values; denitrification leaves the residual NO_3^- pool relatively enriched in both isotopes, and; NO_3^- produced from nitrification results in typically the lowest $\delta^{15}N$ and $\delta^{18}O$ signatures among the considered sources. The expected range of the $\delta^{18}O$ of NO_3^- from nitrification is derived from the relative contribution of O_2 and H_2O to the total O incorporated during the oxidation steps. O_2 contributes one atom during ammonia oxidation to hydroxylamine (NH_2OH), and H_2O contributes the other two O atoms during further oxidation to nitrite (NO_2^-) and NO_3^- (Hollocher et al., 1981; Andersson et al., 1983; Hollocher, 1984; Kendall et al., 2007). The relatively low $\delta^{18}O$ of soil H_2O (-25 to +4‰$_{SMOW}$, Amberger et al., 1987) explains the relative low $\delta^{18}O$ of NO_3^- from nitrification compared to the other sources (Figure 7.1).

In multiple NO_3^- source determination studies the $\delta^{18}O$ of the NO_3^- is low compared with that of atmospheric deposition and fertilizer input, but closer to the range expected from biologically produced NO_3^- through nitrification (-5 to -15‰$_{SMOW}$) (Figure 7.1). It is consequently reasoned that most NO_3^- in these systems (e.g. groundwater, drainage water, or river catchments) is derived from microbial nitrification within the soil system (e.g. Spoelstra et al., 2001; Williard et

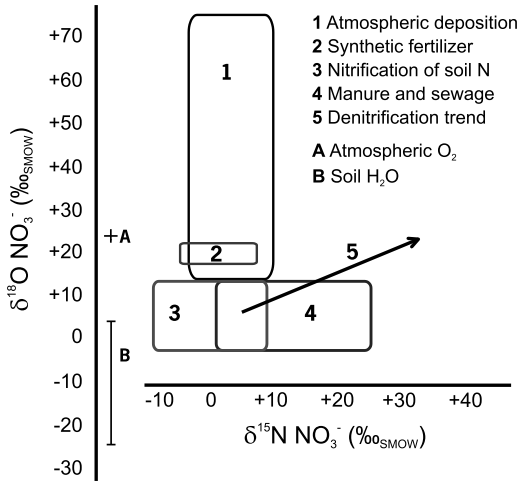

Figure 7.1: Overview of the different O and N isotopic signatures in NO_3^- from various sources (Kool et al. (2007), after Kendall (1998)). Along the y-axis the 18O of atmospheric O_2 and (soil) H_2O are indicated.

al., 2001; Burns et al., 2002; Mayer et al., 2002). However, as mentioned above, soil water has an even lower $\delta^{18}O$ than all above mentioned NO_3^- sources, including nitrification-derived NO_3^- which is still assumed to obtain part of its O from O_2 (at approximately +23.5‰$_{SMOW}$) (Figure 7.1). If exchange of O with H_2O would affect the NO_3^- pool, it would thereby lower the $\delta^{18}O$-NO_3^-. Regardless of the original source of the NO_3^-, its 'net' ^{18}O isotopic signature would partly be defined by the $\delta^{18}O$ of H_2O depending on the extent of the O exchange. As a result, the contribution of nitrification NO_3^- would then be overestimated at the expense of atmospheric deposition and fertilizer input.

Isotope fractionation during denitrification leaves the residual NO_3^- relatively enriched in ^{15}N and ^{18}O, which is used to evaluate the progress of denitrification (e.g. Böttcher et al., 1990; Wassenaar, 1995; Aravena et al., 1998; Groffman et al., 2006; Panno et al., 2006). However, an effect of O exchange would lower the $\delta^{18}O$-NO_3^- (as H_2O is depleted in ^{18}O compared to NO_3^-), which would mask (part of) the enrichment effect of denitrification. As a result, the rate of denitrification might be underestimated.

In summary, multiple sources and processes are taken into account to evaluate the fate and origin of NO_3^- based on the O isotopic signature. However,

consideration of O exchange with H_2O as a defining factor of the $\delta^{18}O\text{-}NO_3^-$ is lacking. If O exchange indeed occurs and affects the $\delta^{18}O$ of the NO_3^- in ecosystems significantly, it would constitute a pitfall for NO_3^- source determination studies.

To identify an effect of O exchange on the O isotopic signature of the NO_3^- pool in an actual ecosystem, one would need to know (i) the exact sources of the NO_3^-, as well as (ii) the isotopic fractionation factors of its production and consumption (mainly nitrification and denitrification, respectively). Therefore, to investigate the potential occurrence and effect of O exchange on the $^{18}O\text{-}NO_3^-$ signature, we carried out a soil incubation experiment in which we were in control of (i) the source of the NO_3^-, i.e. by application, and (ii) the (negligible) effect of isotopic fractionation, i.e. by the use of enriched compounds. By tracing the fate of ^{18}O relative to ^{15}N enrichment of the NO_3^- we studied O exchange as a potential defining factor of the O isotopic signature of NO_3^-.

Methods

Experimental set-up

Three soils were used in our experiment: two silt loam grassland soils from experimental field Easter Bush near Edinburgh, United Kingdom, that differed in fertilizer and grazing intensity (i.e. 'moderate' and 'intensive' management; Gm and Gi respectively), and one sandy, relatively poor, arable soil from experimental farm 'Droevendaal' near Wageningen, The Netherlands (A). Soils were dried at 40°C, sieved (2mm) and stored at 4°C until further use. Soil samples were incubated in glass jars (100 g soil per jar) and treated with 50 mg NO_3^--N kg^{-1} soil. The applied NO_3^- was artificially enriched in either ^{18}O at 2.0 atom% excess (TR1) or ^{15}N at 30.0 atom% excess (TR2). Two times four replicate samples per treatment, allowing for two destructive sampling moments, were pre-incubated for seven days at 40% WHC and 16°C. Moisture content was raised to 80% WHC with the treatment application at the start of the incubation.

After treatment application, soil was destructively sampled for the start (t0) measurement as soon as possible (within 4h after application at the latest). At the end of the incubation 24 hours later, the t24 samples were taken. All samples were processed by KCl extraction (20 g moist soil with 50 ml 1M KCl)

immediately after sampling. The O isotopic enrichment (^{18}O) of the NO_3^- in TR1 was determined on the KCl extracts by the denitrifier method (Casciotti et al., 2002; Xue et al., 2010). For the TR2 samples, the ^{15}N isotopic signature of the NH_4^+ and NO_3^- were derived by microdiffusion technique (Kool et al., 2009b). Isotopic analyses were carried out at the UC Davis Stable Isotope Facility.

Data evaluation

The ^{18}O and ^{15}N enrichments of the NO_3^- at t24 were compared with those at t0. Changes in the enrichments over the incubation period were studied using t-tests, significant differences were identified at $P<0.05$ ($\alpha = 0.05$).

Next to the individual ^{18}O and ^{15}N enrichments, we also evaluated the ^{18}O:^{15}N enrichment ratio of the NO_3^- ($ER_{(NO3)}$) to investigate whether their enrichment relatively to each other changed during the incubation. The ratio at t24 ($ER_{(NO3)t24}$) was compared to t0 ($ER_{(NO3)t0}$) and together defined the enrichment ratio retention $ERR_{(NO3)}$:

$$ERR_{(NO3)}\ (\%) = 100 \cdot ER_{(NO3)t24} / ER_{(NO3)t0} \qquad (eq\ 7.1)$$

$$with\ ER_{(NO3)} = {}^{18}O(NO_3^-{}_{(TR1)}) / {}^{15}N(NO_3^-{}_{(TR2)}) \qquad (eq\ 7.2)$$

where $^{18}O(NO_3^-{}_{(TR1)})$ and $^{15}N(NO_3^-{}_{(TR2)})$ are the ^{18}O and ^{15}N isotopic enrichments of the NO_3^- in TR1 and TR2 respectively, either both determined at t0 for $ER_{(NO3)t0}$ or at t24 for $ER_{(NO3)t24}$. As the $ER_{(NO3)}$s are ratios, we could not directly derive standard errors of the means from the replicates. Standard errors of the $ER_{(NO3)}$s were therefore approximated by a first-order Taylor linearization (Kendall et al., 1977; Kool et al., 2010).

With the use of enriched compounds, the fractionation effects during production and consumption of NO_3^- become negligible. Therefore, the $ER_{(NO3)}$ should not change over the course of the incubation, i.e. the $ERR_{(NO3)}$ should be 100% in the absence of O exchange. O exchange would cause a decrease in the $ER_{(NO3)}$ at t24 compared to t0, represented by a loss in the $ERR_{(NO3)}$.

Results and discussion

Our results show that in all soils the ^{18}O enrichment of NO_3^- decreased significantly over the course of the incubation time (24h), while the ^{15}N

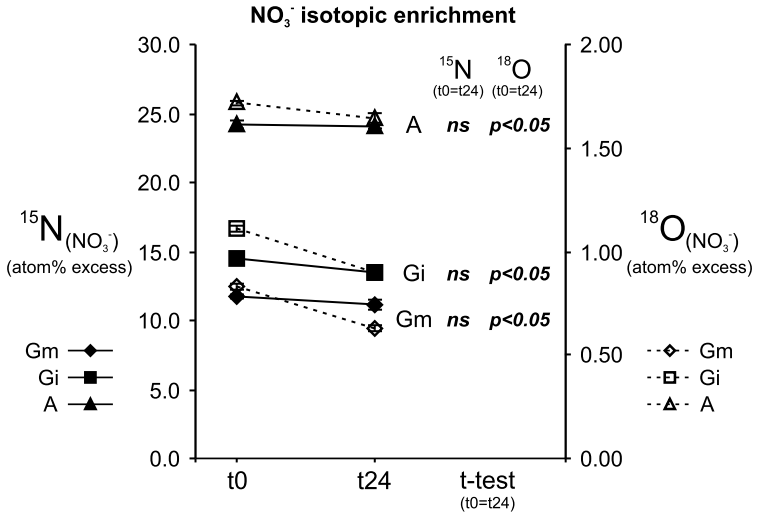

Figure 7.2: The O and N isotopic signatures of NO_3^- (in TR1 and TR2, respectively) for t0 and t24. T-tests evaluated that the differences between isotopic signatures at t0 and t24 were significant for ^{18}O and insignificant for ^{15}N in all soils. Dashed lines with open symbols represent ^{18}O data, solid lines with closed symbols represent ^{15}N. The error bars denote the standard errors (n=4 for all, except for $^{15}N_{(NO3)}$ at t0 of soil A; n=3).

enrichment did not (Figure 7.2, Table 7.1). Moreover, the $ERR_{(NO3)}$ was approximately 80, 88, and 96% for the 3 different soils, confirming a loss in ^{18}O relative to the ^{15}N (Figure 7.3). Overall small standard errors support high accuracy of the isotopic enrichment data. One data point (one replicate of TR2(t0) (^{15}N-NO_3)) appeared to be an outlier and was therefore excluded from the data calculations in Figure 7.2 and 7.3. Results of all data analyses on the isotopic enrichments and the $ERR_{(NO3)}$ are presented in Table 7.1.

Our results suggest that O exchange indeed occurred and affected the O isotopic signature of NO_3^- in at least two of the three soils. To our knowledge, no ecosystem-based study has experimentally identified an effect of O exchange on the O isotopic signature of NO_3^-. Dissimilatory nitrate reduction to ammonium (DNRA) or immobilization and re-mineralization of NO_3^- to NH_4^+ for subsequent nitrification, such as the 'immobilization-mineralization turnover concept' suggested by Mengis et al. (2001), would also maintain the ^{15}N but not the ^{18}O enrichment. Our incubation period was kept deliberately short to exclude significant effects of these processes. Given the short incubation period however,

more severe effects of O exchange might be expected in real ecosystems with longer residence time of the NO_3^-.

As we intend to study the potential effect of O exchange, we should consider whether this may have occurred within our samples after sampling as well. During sample storage or transport, O exchange will likely be minimal due to the high salt concentrations in the sample (KCl extracts) that reduce microbial activity. Also during the analytical procedure of the denitrifier method O exchange is assumed to be minimal: it is one of the selection criteria for the denitrifier strain used in this technique (*Pseudomonas aureofaciens*) (Casciotti et al., 2002). Moreover, this method has shown excellent repeatability and very good comparison with the silver nitrate method (Xue et al., 2010). Most importantly however, we could disregard such uncertainty in our approach as the enrichments are measured at the start as well as at the end of the incubation. Any O exchange after sampling that would affect the obtained ^{18}O signature will thus affect both measurements, and thereby not interfere with our evaluation of the

Table 7.1: The measured ^{18}O and ^{15}N isotopic signatures of NO_3^- (in TR1 and TR2, respectively) and the $ER_{(NO3)}$ and $ERR_{(NO3)}$ derived from those data. T-tests evaluated differences between the isotopic signatures at t0 and t24. Data between brackets denote the standard error of the mean for the isotopic signatures, and the approximated standard error for the $ER_{(NO3)}$. The data of soil A are provided in- and excluding one outlier of TR2 at t0. Also including this data point, the ^{15}N of the NO_3^- did not change significantly over the incubation period (whereas the ^{18}O-NO_3^- did (TR1)).

	^{18}O enrichment (TR1)			^{15}N enrichment (TR2)			$ER_{(NO3)}$		$ERR_{(NO3)}$
	t0	t24	t-test	t0	t24	t-test	t0	t24	t24 : t0
Soil	(atom% excess)		(H_0: t0=t24)	(atom% excess)		(H_0: t0=t24)	(^{18}O:^{15}N ratio)		%
Gm	0.829	0.629	p<0.05	11.74	11.15	*ns*	0.071	0.056	79.8
	(0.013)	(0.014)		(0.16)	(0.37)		(0.001)	(0.002)	
Gi	1.108	0.899	p<0.05	14.50	13.41	*ns*	0.076	0.067	87.8
	(0.013)	(0.017)		(0.38)	(0.34)		(0.002)	(0.002)	
A	1.718	1.639	p<0.05	24.23	24.03	*ns*	0.071	0.068	96.2
	(0.008)	(0.027)		(0.26)	(0.13)		(0.001)	(0.001)	
A[a]	1.718	1.639	p<0.05	27.63[a]	24.03	*ns*[a]	0.062[a]	0.068	109.7[a]
	(0.008)	(0.027)		(3.40)	(0.13)		(0.009)	(0.001)	

[a] Including an outlying data point in TR2(t0)

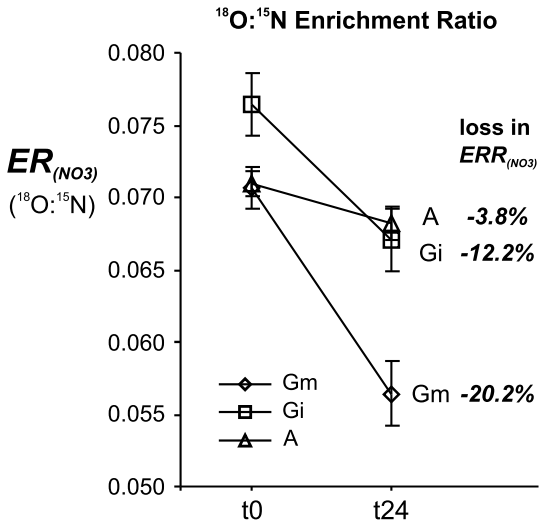

Figure 7.3: The $ER_{(NO3)}$ calculated from the isotope enrichment data for t0 and t24, and the loss in $ERR_{(NO3)}$ from t0 to t24, i.e. from the $ER_{(NO3)t0}$ and $ER_{(NO3)t24}$. The error bars denote the approximated standard errors.

occurrence of O exchange during the incubation period between those measurements.

If O exchange is indeed a defining factor of the O isotopic signature of NO_3^-, this would have significant implications for contemporary NO_3^- source determination. The quantitative contribution of the factors defining the O isotopic signature all entail some uncertainty: the isotopic signatures of the different sources as well as the extent of isotopic fractionation effects during production and consumption of NO_3^- are only known within a certain range (Bedard-Haughn et al., 2003; Kendall et al., 2007). Results from experimental studies at natural abundance can therefore generally be explained within the available conceptual framework, and will not directly lead to suspicion of O exchange without the use of artificially enriched compounds.

Observed discrepancies (both higher and lower) from the $\delta^{18}O$ of NO_3^- which is expected based on reaction stoichiometry have been attributed to fractionation, microscale variability in $\delta^{18}O$ of O_2 and H_2O, and contributions of heterotrophic nitrifiers whose nitrifying mechanism may differ with respect to O incorporation (Mayer et al., 2001; Burns et al., 2002; Kendall et al., 2007). Mengis et al. (2001)

observed ^{15}N signatures of the soil NO_3^- which corresponded to a major contribution of fertilizer as NO_3^- source, but simultaneously found that the ^{18}O-NO_3^- signatures were significantly lower than that of the fertilizer. They suggest that the original $\delta^{18}O$ of the fertilizer NO_3^- in their agricultural soils has faded due to microbial immobilization followed by mineralization and nitrification of the NO_3^-.

In studies on the origin and fate of ecosystem NO_3^-, O exchange is hardly considered as defining factor of the $\delta^{18}O$-NO_3^-. However, the potential presence and effect of O exchange should not come completely unexpected. First, O exchange may occur during nitrification, the production of NO_3^-. The final nitrification step of NO_3^- production from NO_2^- oxidation incorporates H_2O-O and is catalyzed by the enzyme nitrite oxidoreductase (Aleem et al., 1965; Bock et al., 1986). However, reduction of NO_3^- to NO_2^- is found to be brought about by this enzyme as well (Sundermeyer-Klinger et al., 1984; Wood, 1986). In other words, this process is reversible, which may provide the mechanistic explanation of the O exchange. If forward and reverse NO_2^-/NO_3^- transformations take place concurrently, the involvement of H_2O-O in this step implies that all the O in NO_3^- (and NO_2^-) can ultimately be replaced by O from H_2O. Furthermore, in the first steps of nitrification (ammonia oxidation to NO_2^-), microbially mediated exchange may occur between H_2O and nitrite (NO_2^-). Relatively early pure culture studies already associated O exchange with nitrifiers, both ammonia and nitrite oxidizers (Andersson et al., 1982; Kumar et al., 1983; DiSpirito et al., 1986). Recent pure culture studies on four different stains of ammonia oxidizing bacteria (AOB) reported an O exchange of 1 to 25% of the NO_2^--O atoms (Casciotti et al. (2010)). As a result, microbially produced NO_2^- and NO_3^- will exhibit $\delta^{18}O$ values that are closer to that of H_2O than expected based on reaction stoichiometry. Using a multi-box model to evaluate the $\delta^{18}O$-NO_3^- in the ocean, Sigman et al. (2009) also suggested that as a result of O exchange less than one out of six of the O atoms in NO_3^- originates from O_2, which is consistent with at least 50% O exchange. Casciotti et al. (2010) noted the discrepancy between their observed relatively low amounts of exchange (maximally 25%) compared to these model derived exchange rates. They suggest this may in part be explained by the fact that ammonia oxidizing Archaea (AOA) (may) significantly contribute to total nitrification in ocean waters, but may perform nitrification with alternative

reaction mechanisms, e.g. involving different enzymes. However, it has not been considered that O exchange during the *consumption* of NO_3^- may have an effect on the NO_3^- as well.

The O isotopic signature of nitrous oxide (N_2O), produced from ^{18}O enriched NO_3^- or from non-enriched NO_3^- in the presence of ^{18}O enriched H_2O, clearly showed to be affected by O exchange (Kool et al., 2009a). Pure culture studies on denitrifying bacteria have also presented O exchange affecting the substrate NO_2^- (Garber et al., 1982; Shearer et al., 1988). In the stepwise reduction of NO_3^- to N_2O the O exchange is suggested to be mainly associated with the NO_2^- and NO reduction steps (Garber et al., 1982; Kool et al., 2007). Again, the reversibility of this step may well explain the potential occurrence of O exchange. Although studies on O exchange during denitrification have mainly focused on the steps of NO_2^- and NO reduction, an effect on NO_3^- may still be conceived. For studies on NO_3^-, it might appear speculative to suggest that O exchange during NO_3^- reduction might affect the substrate's O isotopic signature. However, for sulfate (SO_4^{2-}) it is generally acknowledged that the $\delta^{18}O$ is affected not only by isotope fractionation, but by (varying degrees of) equilibration with H_2O (Fritz et al., 1989; Böttcher et al., 2001; Farquhar et al., 2008; Turchyn et al., 2010). Oxygen and sulfur isotope effects in SO_4^{2-} during its bacterial reduction process were modeled by Brunner et al. (2005a; 2005b), incorporating the forward and reverse steps in the reduction. With this model observed patterns in isotope data from natural environments and laboratory studies could be better explained. Also for NO_3^- reduction, such a model based on O isotope exchange effects could help to better explain oxygen isotope effects of residual NO_3^- from denitrification.

Conclusion

Altogether, based on observations in pure culture studies and model comparisons, O exchange needs to be considered as a defining factor of the O isotopic signature of NO_3^-. Awareness about this effect on NO_3^- source determination has been limited since experimental ecosystem-based studies which address this potential effect have remained lacking. Our experiment indicates that O exchange may indeed affect the O isotopic signature of NO_3^- in actual soil ecosystems. Studies evaluating the source and fate of NO_3^- in

ecosystems based on its O isotopic signature may have been overestimating microbial nitrification as a source of NO_3^-, and/or underestimating the progression of denitrification. We conclude that this experiment should instigate further and more elaborate ecosystem-based studies to identify the presence and effect of O exchange on the O isotopic signature of NO_3^-. Ultimately, considering O exchange would improve the interpretation of O isotopic analyses in NO_3^- source determination studies.

Acknowledgements

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Chapter 8

General discussion

Recalling the main objectives

A particular deficiency in our understanding of N_2O production in soil concerns the pathway of 'nitrifier denitrification' (Figure 8.1). 'Conventional' autotrophic nitrification and heterotrophic denitrification have generally been considered as the prime sources of biochemical N_2O production in soils (Mosier et al., 1998; Pérez et al., 2001). However, soil-based studies increasingly suggest that nitrifier denitrification, i.e. nitrite reduction (denitrification) by autotrophic ammonia oxidizers (nitrifiers), might contribute significantly to N_2O from soil as well (Granli et al., 1994; Webster et al., 1996; Hütsch et al., 1999; Wrage et al., 2004a; McLain et al., 2005; Ma et al., 2007; Venterea, 2007; Sánchez-Martín et al., 2008). The potential for this pathway of N_2O production has been proven for various ammonia oxidizers in pure cultures (e.g. Hooper, 1968; Ritchie et al., 1972; Colliver et al., 2000; Shaw et al., 2006), but its actual occurrence and contribution to the total N_2O production in natural ecosystems has remained elusive.

To enable the distinction of N_2O produced by nitrifier denitrification, novel methodology was needed. Combined O and N stable isotope tracing has been suggested to offer such methodology. However, an approach based on current understanding of the origin of the O in N_2O from O_2 and H_2O (Wrage et al., 2005) does not adequately consider, let alone account for, the potential interference of O exchange. Consequently, my first objective was (i) to study, identify and quantify the process of O exchange between H_2O and intermediates of the N_2O production pathways, and its effect on the O isotopic signature of N_2O from soil. I subsequently (objective ii) aimed to develop and apply an advanced O and N isotope tracing approach that *could* distinguish nitrifier denitrification from 'conventional' nitrification and denitrification in soil-based studies. Anticipating that nitrifier denitrification would be successfully identified, my final objective (iii) was to evaluate the significance and idiosyncratic character of nitrifier denitrification as production pathway of N_2O in soil.

In this chapter I discuss the main findings of my research and their implications for our understanding of N_2O emissions from soils. The main assumptions underlying my approach are also discussed.

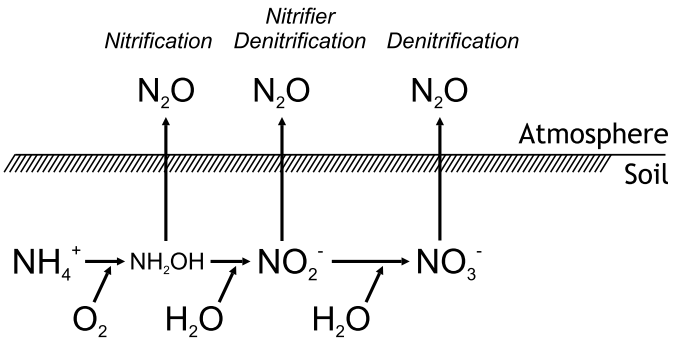

Figure 8.1: Illustration of the incorporation of oxygen (O) from O_2 and H_2O during the production of N_2O through the different pathways, including nitrifier denitrification next to 'conventional' nitrification and denitrification.

Oxygen exchange as defining factor of the O isotopic signature of N_2O

According to reaction stoichiometry, N_2O obtains its O from O_2 and H_2O in ratios distinct for the different pathways of N_2O production (Figure 8.1). It has generally been assumed that the O isotopic signature of the produced N_2O would subsequently reflect the isotopic signature of its substrate (e.g. NO_3^-) and of O_2 and H_2O in the ratios designated by reaction stoichiometry (further only affected by fractionation) (Kim et al., 1990; Pérez, 2005; Wrage et al., 2005; Kendall et al., 2007). In other words, O exchange between H_2O and intermediate compounds of biochemical N_2O production has been assumed to be negligible. My research conclusively falsified this assumption, and I conclude that the presence of O exchange has been inappropriately neglected. My literature review (chapter 2) already suggested that assumptions on the negligibility of O exchange should be approached with extreme caution. In a wide range of soils I experimentally identified that O exchange strongly determined the O isotopic composition of N_2O (chapter 3). These findings necessitated the development of an advanced approach to discriminate the main pathways of N_2O production in soil. In this pursuit, I introduced the use of the *ratio* of the ^{18}O:^{15}N isotopic enrichment of N_2O relative to that of NO_3^-. Tracing their enrichment ratio retention (ERR) allowed to not only identify, but also quantify the O exchange for the denitrification of NO_3^- to N_2O (chapters 3 and 4). Incorporating the effect of O exchange in advanced dual (O and N) isotopic tracing enabled to further distinguish nitrifier

denitrification from 'conventional' nitrification and denitrification as pathways of N_2O production in soil. Additionally, in an exploratory study I demonstrated that next to N_2O, O exchange might affect the O isotopic composition of NO_3^- in soil as well (chapter 7). These findings suggest that NO_3^- source determination studies may also need to recognize O exchange as defining factor of the O isotopic signature to properly interpret the sources and cycling of NO_3^- in ecosystems.

Nitrifier denitrification as pathway of N_2O production in soil: experimentally identified

Applying the advanced dual isotope approach, I studied the occurrence of nitrifier denitrification as N_2O production pathway in soil (chapters 5 and 6). Albeit with an uncertainty range, these studies now present compelling evidence that nitrifier denitrification indeed occurs in soils.

In my studies on a diversity of European soils (chapter 5), total N_2O was dominated by NO_3^- driven denitrification under the moist experimental conditions. The identified minimum contribution of nitrifier denitrification to total N_2O remained low (over 60% for one soil, but less than 10% for the others), and its relative significance might therefore appear small. However, actual contributions may likely have been larger, as this minimum was defined under a rather extreme scenario, maximizing both O exchange and the contribution of nitrification-coupled denitrification to N_2O production. Moreover, assessment of the nitrifier-derived N_2O revealed that the contribution of nitrifier denitrification strongly dominated over the contribution of 'conventional' nitrification. Next, my experiments on a single soil at different moisture levels (chapter 6) showed that at moderate moisture content (50% and 70% WHC), nitrifier denitrification was responsible for more N_2O than 'conventional' denitrification. To conclude, these results show that nitrifier denitrification can constitute a significant contribution to soil-derived N_2O.

Nitrifier denitrification as pathway of N_2O production in soil: environmental controls

After recognizing nitrifier denitrification as distinct pathways of N_2O production

next to 'conventional' nitrification and denitrification, we ultimately aim to understand its environmental controls as well. Prime environmental regulators of N_2O production include soil moisture and O_2 conditions, pH and C availability (Firestone et al., 1989; Paul et al., 1996; Robertson et al., 2007). It is generally accepted that 'conventional' nitrification and denitrification are affected differently by these environmental controls. Although anticipated, for nitrifier denitrification its **idiosyncratic response** has never been experimentally established since proof of its mere presence in soil remained lacking.

In chapter 6 I demonstrate that nitrifier denitrification indeed responds idiosyncratically to soil **moisture content**. Nitrifier denitrification is thought to occur under marginally aerobic and/or short-term anaerobic conditions, as O_2 is needed for preceding ammonia oxidation and the denitrifying pathway would be similar to that of heterotrophic denitrifiers (Wrage et al., 2001). My results suggest that nitrifier denitrification is less repressed by increased aerobicity than 'conventional' denitrification. Under relatively aerobic conditions, N_2O production by nitrifier denitrification could be equally significant as N_2O evolved as by-product of ammonia oxidation.

In chapter 5 I evaluated soil **pH and C content** as possible predictors of the relative pathway contributions to N_2O production. Relative contributions of NH_4^+ and NO_3^- derived N_2O showed to be related with both soil pH and soil C content. However, the overall dominance of NO_3^--driven denitrification under the (relatively moist) experimental conditions complicated the assessment, and effects of these parameters may therefore be less pronounced. With the small contribution of NH_4^+ (i.e. total nitrifier contribution) to total N_2O, and the ability to only partially quantify the different nitrifier pathways, the contribution of nitrifier denitrification was not distinct enough to evaluate its individual relation with the diversity in pH and soil carbon.

It is however possible to speculate about the response of nitrifier denitrification to variations in soil pH and soil carbon content, in comparison with 'conventional' nitrification and denitrification. Bacteria denitrify preferably at higher **pH**, and nitrogen oxide reductases in the stepwise denitrification pathway are thought to be progressively inhibited with decreasing pH (Knowles, 1982). Based on identified similarities in the denitrifying enzymes of autotrophic nitrifiers and heterotrophic denitrifiers, nitrifier denitrification and 'conventional'

denitrification may be hypothesized to be affected alike by pH. However, my results already showed that the observed enzymatic similarity does not necessarily generate similar responses to moisture conditions. Nitrification is observed in ecosystems across a wide range of pH, but based on pure culture studies on AOB nitrification is thought to prefer higher pH as well. Wrage et al. (2001) evaluated that while AOB may favor ammonia oxidation at a pH of 7, at pH 4 they might gain more energy from nitrifier denitrification.

Heterotrophic denitrification requires a, preferably readily available, C source and is thus directly affected by soil **C content** and quality. Autotrophic ammonia oxidizers are indirectly affected by SOC content and quality as it regulates NH_4^+ availability through mineralization and immobilization (Paul et al., 1996; Robertson et al., 2007). Heterotrophic denitrification and autotrophic nitrification will therefore respond differently to variation in SOC, but ammonia oxidation and nitrite reduction by AOB (i.e. nitrification and nitrifier denitrification) might be regulated alike by C availability. However, total soil microbial activity, and therefore O_2 consumption, is C dependent. Increased C availability could thereby reduce O_2 availability for AOB, improving conditions for nitrifier denitrification relative to ammonia oxidation.

Main implications: Oxygen exchange between H_2O and intermediates of N_2O production

Evidently, the presence of O exchange in soil has implications for source determination of nitrogen oxides based on their O isotopic signature.

If O exchange is not accounted for when distinguishing the pathways of N_2O production, we would overestimate the pathways that according to reaction stoichiometry produce N_2O with relatively more O from H_2O: nitrification-coupled denitrification and nitrifier denitrification. Nitrous oxide produced as by-product of 'conventional' nitrification and from denitrification of applied (fertilizer) NO_3^- would be underestimated. The level of O exchange varied across soils and moisture conditions (chapters 3 and 6). This is not surprising, as already in pure cultures large variation in O exchange rates was observed across bacterial strains (Ye et al., 1991; Casciotti et al., 2002). In complex ecosystems such as soils with variability in microbial composition and activity across space and time, O

exchange will also be highly dynamic. This implies that the effect of O exchange will need to be quantified in concurrence with N_2O production in each experiment, i.e. it is an indispensable component of accurate O isotope tracing.

Implications of O exchange should also be considered for natural abundance studies. At natural abundance levels, reaction steps in the production of N_2O fractionate in favor of the lighter isotopes (^{16}O and ^{14}N) resulting in a relatively depleted product (N_2O) compared to the substrate. Likewise, reduction of N_2O to N_2 leaves the residual N_2O relatively enriched with the heavier isotopes (^{18}O and ^{15}N) due to isotopic fractionation. This effect on the N_2O isotopic signature is used to study the process of production and consumption N_2O (e.g. Schmidt et al., 2004; Wrage et al., 2004b; Pérez, 2005; Van Groenigen et al., 2005). If the N_2O production is affected by O exchange, the $\delta^{18}O$ of the N_2O pool would (next to fractionation factors) be further defined by the O isotopic signature of the H_2O involved. This would lower the $\delta^{18}O$ of the N_2O, as H_2O typically has a lower $\delta^{18}O$ value than N_2O and its preceding compounds. As consumption leads to relative ^{18}O enrichment of the N_2O pool, O exchange might lead to overestimation of production relative to consumption of N_2O.

In addition, I present that the implications of O exchange between H_2O and intermediates of N_2O production are not limited to N_2O source determination: O exchange could affect NO_3^- as well (chapter 7). In source determination of NO_3^-, analyses of the $\delta^{18}O$ signature are commonly used to discriminate between NO_3^- derived from e.g. atmospheric deposition, fertilizer, and microbial production (i.e. nitrification) (e.g. Amberger et al., 1987; Durka et al., 1994; Kendall et al., 2007). In these studies, O exchange is (implicitly) assumed to be negligible. The $\delta^{18}O$ of NO_3^- is often relatively low compared to that of atmospheric and fertilizer input, and closer to what would be expected from nitrification. Again, the $\delta^{18}O$ of soil H_2O is even lower, and O exchange would therefore decrease the $\delta^{18}O$ of the total NO_3^- pool. As a result, respective studies in e.g. ground and drainage water and river catchments may have been overestimating the contribution of nitrification-derived NO_3^- at the expense of atmospheric and fertilizer input.

Main implications: Nitrifier denitrification as pathway of N_2O production in soil

Identifying nitrifier denitrification as a distinct and idiosyncratically controlled pathway of N_2O production in soil is a step forward in our process based understanding of N_2O production. Ultimately, such understanding is key to adequately predict and mitigate N_2O emissions to the atmosphere. The acknowledgement of nitrifier denitrification therefore imposes considerable implications for studies that aim to simulate and predict N_2O emissions from soil. Main challenges in such modeling studies result from the fact that N_2O from soil (i) can derive from multiple processes, (ii) is produced and consumed simultaneously, and (iii) is controlled by a large number of environmental variables (Li, 2000). Process-oriented models have been developed that include sub-models describing nitrification and denitrification and their response to environmental controls (DNDC (Li, 2000), DAYCENT (Del Grosso et al., 2005)). Clearly, a distinct role of nitrifier denitrification is not yet considered in these models. The lack of understanding of nitrifier denitrification, both its significance and its idiosyncratic response to environmental controls, may be a reason why current models struggle to adequately simulate and predict N_2O emissions. The distinct response of nitrifier denitrification to environmental parameters is not accounted for and models may consequently fall short in predicting total N_2O emissions. Ultimately, process-based models would need to consider a more diverse set of processes of N_2O production that respond individually to environmental parameters. Incorporating nitrifier denitrification may improve model performance, but without doubt will be a major challenge, specifically as long as the controlling factors remain poorly understood.

Main assumptions

The findings of my research contribute to and have implications for our understanding of N_2O production in soil, as discussed above. However, next to these implications, main assumptions underlying my approach should be discussed. Primarily, it is assumed that (i) N_2O derived as by-product from ammonia oxidation (nitrifier nitrification, NN) does not obtain any O from H_2O; (ii) NO_3^- is the substrate and an obligatory intermediate for 'fertilizer' denitrification and nitrification-coupled denitrification (i.e. total 'conventional' denitrification); (iii) across the pathways where O exchange is considered to

occur, it takes place at the same rate as quantified for denitrification of NO_3^- to N_2O; (iv) the addition of mineral N compounds (NH_4^+ and NO_3^-) needed to obtain the desired enrichment does not severely disrupt the system. Figure 8.2 illustrates these assumptions, which I discuss and assess in more detail below.

Re (i): Nitrous oxide derived from ammonia oxidation is thought to be a by-product of (incomplete) oxidation of hydroxylamine (Hooper et al., 1979; Arp et al., 2003). As the O in hydroxylamine has been shown to originate from O_2 and not from H_2O (Dua et al., 1979; Hollocher et al., 1981), O_2 is assumed to be the sole source of the O in N_2O resulting as by-product from ammonia oxidation (Figure 8.1). However, the exact mechanism of this step from hydroxylamine to N_2O is still unknown, implying some uncertainty about this assumption (chapter 2). Although available literature suggests the validity of this assumption, only a full description of the hydroxylamine-N_2O step would fully verify it.

Re (ii): In the ^{15}N tracing, it is assumed that in nitrification-coupled denitrification NH_4^+ is completely nitrified to NO_3^- which is subsequently reduced by denitrifiers (Figure 8.1). However, these denitrifiers might also directly take up and reduce NO_2^- formed in the first step of nitrification. The contribution of nitrification-coupled denitrification would in that case be underestimated and identified as nitrifier denitrification instead. However, although heterotrophic denitrifiers can reduce NO_2^- directly, NO_3^- is energetically more profitable. Also, the intermediate NO_2^- would need to be released by the nitrifiers and move through the soil to become available for those denitrifiers. In my studies, NO_3^- was abundant (applied) and clearly readily denitrified under the experimental conditions (chapters 3-6). Altogether, I postulate that this justifies the assumption that NO_3^- was intermediate for the large majority of N_2O produced through nitrification-coupled denitrification in the soil incubation studies performed.

Re (iii): The developed methodology quantifies O exchange for the reduction of NO_3^- to N_2O. In the data evaluation, the potential of O exchange during other pathways is considered as well, for which the same exchange rate is assumed. Nevertheless, uncertainty remains whether it indeed occurs during the considered nitrifier pathways, and if so to what extend. These uncertainties are however taken into account through analyzing a range of scenarios regarding the possible occurrence of O exchange in the possible other pathways (chapter 4). The

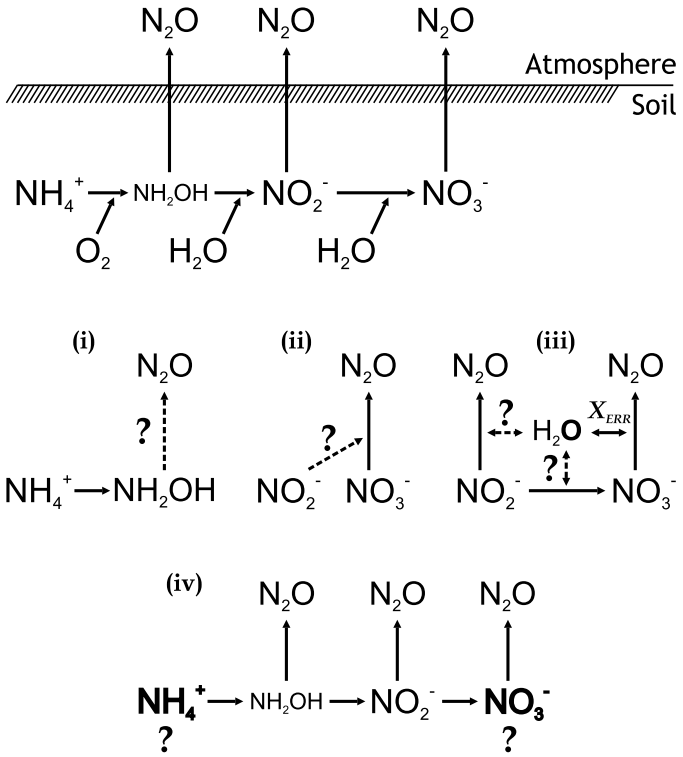

Figure 8.2: Illustration of the main assumptions related to the advanced dual isotope approach that is used to study the main pathways of N_2O production in soil.

most extreme scenario maximizes O exchange: a (minimum) contribution of nitrifier denitrification is thereby only identified when the ^{18}O signature of N_2O could not be explained without it. As a result, this approach quantifies the relative contributions to N_2O production in terms of ranges (i.e. with minima and maxima) with respect to the nitrifier pathways (chapters 5, 6).

Re (iv): A major advantage of the application of enriched compounds with stable isotope tracing approaches is that the effect of fractionation, i.e. the preferential use of the lighter isotope and residual enrichment of the heavier isotope, becomes negligible. However, eliminating the effect of fractionation entails the addition of NH_4^+ and NO_3^-, which may disrupt the experimental system. This approach would therefore not allow to quantitatively determine the in-situ contribution of N_2O production pathways. In stead, it is designed to

provide an assessment of the significance of the different pathways relative to each other, and across soil types and environmental conditions.

In conclusion, I believe that the above argumentation justifies the assumptions underlying my approach, and that multi-isotope tracing provides a powerful tool to improve our understanding of N_2O production pathways in soil. My study provided the first compelling evidence that, next to 'conventional' nitrification and denitrification, nitrifier denitrification is one of the main pathways of N_2O production in soil.

Alternative origins of N_2O production

Apart from conventional nitrification, denitrification and nitrifier denitrification, a wide variety of processes with the potential to produce N_2O is acknowledged in literature. These include dissimilatory nitrate reduction to ammonia (DNRA) (Smith et al., 1981; Stevens et al., 1998), heterotrophic nitrification, co-oxidation of ammonia by methanotrophs (Yoshinari, 1985; Megraw et al., 1989; Mandernack et al., 2009), aerobic denitrification (Lloyd et al., 1987; Bell et al., 1991; Takaya et al., 2003), fungal denitrification (e.g. Bollag et al., 1972; Shoun et al., 1992; Hayatsu et al., 2008) and co-denitrification (e.g. Garber et al., 1982; Tanimoto et al., 1992b; Laughlin et al., 2002) (Figure 8.3). Even though their environmental significance remains topic of debate, the growing awareness of this variety of processes prompts a survey of our current understanding of these potential contributors to N_2O production.

Dissimilatory nitrate reduction to ammonia forms a distinct pathway in the N cycle. The pathway of DNRA is not well understood but it has been shown that N_2O can be produced during ammonification of NO_3^- (Smith et al., 1981; Stevens et al., 1998). Some studies speculate that DNRA could account for a significant part of NO_3^- reduction, also in soils (Caskey et al., 1979; Bonin et al., 1998; Stevens et al., 1998; Huygens et al., 2007; Wan et al., 2009). Disregarding N_2O production by DNRA would overestimate the contribution of denitrification in isotope tracing studies, including the one in this thesis. Two types of DNRA are recognized: the first is coupled to fermentation, the second to sulphur oxidation (Burgin et al., 2007). Nitrate reduction through fermentative DNRA rather than denitrification is thought to be relatively favored in NO_3^--limited systems

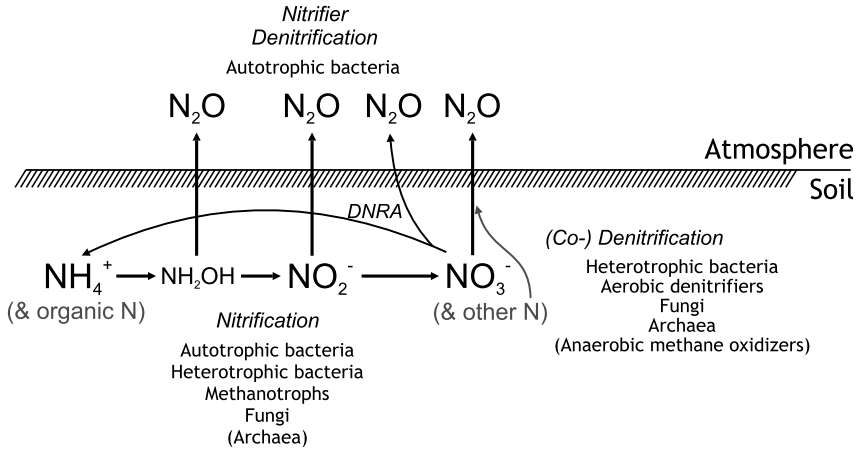

Figure 8.3: Depiction of the diversity of processes and organisms that (may) have the potential to produce N_2O, although their relative significance in soil remains controversial.

(Nijburg et al., 1997; Tiedje 1988) and DNRA coupled to sulphur oxidation is found mainly in aquatic environments (Brettar et al., 1991; Brunet et al., 1996). In our studies, DNRA was therefore unlikely to be significant. This was verified by the insignificant ^{15}N enrichment of the NH_4^+ after application of enriched NO_3^- (chapters 3-6). However, the need remains to check for the absence or presence of DNRA in future N_2O source determination studies. In general, understanding the pathway and role of DNRA in nitrogen cycling remains a future challenge.

Apart from DNRA, most of the above mentioned additional sources of N_2O are distinguished not because they are different biochemical *pathways*, but because they involve different microbial groups capable of similar pathways: i.e. nitrification by heterotrophic bacteria, fungi and methanotrophs, and denitrification by (semi-)aerobic bacteria, fungi, and through co-denitrification. If the substrates and products of these processes are indeed similar, they cannot be individually distinguished with current isotope tracing approaches, including the ones outlined in this thesis. However, various organisms may likely act, react, and be controlled idiosyncratically by environmental factors even while carrying out similar pathways.

Besides autotrophic bacteria, many **heterotrophic bacteria and fungi** are capable of **nitrification** (Robertson et al., 2007; Laughlin et al., 2008). Oxidation of

NH_4^+ by heterotrophic bacteria is thought to be enzymatically similar to that of AOB (Robertson et al., 2007; Hayatsu et al., 2008), and fungi appear to be capable of oxidizing both NH_4^+ and organic N (Robertson et al., 2007; Laughlin et al., 2008). Because autotrophic nitrification is commonly thought to be increasingly inhibited with lower pH (Bock et al., 1986; Haynes, 1986; Stephen et al., 1998), nitrification in acid soils has often been thought to be mainly heterotrophic (De Boer et al., 1991; Paul et al., 1996; Papen et al., 1998). De Boer and Kowalchuk (2001) however stated in a review that although heterotrophs may contribute to some extent, autotrophic bacteria dominate the nitrifying community in soil. Also **methanotrophs** may co-oxidize NH_4^+, resulting in concomitant release of N_2O (Yoshinari, 1985; Megraw et al., 1989; Mandernack et al., 2009). Some studies speculate that the contribution of methanotrophs to nitrification in soil and (freshwater) sediment may be considerable (Roy et al., 1994; Mandernack et al., 2000). Because of their different carbon requirements, the relative contribution of N_2O from autotrophic, methanotrophic, heterotrophic, and fungal nitrification may likely be affected by the supply and quality of SOC. Notably, **Archaea** have been suggested to have a significant role in the NH_4^+ oxidizing community in soils as well (Leininger et al., 2006). Ammonia oxidizing Archaea have not been shown to produce N_2O, but the mechanisms and their contribution to nitrification remain to be further explained (Nicol et al., 2006).

For long, heterotrophic bacteria have been held primarily responsible for denitrification in soils. Next to recognition of denitrification by autotrophic ammonia oxidizers (i.e. nitrifier denitrification), **fungal denitrification** in soil has recently gained more attention (Shoun et al., 1992; Laughlin et al., 2002; Morozkina et al., 2007; Crenshaw et al., 2008; Hayatsu et al., 2008). As fungi often lack N_2O reductase, N_2O appears to be the main product of fungal denitrification (Shoun et al., 1992). This may have important ecological implications as changes in environmental conditions, e.g. nutrient and tillage management, can affect the fungal:bacterial ratio and as such their contribution to total denitrification (Lovell et al., 1995; Frey et al., 1999; Bittman et al., 2005; De Vries et al., 2006). Moreover, O_2 availability has been shown to control fungi and bacteria differently. Where bacterial denitrification generally requires anaerobic conditions and denitrifying enzymes are (from nitrite to nitrous oxide reductase) increasingly inhibited by O_2 (Knowles, 1982; Ferguson, 1994; Zumft, 1997), fungi are reported to be capable of

denitrification under both aerobic and anaerobic conditions and may even need low levels of O_2 (Tanimoto et al., 1992a; Zhou et al., 2001; Hayatsu et al., 2008). Conversely, several **aerobic denitrifying bacteria** have also been identified from diverse ecosystems including soils, suggesting that aerobic denitrification may not be a trivial source of N_2O in soils (Lloyd et al., 1987; Bell et al., 1991; Patureau et al., 2000; Takaya et al., 2003). Another distinguished pathway of N_2O production is **co-denitrification**, where NO_3^- or NO_2^- is combined with other nitrogenous compounds to produce N_2O or N_2. This process is most commonly recognized in denitrifying fungi (Shoun et al., 1992; Tanimoto et al., 1992b; Laughlin et al., 2002; Morozkina et al., 2007), but some studies have also identified bacteria (including actinomycetes) able to carry out co-denitrification (Garber et al., 1982; Kumon et al., 2002). Isotope (^{15}N) labeling studies are suggested to enable the distinction between denitrification and co-denitrification. However, in ecosystems the evident complexity of N-transformations complicates the isolation and discrimination of those two processes from the wide spectrum of other N_2O and/or N_2 producing processes. Adding to the denitrifying community, several **Archaea** have also been shown to carry out dissimilatory reduction of NO_3^- via NO_2^-, NO and N_2O to N_2 (Werber et al., 1978; Volkl et al., 1993; Cabello et al., 2004). This pathway appears similar to the bacterial one (Zumft et al., 2007; Hayatsu et al., 2008), but genome sequencing has revealed differences in the genetic organization, structure and regulation of the genes (Philippot, 2002). Recently, genes encoding for potential homologues of nitrite reductases (NirK) have also been found in ammonia oxidizing Archaea from various environments (including soils) (Bartossek et al., 2010). Altogether the role of Archaea in denitrification and N_2O production in natural ecosystems remains to be elucidated. Even less understood is the process of **denitrification coupled to anaerobic methane oxidation** (i.e. nitrate/nitrite-dependent anaerobic methane oxidation, N-DAMO). Although theoretically feasible, experimental proof and acknowledgement of this process was obtained only recently. Raghoebarsing et al. (2006) identified the first and up to now only microbial consortium that can oxidize methane anaerobically with denitrification serving as electron-acceptor. Understanding the process and ecological significance of N-DAMO, let alone quantifying a potential contribution to N_2O production, is still far from feasible.

While recognizing that in soil ecosystems the role of many of the above

mentioned processes may likely be minor, the above synthesis challenges our conventional understanding of N_2O production. Altogether, the 'conventional' paradigm that addresses 'nitrification and denitrification as main processes of N_2O production in soils' has been attractively simple and convenient, but is no longer be legitimate. Foremost, my research strongly encourages to routinely consider nitrifier denitrification as one of the major sources of N_2O from soil.

Understanding the origin of N_2O and its Oxygen: Future research directions

My research elucidated several aspects of N_2O production in soil, and naturally also raises new questions that point to future research directions. Continued studies on the **process of O exchange** are needed to better understand and account for its control on the O isotopic signature of nitrogen oxides. The occurrence and extent of O exchange in pathways other than NO_3^- reduction to N_2O could not be quantified in my experiments, which imposed assumptions on the data evaluation. Literature contains several studies on O exchange by denitrifiers in pure cultures: future studies could include investigations of O exchange in pure cultures of nitrifiers, ammonia oxidizers as well as nitrite oxidizers. Based on these results, adjusted assumptions could be made on the occurrence of O exchange during nitrifier N_2O production in soil. This would improve the assessment of the relative pathway contributions to N_2O production with the advanced dual isotope approach. Insights in nitrifier-induced O exchange would also be valuable regarding the potential implications for NO_3^- source determination studies. Observations in pure culture studies could further unravel the extent and mechanism of O exchange and its effect on NO_3^-. Ecosystem studies (on e.g. soils, sediments, aquatic systems) could further identify and potentially quantify O exchange with the use of ^{18}O enriched compounds. Effective implications for NO_3^- source determination at natural abundance would need to be assessed subsequently.

Understanding the **pathways of N_2O production** is indispensable for the development of effective mitigation strategies for N_2O emissions to the atmosphere (Mosier et al., 1998; IPCC, 2007). The identification of nitrifier denitrification as distinct major pathway of N_2O production suggests that current

models should adjust their process-based modules and incorporate a more diverse set of N_2O production processes that respond individually to environmental parameters. However, the environmental controls of N_2O production through nitrifier denitrification remain to a large extent unclear. Moreover, with the growing awareness of the wide variety of potential N_2O production processes it is clear that it remains a major challenge to comprehend all pathways and organisms involved. Future research should therefore first be directed to further improve our process-based understanding of N_2O production processes.

Technologies to investigate pathways of N_2O production continue to advance rapidly, including the use of isotopomer ratios and molecular techniques. Analyzing the **isotopomer composition** is increasingly suggested as a promising tool in source determination of N_2O (Schmidt et al., 2004; Toyoda et al., 2005; Sutka et al., 2006; Ostrom et al., 2010). Such an approach evaluates the intramolecular site preference (SP) of the ^{15}N in N_2O, at natural abundance. Where isotope tracing studies need to apply enriched compounds to discount the effect of isotopic fractionation, studying the isotopomer composition can be done without the need to disturb ecosystems with fertilizing compounds. However, ambiguity about the SP for different pathways and microbial communities currently limits the use of isotopomer ratios to assess the contributions of distinct pathways to N_2O production (Schmidt et al., 2004; Well et al., 2006; Ostrom et al., 2007; Ostrom et al., 2010). Future studies could attempt to further characterize distinct SP values and combine this tool with other stable isotope techniques. While recognizing the need for future investigation, recent studies have already suggested the potential of the $\delta^{18}O$/SP fingerprint of N_2O as a tool to identify the dominant production process of N_2O in soil (Well et al., 2008; Well et al., 2009).

Molecular techniques enable to determine the abundance of ammonia oxidizers (AOB and Archaea) and denitrifying bacteria in ecosystems by DNA and mRNA extraction. Successive PCR amplification by specific primers targets the functional genes encoding for specific enzymes that catalyze nitrification and denitrification (Kowalchuk et al., 2001; Philippot, 2002; Wallenstein et al., 2005; Leininger et al., 2006; Sharma et al., 2007). An extensive, solid set of primers is already available, but they do not amplify all variants of the targeted genes (Sharma et al., 2007). Moreover, genes encoding for the enzymes of the

denitrification pathway in ammonia oxidizing bacteria (i.e. for nitrifier denitrification) (Casciotti et al., 2005; Cantera et al., 2007; Garbeva et al., 2007; Norton et al., 2008) and ammonia oxidizing Archaea (Bartossek et al., 2010) appear to be homologous to those in heterotrophic denitrifiers, but it is not clear whether these would be amplified by the same primers. Altogether, such techniques may not cover or differentiate certain distinct pathways. For this, future research could invest in further extension of genetic databases to serve improved primer design, by molecular studies on more diverse pathways and/or organisms involved. Continuous improvement of molecular techniques offers great potential to be combined with stable isotope approaches, to study the relation between microbial community and N_2O production pathways.

To conclude, despite their current constrains, stable isotope, isotopomer, and molecular techniques are promising tools that deserve further development. Their integrated use offers great potential to further unravel the significance and environmental controls of the diverse pathways of N_2O production at a process-based level. A key challenge remains that such process-targeted methodology often sets high specific requirements and/or may influence the system under study. Such technologies currently do not suit the scale and complexity of field studies. Conversely, ecosystem-based approaches allow little in-depth process-based examination of the sources of N_2O production. While 'up-scaling' to increasingly realistic and inherently complex systems, from pure cultures to soil lab-incubations to in-situ field work, we have to settle for a less comprehensive understanding of N_2O production. Ultimately, it is an interdisciplinary research challenge to adopt a complementary approach in search for a joint process- and ecosystem-based understanding on the origin of N_2O.

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Summary

Nitrous oxide (N_2O) is a greenhouse gas that contributes to global warming and to the depletion of stratospheric ozone. To reduce N_2O emissions to the atmosphere it is important to understand how and where it is produced. Currently, many uncertainties remain about the different pathways of N_2O production and their environmental controls. Globally, soils are the major source of N_2O to the atmosphere. With my research I therefore aimed to improve our understanding on the production of N_2O in soil ecosystems. Specifically, the main objective was to identify the presence and prospective contribution of 'nitrifier denitrification' as a distinct N_2O production pathway. Nitrifier denitrification is the stepwise reduction of nitrite (NO_2^-) to N_2O and N_2 by ammonia oxidizing bacteria. In pure culture studies, the potential of these nitrifiers to produce N_2O through this pathway had been well studied. However, experimental proof of the presence of nitrifier denitrification in actual soils remained inconclusive due to the lack of adequate methodology.

A novel dual isotope approach was suggested to enable the distinction of nitrifier denitrification from the conventionally considered two main pathways of N_2O production in soil, nitrification and denitrification. This methodology is based on tracing stable isotopes of oxygen (O) and nitrogen (N) (^{18}O and ^{15}N respectively) from enriched compounds (^{18}O water and ^{15}N ammonium and/or nitrate) into N_2O. Methodology based on ^{15}N tracing has been well-established, but does not enable the distinction between N_2O from nitrifier denitrification and nitrification, as the N_2O-N is in both pathways derived from ammonium. The O in N_2O originates both from O_2 and water (H_2O). Reaction stoichiometry shows that the relative contribution of O_2 and H_2O to the total O in N_2O differs between the pathways of N_2O production. The suggested dual isotope approach was based on the general understanding that next to reaction stoichiometry only isotopic fractionation would affect the O isotopic signal of N_2O. The use of isotopically enriched compound enables to discard the latter effect and to distinguish the relative contributions of the production pathways based on tracing the ^{18}O from H_2O into N_2O.

However, shortly after the start of my research I realized that there might be an additional defining factor of the O isotopic signature of the O in N_2O: oxygen exchange between H_2O and intermediates of the N_2O production pathways. Throughout this thesis, 'oxygen (or O) exchange' is used as short for the exchange

of O between nitrogen oxides and H_2O. As a result of such exchange, relatively more H_2O-O could end up in the N_2O than based on reaction stoichiometry alone. Ignoring O exchange would distort the interpretation of the pathways' relative contributions to N_2O production based on the O isotope signatures.

Against the background of the original main objective, but acknowledging the potential methodological constraints of the dual isotope approach caused by O exchange, the (revised) objectives of my PhD research have been:

(i) to study, identify and quantify the process of O exchange between H_2O and intermediates of the N_2O production pathways, and its effect on the O isotopic signature of N_2O from soil;
(ii) to develop and apply an advanced O and N isotope tracing approach that *could* distinguish nitrifier denitrification from 'conventional' nitrification and denitrification in soil-based studies; and
(iii) to evaluate the significance and idiosyncratic character of nitrifier denitrification as production pathway of N_2O in soil.

In chapter 2 I started my studies on O exchange by reviewing literature on the potential occurrence of O exchange. In many pure culture studies on both nitrifiers and denitrifiers, significant rates of O exchange have been reported. Although hardly considered in ecosystem studies, O exchange could therefore very likely be present in soil and aquatic environments. I concluded that the use of the O isotopic signature in source determination of N_2O, and potentially other nitrogen oxides as well, should therefore be adopted with extreme caution.

I subsequently studied the process of O exchange experimentally, on a variety of soils from across Europe. In chapter 3 I showed that O exchange can indeed strongly determine the O isotopic composition of N_2O. First, I identified O exchange by studying the incorporation of ^{18}O from H_2O into N_2O. In all soils, the amount of O in N_2O derived from H_2O exceeded the amount that could be explained by reaction stoichiometry alone. Second, I quantified O exchange during denitrification of NO_3^- after examining the recovery of ^{18}O from applied ^{18}O enriched NO_3^- into the produced N_2O. In the absence of O exchange, the ratio of the ^{18}O and ^{15}N enrichment of NO_3^- should be retained in the N_2O. However, the ^{18}O:^{15}N enrichment ratio retention, the 'ERR', revealed that the enrichment of

the ^{18}O has strongly declined relative to the ^{15}N. With the loss in ERR I quantified that during the reduction of NO_3^- to N_2O, up to 97% of the NO_3^--O had exchanged with (non-enriched) O from H_2O.

The ERR approach quantified the O exchange for the pathway of denitrification. In chapter 4, I further examined the O isotopic signature of the N_2O from my labeling experiments to assess the potential presence of O exchange during the *other* N_2O production pathways. Assuming the presence or absence of O exchange under a series of scenarios, I evaluated the observed N_2O-O isotopic signature. This assessment revealed that the O exchange during the reduction of NO_3^- alone could not fully explain the observed ^{18}O enrichment of the N_2O: during other pathways of N_2O production, additional O exchange with H_2O must have occurred. Nitrifiers could thus mediate O exchange as well, during nitrifier denitrification, nitrite oxidation to nitrate, or both.

In chapter 5 I developed and applied an advanced dual isotope approach, with the aim to discriminate nitrifier denitrification from 'conventional' nitrification and denitrification as pathways of N_2O production in soil. This approach integrates the quantified O exchange during denitrification and anticipates on the additional presence of O exchange with ^{18}O and ^{15}N isotope tracing. The remaining uncertainty about the presence and extent of O exchange is controlled by adopting various assumptions. As a result, the contribution of the different nitrifier pathways (including nitrifier denitrification) could not be narrowed down to one number. However, it did enable to identify a minimum to maximum range of the contribution of nitrifier denitrification to the total N_2O production. With a minimum of zero, the presence nitrifier denitrification would not be conclusively proven. Yet, a minimum contribution of nitrifier denitrification (larger than zero) was quantified for multiple soils, and thus identified that nitrifier denitrification can indeed be a production pathway of N_2O in soils. In these experiments, the soils were studied under relative high moisture conditions (80% WHC). This likely explains why in those experiments total N_2O production was dominated by NO_3^- driven denitrification. Consequently, the identified minimum contribution of nitrifier denitrification remained low for most soils (over 60% of total N_2O for one soil, but less than 10% for the others). However,

actual contributions may likely have been larger, as this minimum was defined under rather extreme assumptions. Moreover, assessment of the total nitrifier-derived N_2O revealed that the contribution of nitrifier denitrification strongly dominated over that of 'conventional' nitrification.

Environmental controls of N_2O production may likely affect the individual pathways differently. In chapter 5 I evaluated soil pH and C content as possible predictors of the relative pathway contributions to N_2O production. Relative contributions of NH_4^+ and NO_3^- derived N_2O showed to be related with both soil pH and soil C content. However, with the small contribution of NH_4^+ (i.e. total nitrifier contribution) to total N_2O and the ability to only partially quantify the different nitrifier pathways, the contribution of nitrifier denitrification was not distinct enough to evaluate its individual relation with the diversity in pH and soil carbon. However, both in chapter 5 and in my discussion I speculate how nitrifier denitrification may likely respond idiosyncratically to these soil parameters.

In chapter 6 I similarly studied the N_2O production pathways, this time on a single soil at three different moisture levels. Under slightly more moderate moisture conditions in these experiments (50% and 70% WHC), nitrifier denitrification was responsible for more of the total N_2O than 'conventional' denitrification of NO_3^-. Nitrifier denitrification was shown to constitute a significant contribution to soil-derived N_2O. Moreover, with this experiment I demonstrated that nitrifier denitrification indeed responds idiosyncratically to soil moisture content. Compared to 'conventional' denitrification, nitrifier denitrification was less repressed by aerobic conditions. Under relatively moderate moisture conditions, N_2O production by nitrifier denitrification was likely equally significant as N_2O from 'conventional' nitrification.

Next to N_2O, also for nitrate (NO_3^-) the O isotopic signature is commonly used to evaluate its sources and cycling in ecosystems. In chapter 7 I therefore carried out an exploratory study on the potential effect of O exchange on NO_3^-. In this experiment I observed a decrease in the ^{18}O enrichment of the NO_3^-, while the ^{15}N enrichment did not significantly change over the incubation period. This

demonstrates that O exchange might indeed affect the O isotopic signature of NO_3^- in soil.

In conclusion, my studies established that O exchange between H_2O and intermediates of N_2O production processes is a defining factor of the O isotopic signature of N_2O and probably NO_3^- as well. This evidently constitutes implications for source determination studies of N_2O and NO_3^- that are based on the interpretation of the O isotopic signature. Taking the effect of O exchange into account, I developed a novel dual isotope tracing approach to study pathways of N_2O production. Subsequently, my studies identified the presence, significance, and idiosyncratic character of nitrifier denitrification as production pathway of N_2O in soil. The acknowledgement of nitrifier denitrification as distinct N_2O production pathway in soil is an important step forward in our understanding of N_2O production to ultimately enable the development of accurate inventories and effective mitigation strategies for N_2O emissions.

S

Samenvatting

Lachgas (N_2O) is een broeikasgas dat bijdraagt aan de opwarming van de aarde en de afbraak van ozon in de stratosfeer. Om emissies van N_2O terug te dringen zullen we moeten begrijpen hoe en waar het ontstaat. Er is echter veel onduidelijkheid over de verschillende manieren waarop N_2O wordt gevormd, en hoe deze processen worden beïnvloed door de omgeving. Wereldwijd vormen bodems de grootste bron van lachgas naar de atmosfeer. Met mijn onderzoek probeer ik daarom een beter inzicht te krijgen in de productie van N_2O in bodems. Mijn hoofddoel was om de bijdrage van 'nitrifier denitrification' als afzonderlijk proces te bestuderen. In dit proces wordt nitriet (NO_2^-) omgezet in N_2O door ammonia oxiderende bacteriën (AOB), die normaliter juist NO_2^- vormen vanuit ammonia (NH_3). Het reducerende proces tot N_2O wordt normaal gesproken voornamelijk toegeschreven aan andere organismen, de denitrificeerders. 'Nitrifier denitrification' is in studies met reinculturen van AOB echter al enige tijd erkend. Maar doordat onderzoekstechnieken ontoereikend bleken is het tot op heden onduidelijk gebleven of dit proces ook in bodems plaatsvindt.

Recent is er een nieuwe methode ontwikkeld om nitrifier denitrification te onderscheiden van nitrificatie en denitrificatie, traditioneel de twee belangrijkst geachte N_2O vormende processen in de bodem. Deze methode bestudeert de stabiele isotopen van zuurstof (O) en stikstof (N) (respectievelijk ^{18}O en ^{15}N) in N_2O. Het ^{15}N 'signaal' wordt al regelmatig gebruikt, maar kan niet het onderscheid tussen N_2O uit 'nitrifier denitrification' en nitrificatie maken, omdat voor beide processen de N afkomstig is van ammonia. De herkomst van de O in N_2O is wel verschillend voor deze processen en wordt volgens de reactievergelijkingen in verschillende verhoudingen geleverd door zuurstofgas (O_2) en water (H_2O). Verschil in het ^{18}O signaal van O_2 and H_2O resulteert daarmee in verschil in het ^{18}O signaal van N_2O. Volgens deze nieuwe methode zou daarmee de bijdrage van de verschillende processen aan de totale N_2O productie onderscheiden kunnen worden.

Echter, kort na de start van mijn onderzoek kwam ik tot de ontdekking dat nog een ander proces invloed kan hebben op het ^{18}O signaal van N_2O: uitwisseling van O tussen H_2O en tussenproducten van de reacties die N_2O vormen, kortweg 'zuurstof (of O) uitwisseling'. Door zuurstof uitwisseling kan er meer O van H_2O in N_2O terecht komen dan men zou verwachten op basis van de

reactievergelijking. Als dit effect wordt genegeerd zal de bijdrage van de verschillende processen aan de N_2O vorming verkeerd worden geïnterpreteerd. Met aandacht voor de beperking van O uitwisseling voor de voorgestelde nieuwe methode werden de belangrijkste doelen van mijn onderzoek:

(i) het bestuderen van O uitwisseling tijdens de vorming van N_2O in de bodem, en het aantonen en kwantificeren van het effect ervan op het O isotopen signaal van N_2O;
(ii) het ontwikkelen van een aangepaste methode om met behulp van O en N isotopen onderscheid te maken tussen de N_2O vormende processen in de bodem;
(iii) het in kaart brengen van de bijdrage en unieke karakter van 'nitrifier denitrification' als N_2O vormend proces in de bodem.

Mijn onderzoek begint in hoofdstuk 2 met een literatuurstudie naar zuurstof uitwisseling. Verscheidene studies tonen aan dat O uitwisseling kan plaatsvinden met reincultures van zowel nitrificeerders als denitrificeerders. Hoewel er in studies in bodem en aquatische systemen nauwelijks rekening mee wordt gehouden, zou O uitwisseling dus wel degelijk kunnen voorkomen in natuurlijke systemen. Ik concludeerde dat men bij het gebruik van het ^{18}O signaal om de bronnen van N_2O te onderscheiden zeer alert moet zijn op het mogelijke effect van O uitwisseling.

Vervolgens bestudeerde ik O uitwisseling daadwerkelijk in experimenten met verschillende bodems. In hoofdstuk 3 laat ik zien dat O uitwisseling inderdaad een groot effect heeft op het ^{18}O signaal van N_2O. Ten eerste bewees de hoge verrijking van ^{18}O in N_2O na toevoeging van ^{18}O-verrijkt H_2O dat uitwisseling plaats moest hebben gevonden. Ten tweede kon ik de uitwisseling tijdens de omzetting van nitraat (NO_3^-) naar N_2O kwantificeren door ^{18}O en ^{15}N verrijkt NO_3^- te gebruiken. Het verlies van ^{18}O in verhouding tot ^{15}N na de omzetting tot N_2O liet zien dat in sommige gronden bijna alle ^{18}O in nitraat was verwisseld voor (niet-verrijkte) O uit H_2O.

Deze benadering stelde mijn in staat om O uitwisseling tijdens de omzetting van nitraat (NO_3^-) naar N_2O, ofwel denitrificatie, te kwantificeren. In hoofdstuk 4 beschrijf ik hoe het O signaal van de N_2O verdere informatie geeft over O uitwisseling gedurende andere N_2O vormende processen. Ik bereken voor

verschillende scenario's waarin ik in meer of mindere mate rekening hou met O uitwisseling wat het verwachte ^{18}O signaal van N_2O zou zijn, en vergelijk dit met het gemeten signaal. Dit liet zien dat O uitwisseling niet alleen tijdens denitrificatie moet hebben plaatsgevonden, maar ook gedurende nitrificatie en/of nitrifier denitrification.

In hoofdstuk 5 gebruik ik de opgedane kennis over O uitwisseling om een aangepaste methode te ontwikkelen die met gebruik van ^{18}O en ^{15}N alsnog N_2O productie uit nitrifier denitrification kan onderscheiden van nitrificatie en denitrification. Omdat ik daarnaast nog een aantal aannames moet doen kan de bijdrage van nitrifier denitrification niet exact worden gekwantificeerd, maar wel met een marge (een minimum en maximum). Met deze vernieuwde aanpak bestudeerde ik 12 verschillende Europese gronden. De minimum bijdrage van nitrifier denitrification aan N_2O productie was in meerdere van deze gronden groter dan nul. Met andere woorden: hier toon ik voor het eerst aan dat nitrifier denitrification inderdaad plaats kan vinden in de bodem. Over het algemeen leek de relatieve bijdrage van nitrifier denitrification klein ten opzichte van klassieke denitrificatie, maar het was duidelijk hoger dan N_2O productie uit nitrificatie.

Factoren die bepalend zijn voor de productie van N_2O beïnvloeden de afzonderlijke processen wellicht in verschillende mate en op verschillende manieren. In hoofdstuk 5 onderzoek ik daarom ook of de pH en het koolstof (C) gehalte van de bodem de verschillen in de bijdrage van de afzonderlijke processen kan verklaren. De relatieve bijdrage van ammonium (NH_4^+) en nitraat (NO_3^-) bleken gerelateerd aan de pH en het C gehalte van de bodem. Echter, de totale bijdrage van NH_4^+ (nitrificatie én nitrifier denitrificatie) bleef zoals gezegd klein, waardoor de specifieke bijdrage van nitrifier denitrification niet groot genoeg was om een relatie met pH en/of C verder te beoordelen.

In hoofdstuk 6 bestudeer ik op vergelijkbare wijze de lachgasproductie, maar dit keer gebruikte ik slechts één grond om het effect van verschil in vochtgehalte te onderzoeken. Het hoge vochtgehalte in voorgaande experimenten was wellicht de oorzaak van de relatief kleine bijdrage van NH_4^+, en dus van nitrificatie en nitrifier denitrification. In de bodems in dit experiment die iets minder vochtig waren was de bijdrage van nitrifier denitrification aan de N_2O beduidend groter, en belangrijker dan klassieke denitrificatie. Dit toont ook aan dat nitrifier

denitrification idiosyncratisch (dus op unieke wijze) beïnvloed wordt door omgevingsfactoren zoals vochtgehalte: onder (relatief) drogere omstandigheden nam N_2O productie door klassieke denitrificatie van NO_3^- veel sterker af dan productie via nitrifier denitrification. Onder dergelijke omstandigheden was de bijdrage aan de totale N_2O productie van nitrificatie en nitrifier denitrification (beide afkomstig van NH_4^+) van vergelijkbare grootte, terwijl nitrificatie verwaarloosbaar was ten opzichte van nitrifier denitrification onder het hoge vochtgehalte.

Het O isotopen signaal wordt niet alleen gebruikt om de oorsprong van N_2O te bepalen; dit gebeurt ook voor nitraat (NO_3^-) in bijvoorbeeld oppervlakte- en grondwater. In hoofdstuk 7 presenteer ik daarom een verkennende studie naar een mogelijk effect van zuurstof uitwisseling op het zuurstof isotopen signaal van NO_3^-. In dit experiment constateerde ik dat het ^{18}O signaal van NO_3^- in de bodem significant was afgenomen na 24 uur, terwijl het ^{15}N signaal onveranderd was. Dit impliceert dat zuurstof uitwisseling inderdaad ook bepalend zou kunnen zijn voor het isotopen signaal van NO_3^-.

Samengevat laat ik met mijn onderzoek zien dat uitwisseling van zuurstof tussen water en tussenproducten van lachgasproductie een belangrijke factor is in het bepalen van het uiteindelijke zuurstof isotopen signaal van lachgas (N_2O), en wellicht ook van nitraat (NO_3^-). Dit heeft gevolgen voor onderzoek naar de herkomst van N_2O en NO_3^-, omdat het zuurstof isotopen signaal daarin vaak gebruik wordt als indicator. Ik introduceerde een nieuwe methode om lachgasproductie te bestuderen waarin het effect van zuurstofuitwisseling expliciet wordt meegenomen. Daarmee heb ik 'nitrifier denitrification' als N_2O vormend proces kunnen onderscheiden van nitrificatie en denitrificatie, en voor het eerst kunnen aantonen dat ook 'nitrifier denitrification' een belangrijke bijdrage kan leveren aan de N_2O emissie uit de bodem. Ook liet ik zien dat 'nitrifier denitrification' anders wordt beïnvloed door omgevingsfactoren dan andere N_2O vormende processen. De bevestiging dat nitrifier denitrification een afzonderlijk en belangrijk proces van lachgasproductie in de bodem is draagt bij aan ons begrip van de herkomst van lachgas. Inzicht in de vorming van lachgas is essentieel om uiteindelijk de emissies van dit broeikasgas effectief terug te kunnen dringen.

Dankwoord

Het zal de meesten van jullie niet ontgaan zijn dat ik de afgelopen 4 jaar met heel veel plezier aan mijn promotieonderzoek heb gewerkt, met dit boekje als resultaat. Echter, vele handen maken licht werk, en 'mijn' proefschrift was dan ook nooit tot stand gekomen zonder de vele geweldige mensen om me heen. Dit dankwoord is niet genoeg, maar in ieder geval een begin om jullie allemaal enorm te bedanken voor jullie support, in welke vorm dan ook!

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Zechmeister, Dick Brus, Chris Van Kessel: Thank you so much for all your effort, input and concern to improve our work! It was sincerely a great pleasure to work with you all. Dave Harris, I like to specifically express additional thanks to you and your team at the UC-Davis Stable Isotope Facility for analyzing the bulk of my samples. Also Tim Clough (and family), thank you very much for the fantastic opportunity and the wonderful time during my visit to your lab at Lincoln University.

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Ook buiten het werk om zijn natuurlijk vele mensen om me heen enorm belangrijk geweest voor het plezier waarmee ik mijn promotietijd heb beleefd. Het is geweldig om de betrokkenheid van jullie allemaal, dichter bij en verder weg, te ervaren. Linda, Linda, Mirjam, Laura, Floor en Johanna: ook al zijn de meesten Wageningen al ontvlogen, we blijven lekker bijkletsen. Tess, het was top om samen in Davis te beginnen en als startende aio's een huisje te delen. Mieke, het was en blijft heerlijk om er af en toe met één dagje wandelen er helemaal tussenuit te gaan. Titia, jammer dat we niet meer regelmatig een balletje kunnen slaan, maar het blijft super om te kletsen en het zó met elkaar eens te kunnen zijn. Maartje, ik trotseer met veel plezier nog vaak samen de regen, ik vind het super

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Curriculum vitae and publications

Curriculum vitae

Dorien Kool was born in Oosterhout, the Netherlands, on September 6th 1982. She completed her secondary (VWO) in 2000, and in the same year she moved to Wageningen to study 'Soil, Water and Atmosphere'. She specialized in soil science for her BSc. For her first MSc thesis project she studied the oxidation and compaction of tropical peat land in Kalimantan, Indonesia, in cooperation with the BOS Foundation. She worked at Alterra (Wageningen) for her second MSc thesis, on emissions of nitrous oxide (laughing gas) from cattle urine deposition. To finalize her studies she did her internship at the University of California in Davis, where she did research on soil carbon saturation under elevated levels of atmospheric CO_2. In March 2006 she received her MSc degree (*cum laude)* in Soil Science and in Earth System Science. Caught by the ironically serious subject of her second MSc project Dorien started her PhD research on nitrous oxide. During her PhD she received the Best Publication Award 2009 of graduate school PE&RC for her publication in Rapid Communications in Mass Spectrometry, and the Schulzman Award for her presentation at the AGU Fall Meeting 2009.

Dorien Kool werd geboren in Oosterhout, op 6 september 1982. Ze rondde haar atheneum af in 2000 en verhuisde hetzelfde jaar naar Wageningen voor de studie 'Bodem, water en atmosfeer'. Al snel koos ze voor de specialisatie bodemkunde. Voor haar eerste afstudeerproject bestudeerde ze de bodem van een tropisch veengebied in Kalimantan, Indonesië, samen met de BOS Foundation. Ze werkte bij Alterra in Wageningen aan haar tweede afstudeeronderzoek, naar lachgas-emissies uit urineplekken. Ter afsluiting van haar studie liep ze stage bij de Universiteit van Californië in Davis, waar ze onderzoek deed naar koolstof-verzadiging in de bodem onder verhoogde atmosferische CO_2 concentraties. In maart 2006 ontving ze haar diploma (*cum laude*) voor de studies Bodemkunde en Aardsysteemkunde. Geboeid door het toch serieuze onderwerp van haar afstudeerscriptie vervolgde Dorien haar onderzoek naar lachgas. Voortvloeiend uit haar promotieonderzoek ontving ze de Best Publication Award 2009 van onderzoeksschool PE&RC voor haar artikel in Rapid Communications in Mass Spectrometry, en de Schulzman Award voor haar presentatie op de conferentie van de AGU in 2009.

List of publications

Kool DM, Wrage N, Oenema O, Van Kessel C, Van Groenigen JW. Oxygen exchange with water alters the isotopic signature of nitrate in soil ecosystems. Submitted.

Kool DM, Dolfing J, Wrage N, Van Groenigen JW. Nitrifier denitrification as a distinct and significant source of N_2O from soil. Submitted.

Kool DM, Wrage N, Zechmeister-Boltenstern S, Pfeffer M, Brus D, Oenema O, Van Groenigen JW (In press). Nitrifier denitrification can be a source of N_2O from soil: a revised approach to the dual isotope labelling method. European Journal of Soil Science, doi: 10.1111/j.1365-2389.2010.01270.x.

Kool DM, Wrage N, Oenema O, Harris D, Van Groenigen JW (2009). The ^{18}O signature of biogenic nitrous oxide is determined by O exchange with water. Rapid Communications in Mass Spectrometry 23, 104-108.

Kool DM, Müller C, Wrage N, Oenema O, Van Groenigen JW (2009). Oxygen exchange between nitrogen oxides and H_2O can occur during nitrifier pathways Soil Biology and Biochemistry 41, 1632-1641.

Kool DM, Wrage N, Oenema O, Dolfing J, Van Groenigen JW (2007). Oxygen exchange between (de)nitrification intermediates and H_2O and its implications for source determination of NO_3^- and N_2O: a review. Rapid Communications in Mass Spectrometry 21, 3569-3578.

Kool DM, Chung H, Tate KR, Ross DJ, Newton PCD, Six J (2007). Hierarchical saturation of soil carbon pools near a natural CO_2 spring. Global Change Biology 13:1282-1293.

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PE&RC Education Certificate

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Microbial sources of N_2O: the contribution of nitrifier denitrification (2006)

Writing of project proposal (4.5 ECTS)

- Microbial sources of N_2O: the contribution of nitrifier denitrification (2006)

Post-graduate courses (6 ECTS)

- NitroEurope summer school 'Microbial nitrogen turnover and production of greenhouse gases', Vienna, Austria (2006)
- Advanced statistics, Wageningen, the Netherlands (2007)
- Postgraduate course 'Biodiversity and ecosystems services', Wageningen, the Netherlands (2008)

Laboratory training and working visits (4.5 ECTS)

- Visit to Dr. N. Wrage, University of Gottingen, Germany (2008)
- Visit to Dr. D. Harris, Stable Isotope Facility, University of California-Davis, USA (2008)
- Visit to Dr. T. Clough, Lincoln University, New Zealand (2010)

Invited review of (unpublished) journal Manuscript (7 ECTS)

- Reviewer of seven articles submitted to international scientific journals Soil Biology & Biochemistry, Rapid Communications in Mass Spectrometry, Geoderma, Global Change Biology (2007-2010)

Deficiency, refresh, brush-up courses (1.5 ECTS)

– Basic statistics (2006)

Competence strengthening, skills courses (6.4 ECTS)
- PhD competence assessment (2006)
- MSc thesis supervision (2008)
- Communicating your science to the public (2008)
- NWO talent day (2009)
- Teaching methodology and skills for PhD students (2009)
- PhD course 'Mobilizing your scientific network' (2009)
- PhD course 'Career perspectives' (2009)
- NWO talent class 'Subsidies aanvragen' (2010)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.9 ECTS)
- PE&RC days 2007-2009

Discussion groups, local seminars, other scientific meetings (6.4 ECTS)
- CPN discussion group (2006-2007)
- PE&RC discussion group Climate change & soil-water-vegetation interaction (2007-2010)
- COST-SIBAE workshop on stable isotopes in biosphere-atmosphere-earth, Lisbon, Portugal (2009)

International symposia, workshops and conferences (25.3 ECTS)
- Annual meetings of the NitroEurope IP; oral presentations (2006 - 2010).
- DIARP workshop, Wageningen, the Netherlands; oral presentation (2007)
- 15th Nitrogen workshop, Lleida, Spain; oral presentation (2007)
- 13th Meeting of COST-action on denitrification, Llubjana, Slovenia; poster (2007)
- ASA-CSSA-SSSA annual meeting, New Orleans (LA), USA; oral presentation (2007)
- IsoEcol 6, Honolulu (HI), USA; poster (2008)
- AGU Fall meeting 2008, San Francisco (CA), USA; oral presentation (2008)
- Soil Organic Matters meeting, Rothamsted, United Kingdom; oral presentation (2009)
- AGU Fall meeting 2009, San Francisco (CA), USA; oral presentation (2009)

Lecturing, supervision of practical's, tutorials (2.4 ECTS)
- BSc course 'Biological interactions in soils', practical supervisor (2008)
- BSc thesis supervision, daily supervisor (2009)