# Nitrous oxide emission hotspots and acidic soil denitrification in a riparian buffer zone

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2010

Van den Heuvel, Ronald Niels (2010) Nitrous oxide emission hotspots and acidic soil denitrification in a riparian buffer zone. Ph.D. thesis, Utrecht University, Faculty of Science, 128pp.

ISBN/EAN: 9789-90-5335-286-1

Cover design: Ridderprint Offsetdrukkerij BV and Ronald van den Heuvel
Printing: Ridderprint Offsetdrukkerij BV Ridderkerk

# Nitrous oxide emission hotspots and acidic soil denitrification in a riparian buffer zone

Hotspots van lachgas uitstoot en zure bodem denitrificatie in een beekbegeleidende buffer strook
(met een samenvatting in het Nederlands)

**Proefschrift**

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op maandag 14 juni 2010
des middags te 4.15 uur

door

**Ronald Niels van den Heuvel**

geboren op 9 mei 1982 te Voorthuizen

| | |
|---|---|
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# Table of contents

# Chapter 1

# General introduction

**Harmful effects of nitrous oxide ($N_2O$) on climate and ozone layer**

Currently, climate change attracts widespread public attention and international concern. An increase in temperature of about 0.6°C of the atmosphere was observed in the 20th century (Brohan et al. 2006). Other (regional) changes in the past century included increased drought, cyclone activity and heavy precipitation events (IPCC 2007). It is generally assumed that the increase of gases with a positive radiative forcing (greenhouse gases) is responsible for the increase in temperature and other changes of the climate (Hegerl et al. 2007). Dependent on concentration, relative radiative forcing and atmospheric lifetime, $CO_2$, $CH_4$ and $N_2O$ are the most powerful greenhouse gases that have increased as a result of anthropogenic activities. $N_2O$ is estimated to contribute approximately 8% to the enhanced greenhouse effect (IPCC 2007).

Another important harmful effect of $N_2O$ is its capacity to deplete stratospheric ozone which absorbs most of the biologically destructive high frequency ultra violet light. Crutzen (1970) showed that stratospheric ozone concentrations are controlled by nitrogen oxides (NO and $NO_2$) among other gases. $N_2O$ breaks down to NO and $NO_2$ in the atmosphere and can thus play an important role in the depletion of stratospheric ozone. Unfortunately, the stratospheric ozone concentration is decreasing (Molina and Rowland 1974). Other important anthropogenic ozone depleting gases, especially chlorofluorocarbons (CFCs) have decreased since 1987 through regulations on the use and emission of these gases in the Montreal Protocol, but $N_2O$ has not been regulated in this protocol (UNEP, 2009). Recently Ravishankara et al. (2009) showed that $N_2O$ is now the dominant ozone depleting gas and most likely will be for the rest of the 21st century.

Considering the detrimental effects of $N_2O$ on climate and ozone layer, atmospheric concentrations should ideally be maintained at a low level. However, the concentration is rapidly increasing since the industrial revolution (Figure 1). Before the industrial revolution, $N_2O$ sources and sinks were more or less balanced. Increases in $N_2O$ emissions due to anthropogenic activities are responsible for the surplus of emission and therefore contribute significantly to the increase of $N_2O$ in the atmosphere.

**Emission sources of nitrous oxide**

In order to decrease (or balance) atmospheric $N_2O$ concentration, emissions should be reduced. To take appropriate measures, an accurate quantification of the emission sources should be made. Until recently, such a detailed quantification of individual sources

was absent (Bouwman et al. 1995), but the number of published $N_2O$ emission measurements is increasing steadily (Stehfest and Bouwman 2006) making it possible to pin point important sources. Already in 1946, it was suggested that soils could be a source of atmospheric $N_2O$ (Adel 1946). On a global scale, the most important sources of $N_2O$ emissions are: energy/transport (1%), industry (3%), agriculture (16.5%), aquatic ecosystems (35.5%) and natural soils (43.5%) (Seitzinger et al. 2000). Emissions from soils (Bouwman et al. 2002) and water bodies (Naqvi et al. 2000) have increased since the industrial revolution due to the application of increasing amounts of industrial fixed nitrogen in manure and fertilizers on a much larger scale than before. It is now estimated that anthropogenic fixed nitrogen contributes to about 40% of the global nitrogen input (Galloway et al. 2008)

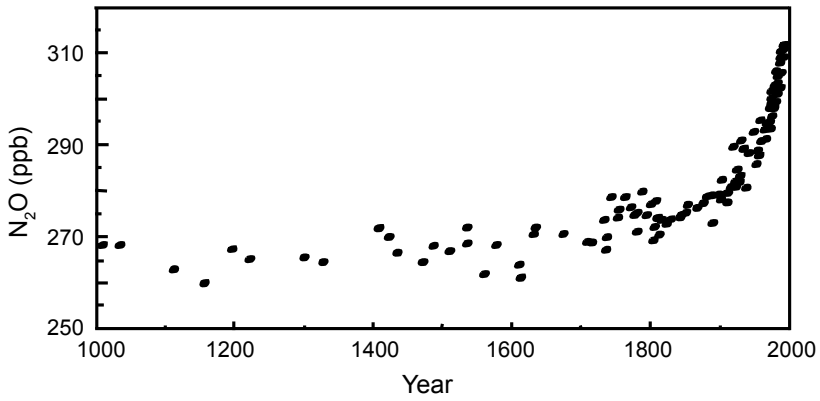


**Figure 1: Atmospheric $N_2O$ concentrations (in ppb) over time (Ehhalt et al. 2001).**

**Soil nitrogen cycle**

The soil microbial nitrogen cycle consists of several processes of which some produce $N_2O$. Nitrification and denitrification are considered to be the main sources of $N_2O$ production in soils (Bremner 1997, Wrage et al. 2001).

Nitrification is a two-step process. First ammonium is oxidized to nitrite, and subsequently nitrite is oxidized to nitrate. Ammonium, and in the second step nitrite, serve as the electron donors for the chemolithoautotrophic microbes catalyzing these processes, while oxygen serves as the electron acceptor. Therefore this process requires oxic conditions. $N_2O$ can be formed as a by-product of ammonium oxidation, which seems to be enhanced by oxygen limiting conditions (Tallec et al. 2006a). In soils with high nitrification rates, significant amounts of $N_2O$ can be produced and may subsequently be emitted. Nitrification-dominated emissions of $N_2O$ from agricultural soils are generally related to fertilizer application and low moisture contents (e.g. Klemedtsson et al. 1988a, Stevens et

al. 1997). Aerobic ammonium oxidizing bacteria (AOB) have another mode of operation: so-called nitrifier denitrification. During this alternative metabolism of AOB, $NH_4^+$ is oxidized to $NO_2^-$ which is subsequently reduced to $N_2O$ or $N_2$ (Bock et al. 1995, Schmidt et al. 2001a, Wrage et al. 2001). $N_2O$ can be produced during both steps of this process and seems to be especially promoted under oxygen limiting (Poth and Focht 1985). The importance of this process in soil $N_2O$ production can be significant (Webster and Hopkins 1996a), but unfortunately, there is only a small number of studies that verify a potential importance of nitrifier denitrification to the $N_2O$ production in a wider range of soils.

Denitrification is a four-step process, defined as the reduction of $NO_3^-$ via the obligatory intermediates $NO_2^-$, NO and $N_2O$ to $N_2$. Alternatively, one or several of the reduction steps have also been defined as denitrification. In this thesis, the term denitrification is used for the reduction of $NO_3^-$ to $N_2O$ or $N_2$. The fact that $N_2O$ is an intermediate of denitrification has important implications when studying soil $N_2O$ emissions. First, instead of $N_2$, $N_2O$ can be the primary end product under certain circumstances (Wijler and Delwiche 1954). Second, $N_2O$ may be reduced via denitrification to $N_2$ resulting in $N_2O$ consumption in soils (e.g. Goldberg and Gebauer 2009). The net $N_2O$ production will be strongly affected by the rate of further reduction to $N_2$ and therefore the $N_2O$:$N_2$ product ratio is an important parameter in determining $N_2O$ production originating from denitrification. Net $N_2O$ production by denitrification in soils has already been reported by Wijler and Delwiche in 1954. Later on denitrification was shown to be the dominant $N_2O$ producing process for a wide variety of ecosystems (Wrage et al. 2004a, Ambus et al. 2006). Especially in anaerobic soils containing high nitrate concentrations, denitrification is mostly assumed to be the main source of $N_2O$ emission (e.g. Hefting 2003).

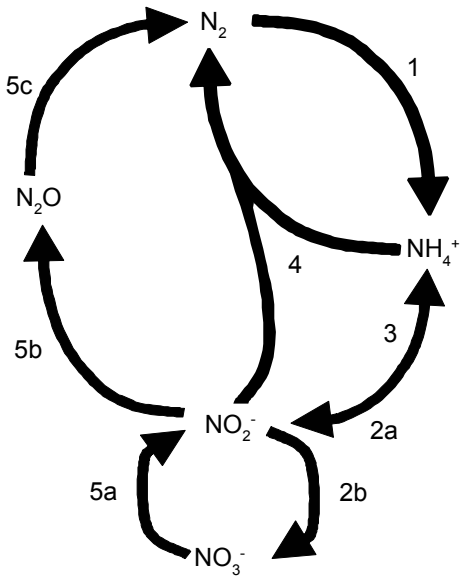


**Figure 2: Schematic microbial nitrogen cycle. Arrows depict possible soil transformations from one form of nitrogen to another. 1: Nitrogen fixation, 2: Nitrification, 2a: Ammonium oxidation, 2b: Nitrite oxidation, 2a + 5b and 5c: Nitrifier denitrification, 5a + 3: Dissimilatory Nitrate Reduction to Ammonium, 4: Anaerobic ammonium oxidation (anammox), 5: Denitrification.**

Other nitrogen transformations of the N-cycle are nitrogen fixation, anaerobic ammonium oxidation (anammox) and dissimilatory nitrate reduction to ammonia (DNRA) (Jetten 2008). To the best of our knowledge, these processes do not produce significant amounts of $N_2O$ (Kartal et al. 2007, Zhong et al. 2009). High rates of these processes may however influence nitrification and (nitrifier) denitrification, by supplying or scavenging substrates of these processes.

**Methods to elucidate the responsible $N_2O$ producing process**

Many studies have attempted to distinguish $N_2O$ production from nitrification or (nitrifier) denitrification. It was shown to be very difficult to determine the relative proportion of the aforementioned processes to $N_2O$ emission (Sutka et al. 2006), especially since these are not strictly segregated under natural heterogeneous conditions (Jetten 2001). Several methods have been applied to determine the relative contributions of these processes to $N_2O$ production.

Determining the concentrations of substrates and products can be an adequate tool for measuring the $N_2O$ production rate for a single process. Determining $N_2$ production is however difficult because of the high atmospheric background (79% $N_2$). A promising method, introduced in the late 1980s, was the selective inhibition of nitrification and $N_2O$ reduction (with 1 Pa and 10 kPa respectively) with acetylene ($C_2H_2$) (Klemedtsson et al. 1988b). Not much later, serious doubts arose about the applicability of this method. Bollman and Conrad (1997) showed that the $C_2H_2$ inhibition method could lead to underestimation of denitrification by means of scavenging NO, while Schmidt et al. (2001b) and Wrage et al. (2004b) showed that denitrification could be overestimated by this method as not all autotrophic nitrifiers were effectively inhibited. Because of these undesired effects, this method should only be applied with care and the limitations should be kept in mind.

An inhibitor independent approach is the use of naturally occurring stable isotopes of nitrogen. The common isotope $^{14}N$ constitutes the bulk of the nitrogen found in nature (99.63%) whereas $^{15}N$ is quite rare (0.37%). This phenomenon can be exploited in three different ways:

First, the heavy stable isotope of nitrogen ($^{15}N$) can be tracked by measuring isotopic signatures of (subsequent) products using mass spectrometry (Schoenheimer et al. 1937). A major limitation of this method is the need for supplying (labeled) nitrogen to the experimental setup, whereby transformation rates can be altered. Furthermore, in a system with heterogeneous conditions, simultaneously operating processes may lead to mixtures of isotopic signatures which are very difficult to interpret. The use of dual-isotope labeling (both $^{15}N$ and $^{18}O$) can prevent some of the limitations of the aforementioned methods

(Wrage et al. 2005), but a major limitation of that method is the rapid oxygen exchange between $H_2O$ and intermediates of $N_2O$ production (Kool et al. 2007).

Second, the so-called isotope fractionation may be traced in natural ecosystems. As microbes generally select for lighter isotopes, an increase in heavier isotopes will appear in substrates, whereas products will contain relatively lighter nitrogen isotopes. The natural isotope fractionation may therefore be used to apportion $N_2O$ to nitrification or denitrification (Webster and Hopkins 1996b). However, Handley and Raven (1992) discussed a number of limitations leading to spatial variation of $^{15}N/^{14}N$ ratio between and within soils and Well and Flessa (2008) showed that $N_2O$ diffusion through the soil also affects isotopic fractionation which makes the interpretation of isotopic ratios of $N_2O$ less reliable.

The third isotopic method is the determination of isotopomer abundance (Yoshida and Toyoda 2000) in the two nitrogen atoms of nitrous oxide. The oxygen atom in the $N_2O$ molecule is attached to only one of the nitrogen atoms during oxidation of $NH_4^+$. If one of the nitrogen atoms represents a $^{15}N$ isotope it can be determined to which of the nitrogen atoms the oxygen atom is bound (Toyoda and Yoshida 1999). Sutka et al. (2006) showed that nitrification and (nitrifier) denitrification produce distinct site preferences for the N-O binding and concluded that the determination of the intramolecular distribution of N isotopes in $N_2O$ can be a quantitative indicator for the originating process of $N_2O$ production. Only few laboratories possess the required specialized mass spectrometer for this method.

All methodologies have their advantages and limitations. In this thesis a set of methods was used, while keeping the limitations of each of the individual methods in mind. The interpretation of the data generated by the various methods was used to reveal the source of $N_2O$ emission in the studied soil.

**Conditions affecting $N_2O$ production and emissions**

A great number of physical, biological and chemical conditions influencing $N_2O$ emissions from soils have been studied in the past. Many of these factors (e.g. soil compaction, tillage system, tree distribution, rainfall events etc.) have only an indirect effect on $N_2O$ production. Although knowledge of conditions indirectly affecting $N_2O$ emissions is important in creating mitigation measures, the focus of research in this thesis was on identifying the fundamental primary controls on $N_2O$ emissions and determining their proportional contributions.

**Physical conditions.** The soil structure directly affects gas exchange between soil and atmosphere. Total $N_2O$ emission is the product of net $N_2O$ production (total production

minus consumption) and the rate of soil-atmosphere $N_2O$ exchange. Clough et al. (2005) summarized a large number of factors including air-filled porosity, tortuosity of soil pores, gas mixture dependant diffusion, the depth of $N_2O$ production, movement of groundwater table and atmospheric pressure changes which will influence exchange rates. Furthermore, net $N_2O$ production is indirectly affected by soil-atmosphere $N_2O$ exchange as it alters the opportunity for $N_2O$ reduction to $N_2$. Finally, groundwater flows may transport $N_2O$ from one site to another.

Water saturation variability poses both a direct and an indirect important control on $N_2O$ production via altering gas diffusion between soil and atmosphere. First, soil water saturation variability will influence oxygen availability (Skopp 1985), which, as explained later, affects most, if not all nitrogen cycling processes. Second, diffusion of $N_2O$ produced in soil to the atmosphere may be limited by high water saturation (Heincke and Kaupenjohann 1999), which prevents immediate emission and thereby creating an opportunity for reducing $N_2O$ to $N_2$. Finally, high water saturation may limit atmospheric $N_2O$ diffusion in the soil and therewith decrease soil $N_2O$ consumption.

Although temperature affects the various nitrogen cycling processes (Powlson et al. 1988, Stark 1996) any effect of temperature on $N_2O$ production and consumption was ignored as spatial variability of soil temperature was considered to be low in the particular wetland ecosystem studied.

**Biological factors** directly influence $N_2O$ production. As explained earlier, $N_2O$ is mainly produced by nitrification and (nitrifier) denitrification. These processes are performed by many different microorganisms.

Nitrification is carried out by a number of autotrophic and heterotrophic bacteria and archaea (Prosser and Nicol 2008). Ammonium oxidation and nitrite oxidation seem to be always performed by distinct clades of microorganisms (Belser 1979). Nitrifier denitrification is assumed to be carried out by autotrophic ammonia oxidizing bacteria (Kuai and Verstraete 1998). Although performing the same process under equal conditions, $N_2O$ production varies between nitrifying species (Anderson and Levine 1986).

The capability to denitrify is widespread among different genera of bacteria and archaea (Philippot 2005) and even some fungi (Shoun et al. 1992, Crenshaw et al. 2008) and foraminifera (Risgaard-Petersen et al. 2006). The denitrification trait is however not similar in all organisms. Some lack parts of the reduction cascade or can even perform only one of the reduction steps so that complete denitrification often relies on co-operating species (Van de Pas-Schoonen et al. 2005). Especially $N_2O$ reduction seems to be a feature lacking in many denitrifiers (Zumft 1997).

As the various reactions in denitrification can be carried out by a large number of species with their specific requirements and capacities, $N_2O$ production is affected by differences in microbial community composition (e.g. Balser and Firestone 2005). Unfortunately, correlations between N-cycling processes and microbial community composition are often unclear or absent (Philippot and Hallin 2005)

Next to effects of microorganisms, plants and fauna can also induce important effects on $N_2O$ emissions. Plant roots can alter soil $N_2O$ emissions via exudation of oxygen and organic compounds (Henry et al. 2008). Furthermore, the presence of earth worms can have a distinct effect on soil $N_2O$ emissions (Karsten and Drake 1997, Bertora et al. 2007). Finally, microbe grazing by nematodes and protozoa can strongly influence microbial community composition and soil nitrogen and carbon cycling (Griffiths et al. 1999, Blanc et al. 2006). Despite the potential importance on $N_2O$ emissions, effects of (macro) fauna and flora were not addressed in detail in this thesis.

**Chemical factors** playing an important role in $N_2O$ production and consumption can be categorized in electron donor availability, electron acceptor availability and acidity.

The effect of electron donor availability is the least known within this category. Kampschreur et al. (2008) and Avrahami et al. (2002) showed increased $N_2O$ emissions after increasing ammonium supply, but this effect was probably attributable to an increased nitrification rate and not to an increased relative $N_2O$ production from the process.

Although electron donors like hydrogen (Smith et al. 1994), iron sulfide (Haaijer et al. 2007), ammonium (Van de Graaf et al. 1995, Kartal et al. 2007) and even methane (Raghoebarsing et al. 2006, Ettwig et al. 2008) can be used for nitrate and nitrite reduction, it is assumed that soil denitrification is mainly driven by slowly released organic compounds from degradation processes. High availability of organic matter may increase denitrification rates and thereby total $N_2O$ production (Parkin 1987, McCarty and Bremner 1992). On the other hand, low availability of organic electron donors may enhance net $N_2O$ production by increasing $N_2O$:$N_2$ ratio (Weier et al. 1993). Furthermore, not only the total availability of organic electron donors plays a role, but also the form of electron donor. Pfenning and McMahon (1997) and Henry et al. (2008) showed differential effects on $N_2O$ production by using various types of organic electron donors. Thus, quantity and quality of organic electron donors may be a key factor in $N_2O$ production from denitrification.

Oxygen is an important electron acceptor, but due to its low solubility may not always be available in water logged systems. Differential effects of oxygen on the $N_2O$ producing processes have already been recognized. Nitrification is an oxygen consuming process and under limited oxygen availability, $N_2O$ production increases (Goreau et al. 1980). In addition to the effects of oxygen on $N_2O$ production from nitrification, oxygen

has direct effects on $N_2O$ production by denitrification. First, denitrification is mainly performed by facultative aerobic microorganisms (Knowles 1982), implying that under aerobic conditions, oxygen will be used as the primary electron acceptor and no denitrification and $N_2O$ production will occur. On the other hand, several species are known which can perform denitrification under aerobic conditions (Robertson et al. 1995, Morley et al. 2008). Generally, relatively high $N_2O$ production is observed with aerobic denitrification. Second, under oxygen limited conditions, $N_2O$ reduction to $N_2$ may decrease (Morley et al. 2008) resulting in higher net $N_2O$ productions. Bonin et al. (1989) showed an example of an aerobic denitrifier of which $N_2O$ reductase was more sensitive to oxygen than nitrate and nitrite reductase. The heterogeneity of oxygen concentrations in soils is further complicating the story. Due to the multitude of the aforementioned effects, soil oxygen concentration is not a straightforward factor in predicting net $N_2O$ emissions.

Other electron acceptors of importance to $N_2O$ production are nitrate and nitrite. Increased concentrations (>10 mM) of nitrite have been shown to negatively affect denitrification rates (Almeida et al. 1995, Baumann et al. 1997), but due to a possible effect of both $N_2O$ production as well as reduction, the effect of nitrite on net $N_2O$ production remains speculative. Nitrate on the other hand, is the main substrate for denitrification in soils and higher availability will lead to increased $N_2O$ production. Furthermore, the work of Blackmer and Bremner (1978) suggests that $N_2O$ reduction is more and more inhibited at increasing levels of nitrate. The effects of nitrite and nitrate on $N_2O$ reduction seem to be especially or solely operational at low pH (Blackmer and Bremner 1978).

The pH of the (soil) environment directly surrounding microorganisms can affect $N_2O$ production in several direct and indirect ways. First, pH may affect organic electron donor availability (Piccolo 2002), which as explained above, will affect denitrification and $N_2O$:$N_2$ product ratio. Second, decreasing pH decreases reduction of nitrate and nitrite to $N_2O$, resulting in decreased $N_2O$ productions (Bandibas et al. 1994). Simultaneously, decreasing pH slows down the reduction of $N_2O$ to $N_2$, leading to increased $N_2O$:$N_2$ product ratios from denitrification (Wijler and Delwiche, 1954, Cuhel et al. 2010). The $N_2O$ product ratio from nitrification increases at decreasing pH too (Morkved et al. 2007). Moreover, pH not only affects rates of (nitrifier) denitrification and nitrification, but also the microbial community composition. Lauber et al. (2009) showed that even on the continental scale soil pH is the best predictor of microbial community composition and diversity. Denitrifier community composition is also controlled by pH (Wallenstein et al. 2006) and, as explained before, differences in denitrifier community composition can be an important factor in denitrifying activity and $N_2O$ production (Cavigelli and Robertson 2001). Nicol et al. (2008) showed that diversity, abundance and transcriptional activity of ammonium oxidizing archaea and bacteria were also influenced by soil pH. Jiang and Bakken (1999)

showed that different ammonium oxidizing bacteria differ in their response to low pH with respect to $N_2O$ production. Finally, chemical reduction of nitrite to nitric oxide and nitrous oxide may occur in slightly acidic soils (pH <5.5) while not at higher pH values (Van Cleemput and Samater 1996), thus possibly increasing nitrous oxide emissions at low pH. In view of the contrasting effects, the net effect of soil pH on net $N_2O$ production is unclear.

Correlations between soil $N_2O$ emissions and soil conditions are often weak or unexpected, due to the complexity and interactions of influencing factors and processes. Explaining variability in $N_2O$ emissions between and within soils requires a fundamental understanding of the responsible mechanisms driving differences in $N_2O$ emission given the aforementioned multitude of physical, chemical and biological factors possibly influencing $N_2O$ emissions.

**Riparian buffer zones and the study site**

Reliable quantitative estimates of total $N_2O$ emissions of natural ecosystems and vegetation types are scarce (Stehfest and Bouwman 2006). Agricultural fields and bordering riparian buffer zones have received ample attention with respect to denitrification and $N_2O$ emissions (Groffman et al. 2000). Relative to whole landscape emissions, riparian buffer zones are considered as high emission ecosystems (Groffman et al. 2000, Verhoeven et al. 2006) and previous studies showed high $N_2O$ emission variability within riparian buffer zones (Dhondt et al. 2004, Hefting et al. 2006a, Clough et al. 2007). Relatively high emissions and high variation are optimal for studying underlying processes of $N_2O$ emission variability. Therefore, a riparian buffer zone, previously shown to exhibit high $N_2O$ emissions (Hefting et al. 2003, 2006a) was chosen as the study site for the research in this thesis.

The study site is located in the eastern part of The Netherlands (N 52$^o$ 25', E 6$^o$ 52') and is called "het Hazelbekke", referring to the Hazelnut trees (*Corylus avellana*) formerly growing along the stream. The ecosystem is located between a first order stream and agricultural cornfields and grasslands with a total width of 30 to 40 meter at the area where the research was performed. The vegetation of the buffer zone consists of alder (*Alnus glutinosa*) with a sparse understorey dominated by blackberry (*Rubus fruticosus*) and stinging nettle (*Urtica dioica*). The soil consists of peaty topsoil (> 10cm) on a sandy substrate and is receiving lateral inflow of $NO_3^-$ rich groundwater. For a more elaborate description of the study site, see Hefting and De Klein (1998), Burt et al. (2002) and Hefting (2003).

**$N_2O$ emission hotspots**

Methods to determine $N_2O$ emissions from soils include micrometeorological measurements or closed chamber measurements. The advantage of micrometeorological measurements is the possibility to determine whole-ecosystem fluxes but uncertainties arise mainly due to within-site spatial heterogeneity (Laville et al. 1999). Total $N_2O$ emissions calculated from the two techniques seem to be rather similar (Laville et al. 1999). Using static flux chambers, many studies found high spatial (and temporal) variation (e.g. Yanai et al. 2003, Hefting et al. 2006a, Jacinthe and Lal 2006, Khalil et al. 2007). Moreover often a typical hotspot pattern is observed, in other words, a few soil spots with strongly increased $N_2O$ emission relative to the emissions of all measured spots (Velthof 1996a, Dhondt et al. 2004, Von Arnold et al. 2005, Hefting et al. 2006a, Mathieu et al. 2006). This observation is of great consequence as a limited number of spots can be responsible for a significant amount of the total ecosystem $N_2O$ emission (see e.g. Velthof et al. 1996a, Hefting et al. 2006a). This typical behavior causes great challenges for prediction (models) of $N_2O$ emissions (Groffman et al. 2009). Furthermore, as long as mechanisms leading to such extraordinary behavior are not understood, mitigation measures may lead to unexpected and undesired results.

A number of studies exist on several aspects of hotspots of denitrification (e.g. Parkin 1987, Groffman et al. 2009) or biogeochemical hotspots in general (McClain et al. 2003) but to our knowledge, to date no studies exist specifically aiming at explaining $N_2O$ emission hotspot behavior.

**Research questions, aim**

The main objective of this research is to find the causal mechanism of $N_2O$ emission hotspot behavior in a riparian buffer ecosystem. Therefore this research focuses on potential differences between hotspots and non-hotspots.

The following research questions are addressed:

- What is the size of a $N_2O$ emission hotspot?
- Is the distribution of hotspots in the ecosystem random?
- Is the elevated $N_2O$ emission by hotspots compared to non-hotspots due to increased diffusion, increased production or decreased consumption of $N_2O$?
- Which N-cycling process is responsible for the $N_2O$ production in hotspots?
- What factors are responsible for elevated net $N_2O$ production in hotspots?
- Can soil denitrification occur at pH 4?
- Which microorganisms are responsible for $N_2O$ production in hotspots?

**Approach and outline**

A critical aspect in the approach of this research is the focus on $N_2O$ emission hotspots and differences between hotspots and non-hotspots. Therefore, set up and measurements are aimed to find potential differences between hotspots and non-hotspots. Whole system average conditions and emissions were therefore not addressed in detail.

**Chapter 2** of this thesis describes a set of field measurements in three large grids. These measurements were used to localize $N_2O$ emission hotspots in the ecosystem. This set up was accompanied by measurements of $N_2O$ emission on various spatial scales in order to characterize and define the spatial scale of hotspots. Complementary laboratory tests with small spatial scale soil cores are described aiming to identify whether potential denitrification and the effect of electron donor and acceptor availability could possibly explain the hotspot behavior.

In **Chapter 3** a set of laboratory incubations is described where hotspot and non-hotspot soils are compared under various conditions. This chapter especially aims at answering the question whether hotspot $N_2O$ emissions are caused by increased $N_2O$ diffusion, increased $N_2O$ production or decreased $N_2O$ consumption relative to non-hotspot soils. Furthermore, the role of $NO_3^-$ and $O_2$ in explaining hotspot behavior is elucidated.

The hypothesis of **Chapter 4** is that soil pH controls $N_2O$ emission variability. In this chapter, laboratory incubations of soil slurries are used to elucidate the role of pH on $N_2O$ production and consumption, $N_2$ production and $NO_3^-$ reduction. The results from these incubations are used to explain a field observed relation between pH and $N_2O$ emissions.

In order to create a soil-comparable but controlled niche in the laboratory, a bioreactor approach is used in **Chapter 5**. The process of denitrification and enrichment of a bacterial species at low pH is described. Effects of pH and electron donor availability on growth, activity and net $N_2O$ production are described. The possibility of soil denitrification at low pH is discussed.

**Chapter 6** summarizes the main results of chapters 2 to 5. The results from the separate chapters are placed in perspective and an overall explanation on $N_2O$ emission hotspot behavior in the studied ecosystem is given. Hypotheses are posed on the source of soil pH variability and the mechanism of the effect of pH on $N_2O$ reduction. The implications for model development and management tools are discussed with respect to the generalization of findings.

# Chapter 2

## $N_2O$ emission hotspots at different spatial scales and governing factors for small scale hotspots.

*Science of the Total Environment 407: 2325-2332*

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**Abstract**

Chronically nitrate-loaded riparian buffer zones show high $N_2O$ emissions. Often, a large part of the $N_2O$ is emitted from small surface areas, resulting in high spatial variability in these buffer zones. These small surface areas with high $N_2O$ emissions (hotspots) need to be investigated to generate knowledge on the factors governing $N_2O$ emissions. In this study the $N_2O$ emission variability was investigated at different spatial scales. Therefore $N_2O$ emissions from three 32 $m^2$ grids were determined in summer and winter. Spatial variation and total emission was determined on three different scales (0.3 $m^2$, 0.018 $m^2$ and 0.0013 $m^2$) at plots with different levels of $N_2O$ emissions. Spatial variation was high at all scales determined and highest at the smallest scale. To test possible factors inducing small scale hotspots, soil samples were collected for slurry incubation to determine responses to increased electron donor/acceptor availability. Acetate addition did increase $N_2O$ production, but nitrate addition failed to increase total denitrification or net $N_2O$ production. $N_2O$ production was similar in all soil slurries, independent of their origin from high or low emission soils, indicating that environmental conditions (including physical factors like gas diffusion) rather than microbial community composition governed $N_2O$ emission rates.

**Introduction**

Since the 1970's it has been recognized that nitrous oxide ($N_2O$) acts as a greenhouse gas (Kroeze 1994, Ramanathan 1998) and depletes stratospheric ozone (Crutzen 1970). Atmospheric $N_2O$ concentrations have increased since the industrial revolution, but an accurate quantification of the emission sources is lacking (Stehfest and Bouwman 2006). Natural and agricultural soils emit the majority of the total $N_2O$ released to the atmosphere. However, with the current knowledge, detailed prediction of $N_2O$ emission from agricultural and natural sources is subject to large uncertainties (Bouwman et al. 1995). Detailed predictions of emissions at large scales are therefore very hard to make.

Riparian buffer zones emit relatively high $N_2O$ amounts compared to many other ecosystems; Hefting et al. (2006a) and Dhondt et al. (2004) found emissions of 8.2 and 0.4 mg $N_2O$-N $m^{-2}$ $d^{-1}$ in riparian buffer zones while the following average values are reported for pastures: 0.06 mg $m^{-2}$ $d^{-1}$ (Stehfest and Bouwman 2006), maize: 2.6 mg $m^{-2}$ $d^{-1}$ (Chen et al. 1997), meadow: 0.08 mg $m^{-2}$ $d^{-1}$ (Huang et al. 2003), tropical forests: 0.3 mg $m^{-2}$ $d^{-1}$ (Kreutzwieser et al. 2003), deciduous forests: 0.16 mg $m^{-2}$ $d^{-1}$ (Pilegaard et al. 2006) and temperate grasslands: 0.06 mg $m^{-2}$ $d^{-1}$ (Huang et al. 2003). McClain et al. (2003) defined biogeochemical hotspots as "patches that show disproportionately high reaction rates relative to the surrounding area" (matrix). Therefore riparian buffer zones might be classified as hotspots for $N_2O$ emission at the landscape scale (Groffman et al. 1998, McClain et al. 2003). However, large spatial variability is not only observed between ecosystems, but can also be observed within these ecosystems. Coefficients of variance of $N_2O$ fluxes in riparian buffer zones can often be as high as 200% (Yanai et al. 2003, Hefting et al. 2006a, Jacinthe and Lal 2006). Typically $N_2O$ emission hotspots are found in agricultural areas (e.g. Velthof et al. 1996a, Mathieu et al. 2006), natural areas like boreal forests (Von Arnold et al. 2005) and riparian buffer zones (Dhondt et al. 2004; Hefting et al. 2006a). A detailed insight into at what spatial scales $N_2O$ emission hotspots are apparent is not yet available. More knowledge on the spatial variability of $N_2O$ emission will give us additional information on the factors controlling $N_2O$ emission and could eventually lead to generating knowledge on potential measures to counteract this emission.

Several studies have examined the factors governing spatial variability of $N_2O$ emission at different levels. In many studies, soil processes and/or properties showed no or only very weak correlation with $N_2O$ fluxes (e.g. Ball et al. 1997, Van den Pol-van Dasselaar et al. 1998, Von Arnold et al. 2005, Mathieu et al. 2006). Other studies came up with a variety of governing factors for $N_2O$ fluxes. At the continent level, Klemedtsson et al. (2005) showed that higher $N_2O$ emissions were strongly correlated to a lower soil C/N ratio in a study comparing boreal forests in various European countries. At the field level, Yanai et al. (2003) reported that organic matter related variables could explain 20% of the

variance of $N_2O$ emission in a 100x100 m arable plot. A positive correlation of nitrate and moisture content with $N_2O$ emission along a 400 m grassland transect was observed by Velthof et al. (2000). Using plot sizes of 200 and 6000 $m^2$, Ball et al. (1997) found negative correlations of pH with $N_2O$ emission. Variation of $N_2O$ emissions within riparian buffer zones was shown to be caused by variations in groundwater nitrate content (Hefting et al. 2003, Dhondt et al. 2004). Taken together these studies show that a large variety of factors influence $N_2O$ emissions and suggest that at specific spatial scales different factors can be controlling $N_2O$ emission.

Several microbial processes can contribute to $N_2O$ production in soils depending on the environmental conditions (e.g. Robertson and Tiedje 1987, Webster and Hopkins 1996a). Nitrification and nitrifier-denitrification (both dependent on ammonium or nitrite) can contribute significantly to nitrous oxide production in ammonium-rich agricultural soils (Blackmer et al. 1980, Webster and Hopkins 1996a) and waste water treatment systems (Tallec et al. 2006b, Cebron et al. 2005, Kampschreur et al. 2006). In forested riparian buffer zones on the other hand $N_2O$ emissions are mostly assumed to be produced by denitrification (Hefting et al. 2003, Dhondt et al. 2004) since these organic soils experience wet (and therefore oxygen-limited) and nitrate rich (from agricultural run-off) conditions. Hefting et al. (2004) showed that denitrification (and ammonification) were the dominant nitrogen cycling processes in riparian buffer zones when groundwater levels were within 30 cm of the soil surface, while nitrification became important only if groundwater levels were below 30 cm of the soil surface. In such buffer zones, denitrification was shown to be very patchy with typical hotspot behavior in many cases (Jacinthe et al. 1998, Hefting et al. 2006a). Within small patches, high denitrification rates might have been induced by high concentrations of electron donor like organic carbon which may have resulted in rapid oxygen depletion and favorable conditions for denitrification (Parkin 1987, Jacinthe et al. 1998). While small scale denitrification hotspot behavior seems to be governed by electron donor availability, the factors for $N_2O$ emission hotspot behavior are, to our knowledge, not well understood yet. This is partly due to the fact that the ratio between $N_2O$ and $N_2$ produced by denitrification can differ significantly under varying conditions. The amount of $N_2O$ produced can vary from almost 0 to over 90% of the total N-gases produced (e.g. Weier et al. 1993, Mathieu et al. 2006). Different conditions of electron acceptor/donor availability, soil moisture content and possibly pH can lead to other $N_2O$:$N_2$ ratios (Weier et al. 1993, Bandibas et al. 1994). Furthermore the $N_2O$ produced at a certain location can be consumed at another location, for instance in an overlying soil layer. Finally the $N_2O$ produced can be trapped in the soil for a period of time and might therefore not (directly) be emitted (Clough et al. 2005).

In this study, the spatial variability of $N_2O$ emission was studied using observations under field conditions. The objective of this study was to identify the spatial scale of $N_2O$ hotspot behavior using flux chambers at different scales. Complementary laboratory tests were used to identify if potential denitrification and the effect of electron donor and acceptor availability could possibly explain the hotspot behavior. The criterion for hotspot used in this study was the definition of extreme cases for boxplots in SPSS (SPSS 12.0.1, 2003, SPSS Inc. USA); plots with emissions more than three box lengths from the upper edge of the 50% percentile box of the emission of all plots. The matrix was defined as plots with emission within the 50% percentile box.

We expected small scale $N_2O$ emission hotspots to be governed by patches with high electron donor availability which would increase denitrification rates. Therefore we expected to find hotspot behavior to be most pronounced at a small scale ($cm^2$). Furthermore, we expected high emission soils to show higher denitrification rates than matrix soils in laboratory incubations, while matrix emission soils were expected to show a strong increase in $N_2O$ production after addition of an electron donor. Controlling factors for larger scale hotspots were not investigated but were assumed to be controlled by gradients in soil moisture content or nitrate concentration.

**Methods**

*Study area*

A riparian buffer zone in the eastern part of the Netherlands "Het Hazelbekke" (N 52° 25', E 6° 52') was selected as a study site (Hefting and De Klein 1998). The vegetation of buffer zone consisted of alder (*Alnus glutinosa*) with a sparse understorey dominated by blackberry (*Rubus fruticosus*) and nettle (*Urtica dioica*). The buffer zone was located along a first-order stream (Hazelbekke) in an agricultural region with grasslands and maize fields. The selection of this site for the nitrous oxide emission measurements was based on previous results by Hefting et al. (2006a). Soil pore water was extracted using 10 cm rhizons (Eijkelkamp Agrisearch Equipment BV, the Netherlands) during the experiment in the experimental grids (see below). Nitrate concentrations of the soil pore water were on average 10.7 mg $NO_3^-$-N $L^{-1}$, ammonium concentrations were 1.3 mg $NH_4^+$-N $L^{-1}$ on average (determined by using an autoanalyser (SA-40, Skalar Analytical BV, the Netherlands)). Soil pore water pH values ranged between 3.8 and 6.6 in the ecosystem. Soil moisture contents were on average 73% in summer and 78% in winter (w/w). In winter, the groundwater level was 25 cm below the soil surface in the highest parts and around soil surface level at the lower parts, while in summer the groundwater levels were 75 cm and 10 cm below surface level in the highest and lowest parts respectively.

*$N_2O$ flux measurements*

Closed flux chambers (0.31 $m^2$ diameter, height 25 cm) were placed in the soil to 2 cm depth. $N_2O$ concentrations in the flux chambers were determined at 8-minute intervals for 48 minutes directly after installation of the flux chambers using a photo-acoustic infrared gas analyzer (type 1302, Bruel & Kjaer, Denmark). A CBISS MK2 multipoint sampler (4-channel, CBISS Ltd. England) was used to determine $N_2O$ concentrations in 4 or 8 flux chambers at the same time.

Nitrous oxide emissions were determined from three grids of 72 plots of 65 x 65 cm (in total 8 by 4 m) with 0.31 $m^2$ flux chambers. Grids were located perpendicular to the stream between 1 m and 9 m from the stream. Flux measurements of the plots were performed randomly over the grid. Fluxes of $N_2O$ were determined from the exact same locations in summer 2006 and winter 2006/2007. All plots (except for the ones which were obstructed by alder trees or branches) within a grid were sampled within two (summer) or one (winter) day(s). Sampling dates of grid 1, 2 and 3 were August 8/9$^{th}$, July 17/18$^{th}$ and 25/26$^{th}$ 2006 and February 26$^{th}$, 12$^{th}$ and 19$^{th}$ 2007 respectively. Additional sampling from 36 plots (placed stratified random in the area within 5 meters from the stream) was carried out using the same method on September 5$^{th}$ and 7$^{th}$ 2006.

*$N_2O$ flux measurements at different spatial scales*

During the winter campaign, the three plots in each grid with the highest emission were selected as hot spots (0.31 $m^2$), while three median emission plots representing the matrix were selected on basis of interquartile emission rates (within 50% of the data range) neighboring the selected high emission plots. The next day, emissions from high emission and matrix plots were determined again with the 0.31 $m^2$ flux chambers. Eight smaller flux chambers (0.018 $m^2$, height 25 cm) were subsequently placed in the selected plots and emissions were determined with these smaller flux chambers (figure 1 shows a schematic representation of part the sampling site). On the basis of the emission rates measured, the plots with the highest emissions (in the 0.31 $m^2$ hot spots) and median emissions (in the 0.31 $m^2$ matrix spots) were selected. In each of these selected (0.018 $m^2$) plots seven flux chambers (0.0013 $m^2$ diameter, height 25 cm) were placed. After 48 minutes, a gas sample was taken from these 0.0013 $m^2$ flux chambers. The $N_2O$ concentrations were determined using a gas chromatograph (GC Hewlett Packard 5890) equipped with an electron capture detector (ECD $^{63}Ni$) and Hayesep Q columns. Cores (0.0013 $m^2$, 10 cm depth) were taken from the selected plots.

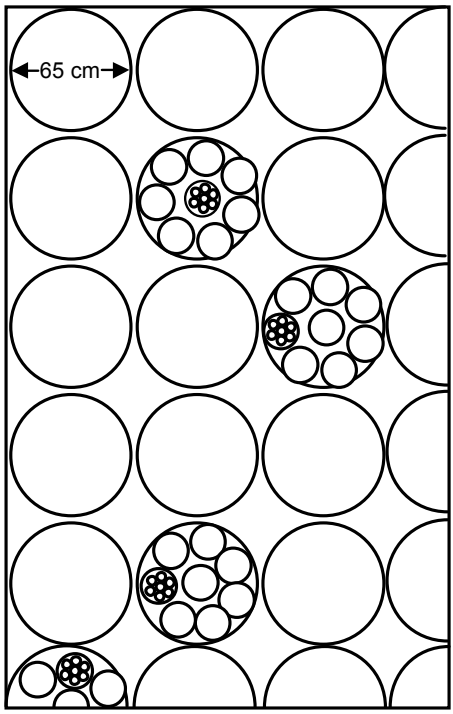


**Figure 1: Schematic top view of the sampling site. Part of one of the three grids is shown. Large circles represent large (0.31m$^2$) flux chambers, while smaller circles show how smaller flux chambers (0.018 and 0.0013m$^2$ respectively) were located.**

*$N_2O$ production in batch slurries*

The 0.0013 m$^2$ diameter cores were submerged in demineralised water (1:1) and shaken vigorously to obtain homogeneous slurries. 10 ml of slurry was added to 40 ml jars. Control, acetate (added as $NaCH_3COO$, 4 mg C g dry weight$^{-1}$), nitrate (added as $KNO_3$, 10 µg N g dry weight$^{-1}$) or nitrous oxide (150ppm (v/v) $N_2O$) treatments were performed. Flasks were made anaerobic by changing the gas phase two times with $N_2$. All treatments were performed both with and without acetylene (10 % (v/v) $C_2H_2$) to determine both net $N_2O$ production and total denitrification. After 4 and 8 hours headspace $N_2O$ concentrations were determined using gas chromatography. $N_2O$ production was calculated in the mentioned 4-hour interval and production was corrected for water dissolved $N_2O$. Slurries were dried and dry weight was measured in order to calculate activities on the basis of dry weight.

*Statistical analysis*

All data were tested for normality and homogeneity of variance. Not all data met the requirements and therefore non-parametric tests were used. Difference between 1st (summer 2006) and 2nd winter (2007) emissions were tested using the Mann-Whitney test. Testing on differences in emissions at different scales was performed with the Friedman test and if differences were significant, a Wilcoxon test was used. A Friedman test was performed to test upon differences of variability between the different scales. The Wilcoxon test was used to test for differences between emissions of consecutive days. All statistical analyses were performed using SPSS 12.0.1 (2003, SPSS Inc. USA)

## Results

*Spatial variation of $N_2O$ fluxes at the plot scale in the riparian zone*

Mean $N_2O$ fluxes of the 199 plots in the summer of 2006 (1.44 mg N $m^{-2}$ $d^{-1}$) were higher ($p<0.001$) than the mean winter fluxes (0.41 mg N $m^{-2}$ $d^{-1}$, 190 plots) at the same location. Emission as well as net uptake of $N_2O$ was observed in summer and winter. Fluxes showed a skewed/lognormal distribution, both in the summer and winter measurements (Figure 2). Variability was high with a coefficient of variance of 258% in summer and 320% in winter.

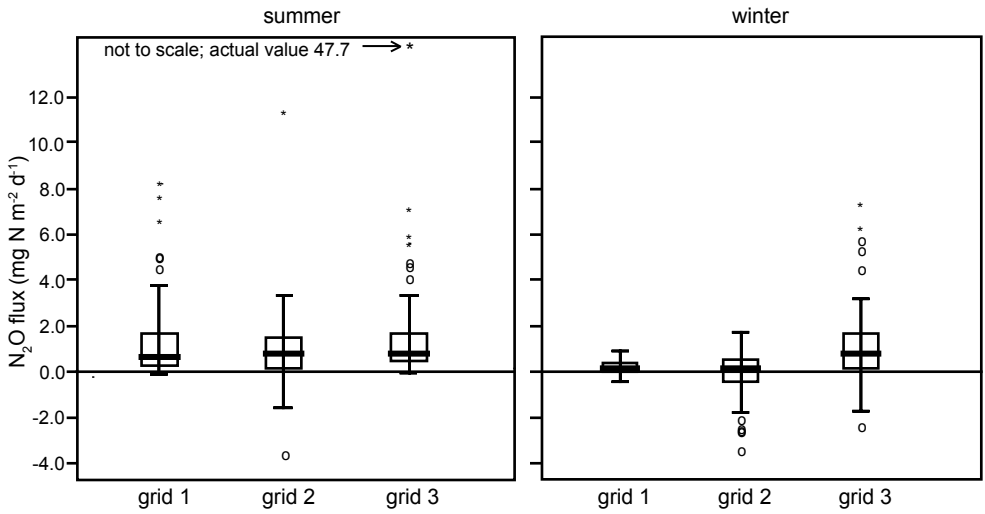


**Figure 2: Boxplots of data range of $N_2O$ emissions from 0.65 x 0.65 m plots in 3 grids in summer (left) and winter (right). Asterisks mark extreme cases (more than 3 box lengths from upper edge interquartile range), circles mark outliers (1.5 box lengths from upper or lower edge of interquartile range).**

During the summer measurements, eight plots showed emissions which met the stringent hotspot criterion (more than three box lengths from the upper edge of the 50% percentile box of the emission of all plots of one grid), while in the winter measurement only two plots qualified as hotspot. All hotspots were located within 5.5 meter from the stream (Figure 3). Especially during the summer measurements all high emissions measured were located close to the stream (less than 6 m). The contribution of the eight hotspots to the total emissions of the summer measurements was 33%, the two hotspots in winter were responsible for 17% of the total emissions, while the surface area of the hotspots accounted for only 4% and 1% of the total area, respectively.

*Spatial variation within high emission soils*

During the winter campaign, the three plots with the highest emissions per grid (i.e. nine in total) were selected for studying the spatial variation of high emission at different scales. Only two of the nine locations selected actually met the stringent hotspot criterion, therefore these nine locations will be called high emission plots from this point on.

Emission rates from the smaller subplots, within the selected high emission plots, showed significantly higher emissions ($p<0.01$) than when measured at the larger scales (Figure 4). Also the plots adjoining the high emission plots showed higher $N_2O$ emissions at the smaller scale.

The hotspot behavior was visible by comparing the high emission plots with their adjoining plots. The relative difference between high emission plots and their adjoining plots was the highest at the smallest scale; the high emission plots emitted 3.2 times more $N_2O$ than their adjoining plots, while for the 0.018 and 0.31 $m^2$ scale this was only 2.6 and 2.5 times respectively.

*Activity of high-emission and matrix soil samples in batch incubations*

In the laboratory slurry experiments, the $N_2O$ production was considerably higher in the acetylene amended batch incubations (total denitrification) compared to non-acetylene amended batches (net $N_2O$ production) irrespective of treatment (Figure 5). Total denitrification was not significantly increased after addition of either nitrate or acetate compared to (acetylene amended) controls (Figure 5). Addition of nitrate (in non-acetylene amended batches) did not increase the $N_2O$ production rate compared to the control treatment. Addition of acetate (non-acetylene amended) on the other hand, increased the $N_2O$ production to a significantly higher value ($p=0.006$). Addition of $N_2O$ did neither change total denitrification, nor net $N_2O$ production significantly.

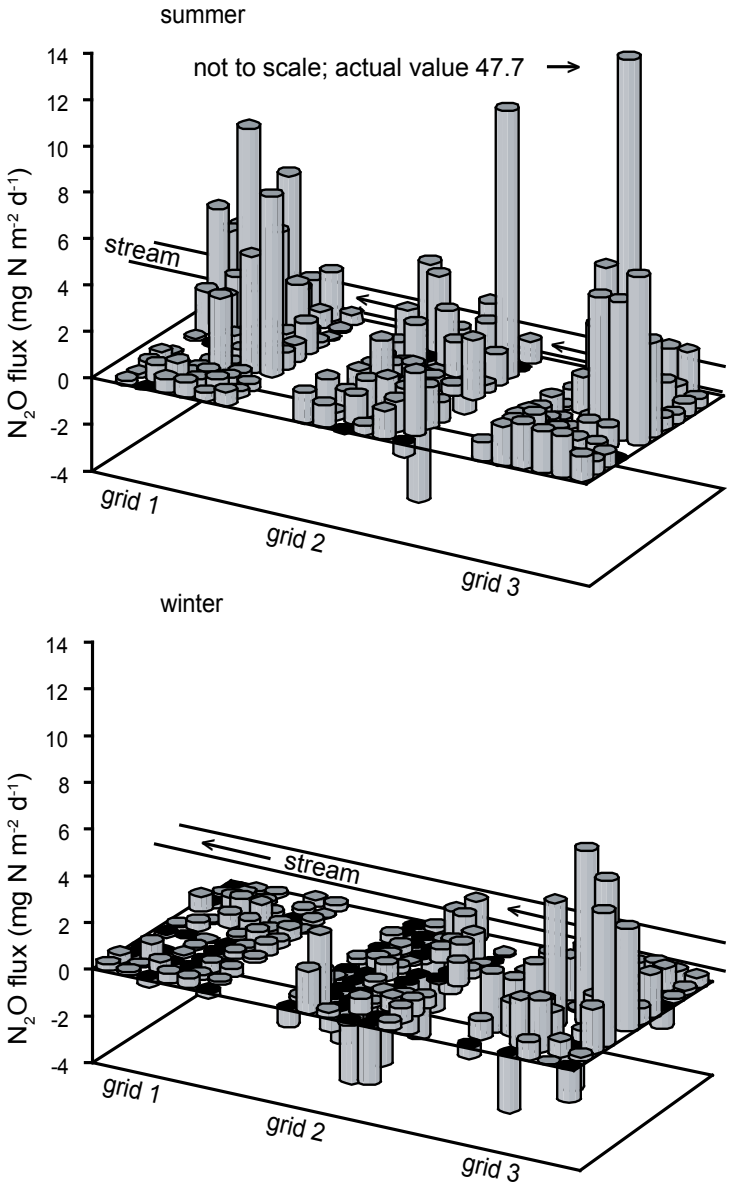


**Figure 3: $N_2O$ fluxes (single measurements) from 0.65 x 0.65 m plots in the three grids, determined in summer (top) and winter (bottom). The grids measured 8 x 4 m and were located perpendicularly to the stream (upper right hand side). Space between grids is not to scale, but was approximately 10 m.**

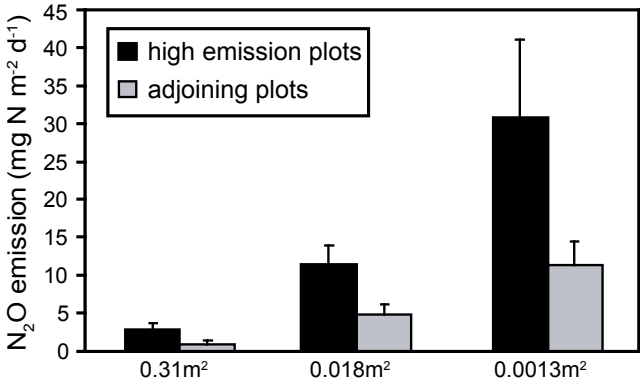


**Figure 4: $N_2O$ emissions of selected high emission plots (black bars) and adjoining plots (gray bars) on various spatial scales (0.31 $m^2$, 0.018 $m^2$ and 0.0013 $m^2$). Error bars show standard error of the mean.**

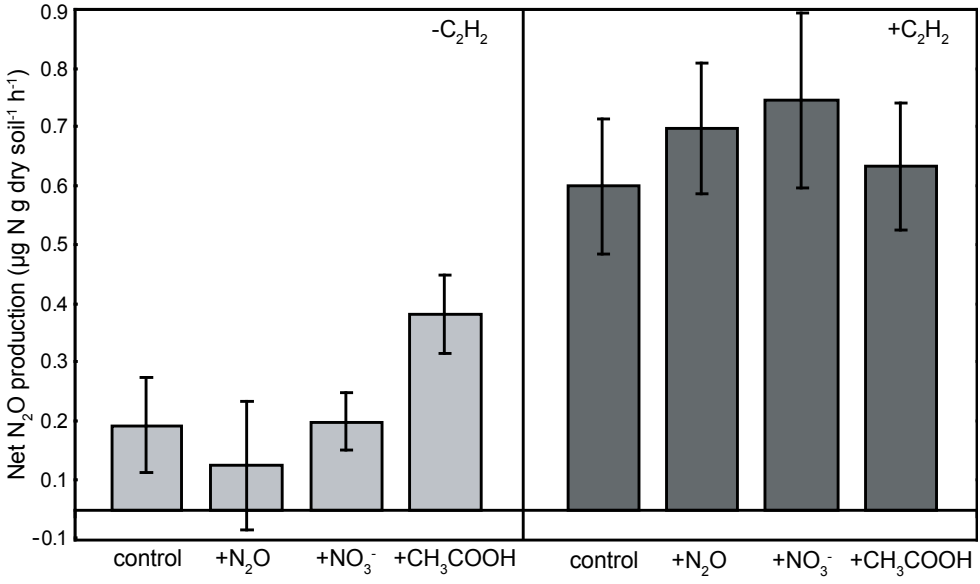


**Figure 5: Net $N_2O$ production (y-axis, µg N g dry $soil^{-1}$ $h^{-1}$) in slurries with different treatments (x-axis). Treatments without acetylene addition are shown on the left hand side, treatments with acetylene addition are shown on the right hand side.**

$N_2O$ production was determined both for high-emission and matrix soils. The results mentioned in the previous paragraph were true for both high-emission and matrix soil slurries. Slurries of high-emission soils did not show significantly higher $N_2O$ production rates than the matrix soils in any of the treatments (Figure 6). Matrix soils showed a significantly higher net production (non-acetylene amended) of $N_2O$ compared to high-emission soils after addition of nitrate ($p=0.042$), but this production was not different from the control treatment.

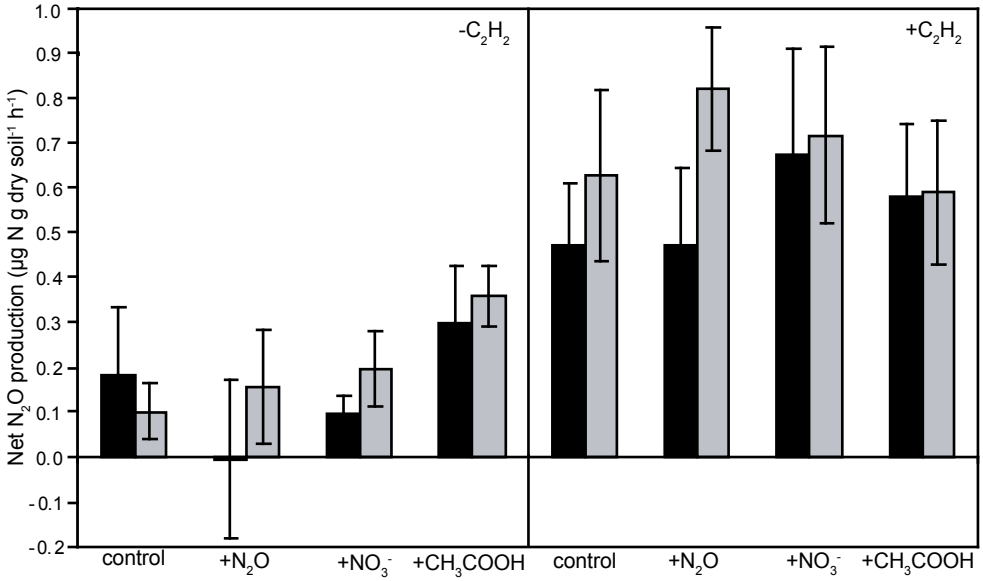


**Figure 6: Net $N_2O$ production (y-axis, µg N g dry soil$^{-1}$ h$^{-1}$) in slurries from either high-emission soil (black bars) or matrix soil (gray bars) with different treatments (x-axis). Treatments without acetylene addition are shown on the left hand side, treatments with acetylene addition are shown on the right hand side.**

*Consecutive day emissions*

In the spatial variation field set up where the $N_2O$ emissions were determined with 0.31 $m^2$ flux chambers on consecutive days, an increase of 1.47 to 6.33 mg N $m^{-2}$ $d^{-1}$ was observed between the first and second day. Seventeen (out of eighteen) plots showed an increase in emissions in the second measurement series (Figure 7). Using the $N_2O$ flux measurements in autumn, this effect was tested by determining $N_2O$ emissions on one day and two days later in 36 plots (0.31 $m^2$). Of these plots, 31 showed an increase in $N_2O$ emissions (Figure 7 shows net production of $N_2O$ on both days). On average the 36 plots showed a significant increase ($p<0.001$) from an average emission of -0.004 to an average emission of 0.11 mg N $m^{-2}$ $d^{-1}$.

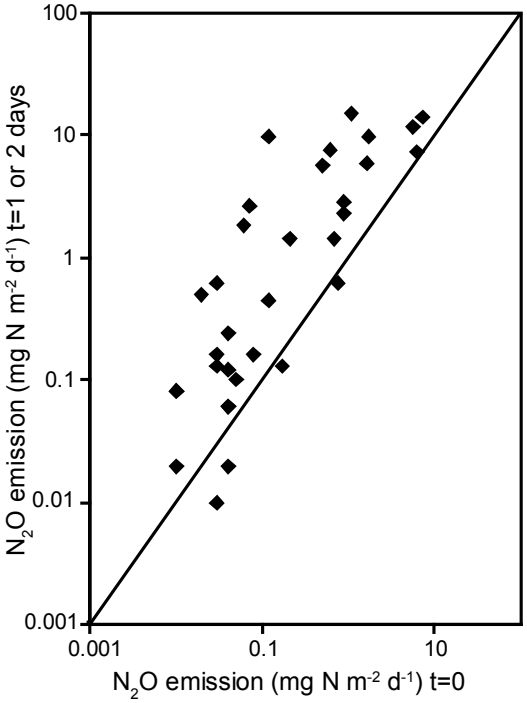


**Figure 7: Comparison of $N_2O$ emission (in mg N $m^{-2}$ $d^{-1}$) on t=0 (x-axis) and t=1 or 2 days (y-axis). Axes are in logarithmic scale.**

## Discussion

The aim of the present study was to identify the spatial scale of $N_2O$ hotspot behavior and to determine if potential denitrification and electron donor/acceptor availability could explain this behavior. A riparian buffer zone was chosen as the study site based on previous investigations (Hefting et al. 2006a). Emissions from three 32 $m^2$ grids were determined twice (summer 2006 and winter 2007). High emissions were only observed in the parts of the grids closest to the stream. Spatial variability was high and present at all scales determined. Apparently on the ecosystem level, large scale gradients (e.g. in soil moisture content or nitrate concentration) are important drivers for providing the right conditions for high emission spots (see also McClain et al. 2003). Total $N_2O$ emissions in the grid areas were lower compared to previous studies in the same area in 1999-2000 (Hefting et al. 2003, Hefting et al. 2006a), possibly due to a gradual desiccation (personal observations) of the area over the years. Total emissions were in the same range as other comparable buffer zones (e.g. Dhondt et al. 2004, Teiter and Mander 2005). Spatial variability of $N_2O$ emission is high in nearly every study; coefficients of variation (CV) are mostly in the range of 50 to 200% (e.g. Yanai et al. 2003, Hefting et al. 2006a, Jacinthe and

Lal 2006, Khalil et al. 2007). The CV's measured in this study (258 and 320%) indicate a high variability compared to other studies and therefore this area was ideal to study spatial variation of high-emission spots. After the second full scale field emission measurement (winter 2007), three different scales were analyzed for spatial variability of $N_2O$ emission within the high emission parts. Spatial variability within each scale was high and highest in the smallest spatial scale. The large differences between high-emission and matrix plots did not disappear if a higher resolution (down to square centimeters) was applied. The classic study of Parkin (1987) already showed hotspots at the scale of millimeters, McClain et al. (2003) described the hotspot concept using spatial scales from large river basins down to soil profiles, this study shows that also on the intermediate level $N_2O$ emission hotspot behavior is apparent.

In the first paragraph of the results section, it was shown that hotspots contribute a very significant amount to the total emission from an ecosystem. Therefore, causes of this hotspot behavior should be investigated. Our aim was to determine whether electron donor (acetate) or acceptor (nitrate or nitrous oxide) availability for denitrification was causing differences in emission between high emission and matrix soils on the square centimeter level. In the slurry incubations, denitrification potential was shown to be present consistently (as determined with the acetylene inhibition method). Potential denitrification in slurry incubations was in the same range as reported values in other studies in similar soils (e.g. Jacinthe et al. 2002, Cosandey et al. 2003, Dhondt et al. 2004). Total denitrification did not increase after addition of either acetate or nitrate. Therefore total denitrification was not strongly limited by electron donor or acceptor availability. Net $N_2O$ production was increased by addition of acetate. As addition of acetate did not increase total denitrification, it is concluded that addition of acetate only promoted nitrate reduction to $N_2O$. A sudden increase in available electron donor could therefore under natural circumstances with a high nitrate concentration lead to a high $N_2O$:$N_2$ ratio and eventually increase $N_2O$ emission. With this information it was tempting to conclude that differences in electron donor availability caused small scale high emission spots in the natural situation. But, high emission soils showed neither higher production of $N_2O$ nor higher total denitrification in the slurry incubations than the matrix soils did. So, soil from high emission spots did not have a different potential activity than the matrix soils did. Apparently, the driving factors causing the differences in the field emissions were not (solely) the factors governing $N_2O$ production under controlled incubation conditions.

An unexpected result from this study was the finding that disturbance of the soil was followed by higher $N_2O$ emissions. This was observed when field $N_2O$ emissions were determined several times on subsequent days on the same location and also when field $N_2O$ emissions were compared with emission from soil cores (data not shown). The exact form

of disturbance was not studied, but probably (gas) sampling and other activities in the riparian zone during the research were responsible for the emission increase. An increase in emission up to a hundred times was observed irrespective of the initial emission. This effect does not seem to be well documented as other studies aiming at temporal variation mostly focus on seasonal changes in emission (e.g. Velthof et al. 1996b, Hefting et al. 2003, Dhondt et al. 2004). We suspect that this effect can only be explained due to a change in the exchange rates of soil and atmosphere gases. Increased diffusion or convection of (a mixture of) soil gases including $N_2O$ to the atmosphere is the most likely hypothesis to explain this and is also used by other authors to explain variability in $N_2O$ fluxes (Clough et al. 2005), but one would expect other gases like $CO_2$ and $CH_4$ to increase as well. These gases were also monitored and did not show increased emissions (data not shown). Alternatively, the increased $N_2O$ emissions could have been caused by a decrease in the rate of $N_2O$ reduction to $N_2$. It is known that denitrifiers decrease $N_2O$ reduction under aerobic conditions and Cavigelli and Robertson (2001) showed that oxygen levels of > 0.5 % already decreased rates of $N_2O$ reduction. Thus increased oxygen diffusion into the soil as a result of soil disturbance could have reduced $N_2O$ reduction rates and therewith increase $N_2O$ emissions. Differences in diffusion, as a driving factor for hotspots of $N_2O$ emission, could also explain the other (somehow unexpected) results; diffusion could be operating different at different scales. Furthermore, if diffusion determines the extent of emission, it is not expected to see differences in net production between high emission and matrix soils. In this study the effect of earthworms on either diffusion or $N_2O$ emission of the soil wasn't tested. Other studies have shown that earthworms can have a positive effect on $N_2O$ production in and emission of certain soils (e.g. Borken et al. 2000, Bertora et al. 2007).

The results presented in this paper show that hotspot behavior is apparent at various spatial scales. This implies that environmental controls on $N_2O$ need to be explicitly investigated at different spatial scales. In addition to that it is important to acquire knowledge on the timeframe of initiation and duration of effects induced by different environmental controls. Together, this can provide essential information for explaining spatial (and temporal) variability of $N_2O$ emissions from soils. In this study neither the availability of electron donor or acceptor nor potential denitrification rates could explain $N_2O$ emission hotspot behavior. Disturbance of the soil was shown to increase $N_2O$ emissions. This is possibly caused by changes in gas diffusion. The effects of gas diffusion in the soil need therefore to be better documented with regard to the different steps of denitrification in the environment.

**Acknowledgements:**

The authors would like to acknowledge “Vereniging Natuurmonumenten” for allowing us to perform this research in Het Hazelbekke. The authors would like to thank Philippe Van Cappellen for advice, discussion and critically reviewing this manuscript. This research was funded by the Centre for wetland ecology (CWE) and by the Netherlands Organization for Scientific Research (NWO, grant number: ALW-836.03.040).

# Chapter 3

## Incomplete denitrification causes $N_2O$ emission hotspots in nitrate loaded wetlands

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**Abstract**

Soils are a source of $N_2O$, a greenhouse gas also linked to stratospheric ozone depletion. $N_2O$ emissions from soils are highly variable and heterogeneous. Particularly for wetland soils, a large part of the total ecosystem $N_2O$ emission may originate from a limited number of small areas (hotspots). In this study, field measurements of $N_2O$ emission rates were combined with slurry incubations of soils from hotspots and non-hotspots in a riparian buffer zone receiving high nitrate input from a nearby agricultural area. $N_2O$ production and consumption were determined in these incubations to determine whether hotspots emit much $N_2O$ because of high production or low consumption. Soils from $N_2O$ emission hotspots showed high net $N_2O$ production rates in laboratory incubations, implying that the *in situ* soil conditions in hotspots favor high $N_2O$ emissions. Neither $O_2$ nor $NO_3^-$ concentrations explained the differences in net $N_2O$ production between hotspot and non-hotspot soils. Rather, the differences were related to imbalances between $N_2O$ production and consumption during denitrification. The underlying factor for the variation in $N_2O$:$N_2$ production ratios remains obscure and object of further study as $O_2$ and $NO_3^-$ levels could not explain this variation.

**Introduction**

Since the 1970s it has been recognized that nitrous oxide ($N_2O$) depletes stratospheric ozone (Crutzen 1970) and acts as a greenhouse gas (Kroeze 1994, Ramanathan 1998). Atmospheric $N_2O$ has been rising since the industrial revolution, but an accurate quantification of the sources is lacking. Natural and agricultural soils are considered to be responsible for the major proportion of $N_2O$ emitted to the atmosphere. However, with the current knowledge, detailed prediction of $N_2O$ emission from agricultural and natural sources is subject to large uncertainties (Bouwman et al. 1995). Reliable predictions of emissions at large spatial scales are nearly impossible because of high variability of emissions among and within ecosystems. Riparian buffer zones, for example, typically exhibit significantly higher $N_2O$ emissions than adjacent soil environments (Groffman et al. 2000).

Differences in soil $N_2O$ emissions have been found to be correlated to differences in soil water $NO_3^-$ concentration (Hefting et al. 2006a), organic matter C/N ratio (Klemedtsson et al. 2005), soil moisture (Velthof et al. 2000), and soil pH (e.g. Ball et al. 1997). Specifically in riparian zones, $N_2O$ emissions have been linked to the local availability of $NO_3^-$ (Dhondt et al. 2004, Hefting et al. 2006a, Ullah and Zinati 2006). The denitrifying community composition has also been suggested as a possible factor explaining variations in soil $N_2O$ emissions (Holtan-Hartwig et al. 2000, Wallenstein et al. 2006). However, most of the reported correlations are fairly weak and are hardly ever linear.

Nitrous oxide can be produced by several nitrogen-transforming processes, including denitrification, nitrification and nitrifier denitrification, and possibly dissimilatory nitrate reduction to ammonium, anammox and N-fixation (e.g. Wrage et al. 2001, Jetten 2008). As far as we know, $N_2O$ consumption only takes place during denitrification, the process which reduces $NO_3^-$ to $N_2$ via several obligatory intermediates, including $N_2O$. Because of the multitude of processes involved, it is hard to assess the causal relationships responsible for the reported correlations between $N_2O$ emissions and environmental factors.

In order to further constrain the mechanism(s) responsible for the large variability in soil $N_2O$ emissions, we focused on soils in a riparian buffer zone with a high nitrogen loading. In these environments, $N_2O$ emissions are characterized by the occurrence of hotspots, that is, highly localized areas exhibiting much higher emission rates than the surrounding soil (Hefting et al. 2006a, Van den Heuvel et al. 2009). In a recent study, we showed that these hotspots are responsible for a large fraction of the total ecosystem $N_2O$ emission (Van den Heuvel et al. 2009). To our knowledge, a satisfactory explanation for the existence of the hotspots has not been presented so far. Existing studies rarely combine field measurements of $N_2O$ emissions with laboratory assays in which the potential rates of

$N_2O$ production and consumption of the soils are measured. It is the balance between production and consumption rates that determine the net $N_2O$ production, which, together with the soil-atmosphere gas exchange, yields the $N_2O$ emission rates from the soil.

The aim of this study was to determine whether $N_2O$ emission hotspots are caused by either enhanced $N_2O$ production rates or, alternatively, by low $N_2O$ consumption rates. Therefore, potential $N_2O$ production and consumption rates were determined using soil sampled inside and outside of $N_2O$ emission hotspots in the riparian buffer zone. Furthermore, the effects of $O_2$ and $NO_3^-$ concentrations on net $N_2O$ production were assessed to delineate their role in soil $N_2O$ emission hotspot behavior. We hypothesized that high $NO_3^-$ concentrations would increase the $N_2O$ production rate by increasing denitrification rates (e.g. Velthof et al. 1997, Hefting et al. 2003). In this way, the occurrence of $N_2O$ emission hotspots in the field would be regulated by fluctuations in soil water $NO_3^-$ concentrations.

**Methods**

*Site description*

A riparian buffer zone in the eastern part of the Netherlands "Het Hazelbekke" (N 52° 25', E 6° 52') was selected for this study. The vegetation consisted of alder (*Alnus glutinosa*) with a sparse understorey dominated by blackberry (*Rubus fruticosus*) and nettle (*Urtica dioica*). The buffer zone is located along a first-order stream in an agricultural region with grasslands and maize fields. The selection of the sampling site was based on previous measurements of $N_2O$ emissions by Hefting et al. (2006a) and Van den Heuvel et al. (2009). Sampling took place in the water saturated zone within 5 meters from the stream.

*Flux measurements and sample selection*

Field $N_2O$ emission rates were determined with closed flux chambers (13 $cm^2$ diameter, 25 cm height). $N_2O$ concentrations in the flux chambers were determined every eight minutes for 48 minutes using a photo-acoustic infrared gas analyzer (type 1302, Bruel & Kjaer, Denmark). Repetitive field soil sampling took place for each of the three batch experiments separately. Paired soil samples were taken in the field, one from the soil with the highest and one from soil with the lowest $N_2O$ emission rate based on at least 38 consecutive measurements at each sampling date (2007: Aug 17$^{th}$ and 28$^{th}$, Oct 22$^{nd}$ and 30$^{th}$, Nov 5$^{th}$ and Dec 11$^{th}$. 2008: Jan 28$^{th}$, Feb 21$^{st}$, Mar 18$^{th}$ and June 5$^{th}$). Soil cores (13 $cm^2$, 10 cm depth) were collected and mixed with local surface water (1:1 ratio) in a glass bottle. The slurries were stored cool overnight.

*Batch experiments*

The slurries were homogenized and distributed equally over 60 ml glass bottles capped with gastight rubber stoppers. Each bottle contained 30 ml of slurry. Headspace gas was replaced seven times with $N_2$. The oxygen concentration was monitored during the experiments using (TCD) gas chromatography (Agilent 6890, equipped with a Porapack Q column and a molecular sieve, Agilent technologies, USA). Several treatments were performed in the batch incubations. In a first series of experiments, $O_2$ (in air) was varied to obtain incubations with 5%, 2%, 1% and 0% headspace $O_2$ (resulting in dissolved $O_2$ concentrations of respectively 96, 38, 19 and 0 µM $O_2$ at equilibrium. 0.5 mM $NO_3^-$ was added (as $NaNO_3$) to the slurries in the $O_2$ dependence experiment. In a second series, $NO_3^-$ concentrations of 0.15, 0.30 and 0.50 mM were added (as $NaNO_3$) to anaerobic slurries (100% $N_2$). In a third series of incubations, successive additions of 50, 60 and 100 µM $N_2O$ (in $N_2/N_2O$ 10:1 mixture) were performed in the absence of $NO_3^-$ additions. All treatments were done in duplicate and control treatments (no additions) were used in all experiments. Additionally several pilot experiments were performed to confirm the reproducibility of the results of the separate series of experiments (data not shown). Glass bottles were incubated at 25°C and continuously shaken at 200 rpm. Gas equilibrium between shaken slurry and headspace was assumed under these conditions.

*Laboratory measurements*

Headspace gas samples were taken at 2 hour time intervals (unless otherwise specified) after the start of the incubation. The $N_2O$ concentration was determined by ECD gas chromatography (Agilent 6890, Porapack Q column, Agilent technologies, USA). Slurry samples were collected every 4 hours (unless otherwise specified). Slurry samples were centrifuged for 5 minutes at 13000 rpm and supernatant was filtered through C-18 Seppak filters (Waters Corporation, USA) for further analysis. $NO_3^-$ and $NO_2^-$ concentrations were determined by HPLC (HP1050, Agilent technologies, USA). $NH_4^+$ concentrations were determined colorimetrically with sodium-nitroferricyanide (140 mg/l) and phenol (14.1 g/l) in NaOH (5.2 g/l) with sodium-hypochloride (15% $Cl^-$, 3.07 ml/l). Soil moisture content and dry weight were determined gravimetrically by drying the soil slurries at 60°C for at least 48 hours.

*Statistical analyses*

Production and consumption rates were calculated from the linear portions of the concentration versus time plots of products and reactants, respectively. Paired sample t-tests (SPSS 16.0, SPSS inc. USA) were used to check for significant differences between rates or concentrations between high and low $N_2O$ flux samples.

## Results

*Field $N_2O$ emission fluxes and net laboratory $N_2O$ production rates*

Depending on the measured field $N_2O$ emission rates, the soil samples were classified as either low-flux sample or high-flux sample. Low-flux sample field emissions ranged from -3 to -94 µmol $N_2O$-N $m^{-2}$ $day^{-1}$ (the minus sign indicates that net consumption took place), while high-flux sample emissions ranged from 83 to 476 µmol $N_2O$-N $m^{-2}$ $day^{-1}$. In the anaerobic batch incubations (no $NO_3^-$ added), the high-flux sample produced 0.10 µmol $N_2O$-N $g^{-1}$ dw $hr^{-1}$ on average, while net $N_2O$ production of low-flux sample was often below the detection limit and always less than 0.04 µmol $N_2O$-N $g^{-1}$ dw $hr^{-1}$ (Table 1). Assuming that the top 10 cm of the soil were responsible for all of the $N_2O$ emission, high-flux sample produced on average 205 times more $N_2O$ in the batch incubations compared to the corresponding field emission rates.

**Table 1**

**Field $N_2O$ emission and production and consumption rates of $NO_3^-$ and $N_2O$ in anaerobic slurry incubations of high and low flux samples. Standard error of mean in parentheses.**

| Sample origin | Field $N_2O$ emission[a] (µmol N-$N_2O$ $m^{-2}$ $day^{-1}$) | Net $N_2O$ production[a] (µmol N-$N_2O$ g $dw^{-1}$ $hr^{-1}$) | Potential $N_2O$ consumption[b] (µmol N-$N_2O$ g $dw^{-1}$ $hr^{-1}$) | $NO_3^-$ consumption[c] (µmol N-$NO_3^-$ g $dw^{-1}$ $hr^{-1}$) |
|---|---|---|---|---|
| High flux sample | 234 (57) | 0.132 (0.037) | 0.43 (0.11) | 1.36 (0.65) |
| Low flux sample | -39 (13) | 0.017 (0.014) | 0.22 (0.05) | 1.66 (1.17) |

**[a] n=6, [b] n=4, [c] n=3**

*Effect of $O_2$ concentration on net $N_2O$ production*

In the batch experiment testing the effect of oxygen concentration, all treatments showed a steady net production of $N_2O$ followed by a period of net consumption, with a large difference in production between high-flux sample and low-flux sample (Fig. 1). Different $O_2$ concentrations did not affect net $N_2O$ production rates in the incubations during the first 10 hours. Higher $O_2$ concentrations did lead to a delay of the moment when net $N_2O$ consumption started, however. Hence, the total amount of net $N_2O$ produced was higher at the higher $O_2$ concentrations. Note, however, that the differences in net $N_2O$ production and consumption rates between experimental treatments were small in comparison to differences between high and low-flux sample. Oxygen concentrations dropped rapidly during the first hours of incubation, with about 50% of the original concentration in the first 2.5 hours followed by $O_2$ consumption rates of respectively 0.98, 0.51 and 0.16 µM $O_2$ $hr^{-1}$ for the 5%, 2% and 1% treatment.

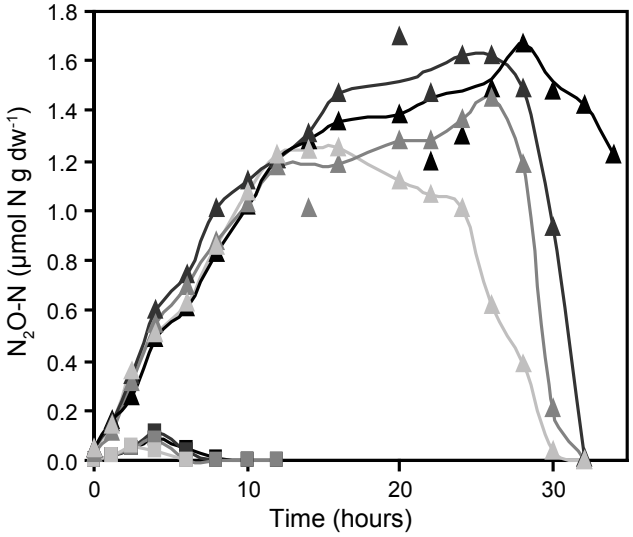



**Figure 1: Oxygen dependence of net $N_2O$ production rates of high- and low-flux samples in closed batch flasks. Darker lines/symbols show higher (start) $O_2$ concentrations. $N_2O$ concentrations (µmol $N_2O$-N g $dw^{-1}$) over time (hours). Squares represent low-flux samples, triangles represent high-flux samples.**

*$NO_3^-$ consumption*

Initial $NO_3^-$ concentrations were higher ($p<0.05$) in high-flux sample than low-flux sample (on average 0.24 and 0.10 mM, respectively). In non-amended batch experiments, the $NO_3^-$ concentration decreased to values below the detection limit in less than 4 hours. Additions of $NO_3^-$ (of 0.5 mM) showed that both low and high-flux sample were capable of reducing $NO_3^-$ concentrations of at least three times the *in situ* $NO_3^-$ concentrations within 10 hours (Fig. 2). The $NH_4^+$ concentrations increased linearly with time, but the increases were not stoichiometrically correlated to the $NO_3^-$ concentration decreases in either low or high-flux sample.

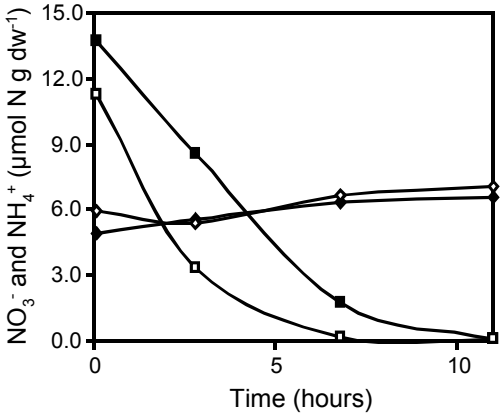



**Figure 2: $NO_3^-$ and $NH_4^+$ concentrations in anaerobic $NO_3^-$ amended batch incubations. $NO_3^-$ concentrations (µmol $NO_3^-$-N g $dw^{-1}$, squares) and $NH_4^+$ concentrations (µmol $NH_4^+$-N g $dw^{-1}$, diamonds) in high-flux samples (filled symbols) and low-flux samples (open symbols) over time (hours).**

*Effect of $NO_3^-$ concentration on net $N_2O$ production*

Higher added $NO_3^-$ concentrations (0, 0.15, 0.30 and 0.50 mM) resulted in higher amounts of net $N_2O$ produced, but had no effect on the initial $N_2O$ production rates (Fig. 3). Net $N_2O$ production was observed when $NO_3^-$ was present, net consumption of $N_2O$ started after $NO_3^-$ was depleted to very low concentrations (< 0.05 mM), both in high and low-flux sample (Fig. 3). High-flux sample produced more $N_2O$ compared to low-flux sample at all concentrations of added $NO_3^-$; in the treatment with the highest added $NO_3^-$ concentration the total net $N_2O$ production was around 2.5 times higher for high-flux sample compared to low-flux sample. The percentage of total $NO_3^-$ consumed recovered as $N_2O$, decreased with increasing added $NO_3^-$ concentration in the high-flux sample, from 44% to 26%.

*$N_2O$ production upon multiple $NO_3^-$ additions*

Both high and low-flux sample were capable of reducing successive $NO_3^-$ additions (data not shown). In low-flux sample incubations, repetitive $NO_3^-$ additions resulted in comparable net $N_2O$ production; only 4.6% (± 0.4) of the amended $NO_3^-$ was recovered as $N_2O$. In high-flux sample incubations, successive $NO_3^-$ additions led to progressively lower net $N_2O$ production; the $NO_3^-$ recovered as $N_2O$ decreased from 41% to 10% (Fig. 4a).

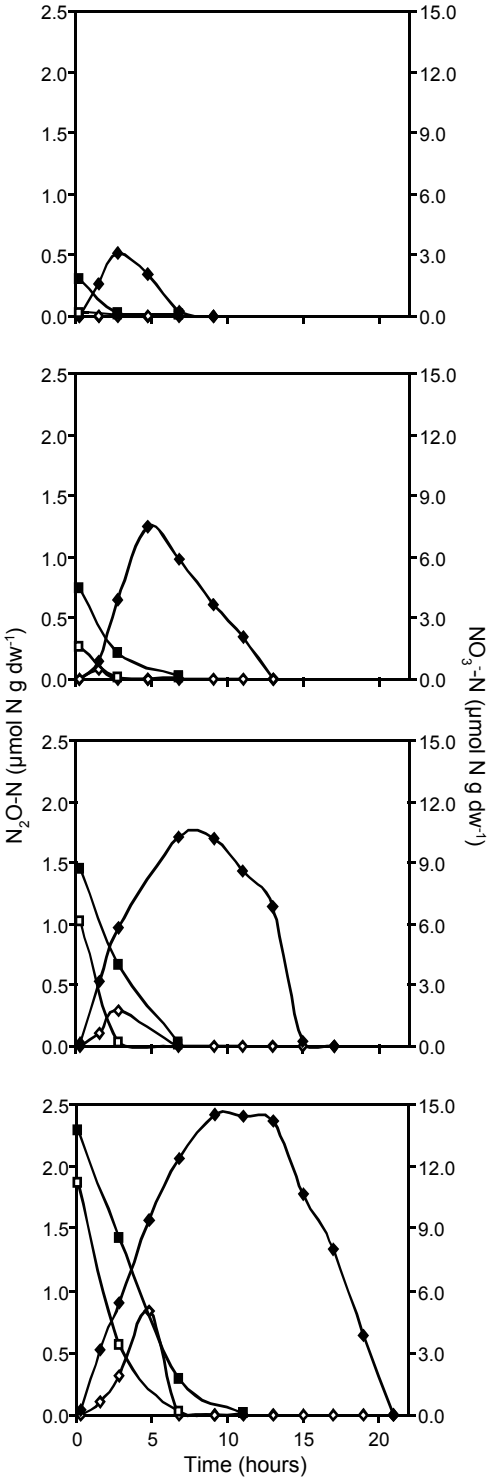



**Figure 3: Nitrate dependence of net $N_2O$ production rate of high- and low-flux samples in closed batch flasks. $N_2O$ (µmol $N_2O$-N g $dw^{-1}$ ) and $NO_3^-$ (µmol $NO_3^-$-N g $dw^{-1}$) concentrations over time (hours). Filled diamonds show $N_2O$ concentrations in high-flux samples, open diamonds in low-flux sample incubations. Filled squares show $NO_3^-$ concentrations in high-flux samples, open squares in low-flux sample incubations. Figures from top to bottom show increasing $NO_3^-$ additions (0, 0.15, 0.30 and 0.50 mM $NO_3^-$ respectively).**

*Potential $N_2O$ consumption rates*

Initial $N_2O$ consumption rates were 0.10 and 0.29 µmol $N_2O$-N $g^{-1}$ dw $hr^{-1}$ for low and high-flux sample, respectively. Potential $N_2O$ consumption rates were determined by repeatedly supplying $N_2O$ to the headspace of the incubation flasks. No lag phase was observed in the consumption of $N_2O$ if $NO_3^-$ was absent (Fig. 4b). Repeated additions of $N_2O$ increased the $N_2O$ reducing capacity of both low and high-flux sample considerably, to 0.33 and 0.73 µmol $N_2O$-N $g^{-1}$ dw $hr^{-1}$, respectively. Consumption rates of externally supplied $N_2O$ were significantly higher ($p<0.05$) in high compared to low-flux sample incubations.

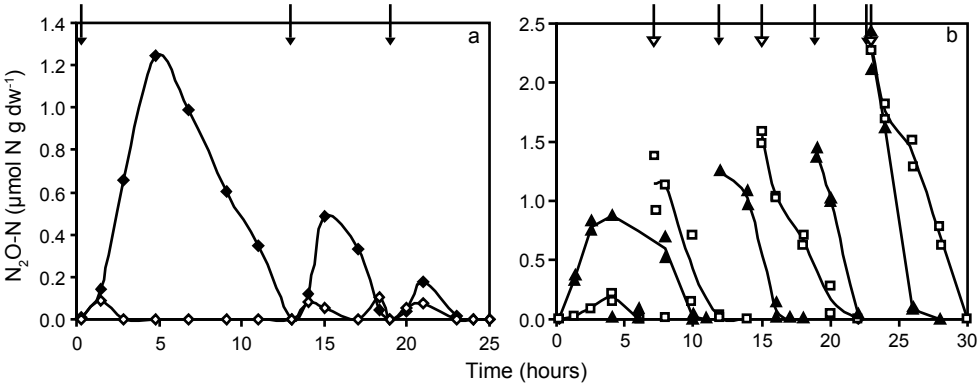



**Figure 4a and 4b: Effect of successive $NO_3^-$ and $N_2O$ additions on net $N_2O$ production and consumption respectively. $N_2O$ concentrations (µmol $N_2O$-N g $dw^{-1}$) over time (hours). Filled symbols show high-flux sample results, open symbols show low-flux sample results. Arrows in figure a) indicate successive 0.15 mM $NO_3^-$ additions, filled and open arrows in figure b) indicate successive $N_2O$ additions to high- and low-flux samples respectively.**

*Comparison of potential and net $N_2O$ production and consumption rates*

In low-flux sample, the average nitrate consumption rate was 1.66 µmol $NO_3^-$-N $g^{-1}$ dw $hr^{-1}$ (Table 1). As no significant amounts of $NH_4^+$ were produced, and only 1% of the $NO_3^-$ was recovered as $N_2O$ production (see Table 1), the $NO_3^-$ consumed was assumed to be completely denitrified to $N_2$. This would imply an average total rate of $N_2O$ reduction to $N_2$ of 1.66 µmol $N_2O$ $g^{-1}$ dw $hr^{-1}$. When $N_2O$ was supplied to the headspace, potential $N_2O$ consumption rates for low-flux sample were on average 0.22 µmol $N_2O$-N $g^{-1}$ dw $hr^{-1}$ (Table 1). Thus, on average, the potential consumption rate equaled only 13% of the $N_2O$ consumption rate derived from the $NO_3^-$ consumption.

In high-flux sample, on average 1.36 µmol $NO_3^-$-N $g^{-1}$ dw $hr^{-1}$ (Table 1) was consumed. Average net $N_2O$ production in high-flux sample was 0.13 µmol $N_2O$ $g^{-1}$ dw $hr^{-1}$. The $N_2O$ production therefore accounted for about 10% of the $NO_3^-$ consumed. The

average potential $N_2O$ consumption rate upon supplying $N_2O$ was 0.43 μmol $N_2O$-N $g^{-1}$ dw $hr^{-1}$ (Table 1), that is, about 32% of the $NO_3^-$ consumption rate. Together, net $N_2O$ production and potential consumption rates could thus account for about 42% of the $NO_3^-$ consumption.

**Discussion**

*Field $N_2O$ emission and laboratory $N_2O$ production similarity*

To further explore the causes of the high variability in soil $N_2O$ emissions at a small spatial scale (cm-dm) in the riparian buffer zone "het Hazelbekke" (Van den Heuvel et al. 2009), soils exhibiting low and high field $N_2O$ emissions were sampled to perform laboratory activity assays and derive $N_2O$ production and consumption parameters. The incubations exhibit a clear contrast in net $N_2O$ production between soil samples originating from locations with high and low field $N_2O$ emissions, respectively. The field $N_2O$ emission and laboratory $N_2O$ production rates thus yield internally consistent trends for hotspot versus non-hotspot locations in the riparian buffer zone.

The fate and transport of $N_2O$ through soils is still poorly known (Clough et al. 2005). Physical properties like soil moisture content, bulk density, aeration and soil structure are likely to affect $N_2O$ movement through the soil. Potentially, these factors play a role in the $N_2O$ emission variability. However, the observed patterns in the laboratory experiments show striking similarity with $N_2O$ emissions measured in the field. The nitrous oxide production in batch can be considered as a potential net $N_2O$ production measurement. In the incubations physical parameters like soil structure, soil moisture and soil aeration are very similar between high and low emission samples. From this, it can be concluded that these parameters are probably not the key factors in explaining $N_2O$ emission variability and $N_2O$ emission hotspot behavior. Although diffusion of $N_2O$ through the soil is prerequisite for $N_2O$ emission (Clough et al. 2005), increased or decreased values of diffusion did not appear to be responsible for $N_2O$ emission behavior. It is more likely that the observed emission patterns in the field reflect the production and consumption dynamics of the soil.

*Denitrification as $N_2O$ producing process*

Nitrification and denitrification are mentioned as the processes for microbial $N_2O$ production (Davidson et al. 1986). In the process of nitrification, $NH_4^+$ is converted to $NO_2^-$ and further to $NO_3^-$ under aerobic conditions. Denitrification takes place under anaerobic conditions and converts nitrate via nitrous oxide to dinitrogen gas. Earlier studies of the wet parts of the riparian buffer zone reported low nitrification and high denitrification potentials (Hefting et al. 2004). The study area is permanently wet (and thereby probably oxygen

limited), the soil is high in organic matter content (thereby providing electron donors) and there is a constant $NO_3^-$ influx; which are all favorable conditions for denitrification. In the incubations, a small amount of $NH_4^+$ production (so no consumption) was observed. As the production was not stoichiometrically correlated to $NO_3^-$ consumption, the increase was likely due to mineralization. The $NO_3^-$ on the other hand was consumed within a few hours and reached concentrations below the detection limit. It was observed that $N_2O$ production is only observed during $NO_3^-$ consumption, while at $NO_3^-$ limited conditions, $N_2O$ consumption takes place. This indicates that the $N_2O$ is being produced by denitrification. The observations are therefore in agreement with earlier studies and the expectations on denitrification potential.

The availability of $NO_3^-$ is often mentioned as the key parameter for $N_2O$ emission (e.g. Jordan et al. 1998, Hefting et al. 2003) during denitrification. The explanation for this phenomenon would be that higher $NO_3^-$ concentrations lead to higher denitrification rates which in turn would lead to higher gaseous nitrogen (including $N_2O$) production. Higher $NO_3^-$ concentrations did indeed lead to higher $N_2O$ production over time. However, the $N_2O$ production rates were similar over the range of $NO_3^-$ concentrations tested. Furthermore, low-flux sample exhibited higher $NO_3^-$ consumption rates than high-flux sample and at the same time produced less $N_2O$ (Table 1). Additionally, $N_2O$ recovery rates were lower at higher $NO_3^-$ additions. These observations do not match the idea that higher denitrification rates produce higher $N_2O$ production rates, but are more an indication that lower denitrification rates lead to higher emissions. This is in agreement with the general hypothesis that $N_2O$ production becomes more favorable than $N_2$ production under denitrification at limiting conditions (Van Cleemput 1998). The often observed relation between $NO_3^-$ concentration and $N_2O$ production is therefore likely in many cases not a causal relationship of $NO_3^-$ leading to more denitrification and therefore $N_2O$, but a co-occurrence of impeded denitrification leading to low $N_2O$ reduction rates in a system with a constant $NO_3^-$ influx.

*Incomplete denitrification leads to differences between high and low flux samples*

The above relates well to the idea that $N_2O$ reduction is an independent module within the denitrification pathway (Zumft 1997). In case of net $N_2O$ production, the $NO_3^-$ to $N_2O$ module works faster than the $N_2O$ to $N_2$ module. In this study, recovery levels of N as $N_2O$ were lower at higher $NO_3^-$ concentrations which could be explained by the rates of the two modules becoming more balanced probably due to an increased $N_2O$ reduction. Successive $NO_3^-$ and $N_2O$ additions resulted in lower net $N_2O$ production and higher consumption rates, respectively. Increasing $N_2O$ reduction activity is hypothesized to be caused by an increased number of denitrifying bacteria , an increased amount of $N_2O$

reducing enzyme expression ($N_2OR$), an increased enzyme activity or a combination of these. Increased expression rates are the most plausible explanation to our opinion, as numbers of denitrifiers and enzyme activity rates are not likely to significantly increase over the course of hours. The $N_2O$ reduction increase seemed to be enhanced by successive $N_2O$ additions, which indicates that $N_2O$ reduction may be regulated by $N_2O$ or by one of the other N-compounds. According to Zumft (1997) the main exogenous regulators for denitrification are low oxygen tension and presence of respirable N oxide. $N_2OR$ is more inhibited by oxygen availability than the other denitrification enzymes (Morley et al. 2008). However, in this study, an effect of oxygen on net $N_2O$ production during $NO_3^-$ consumption was not observed, although it took longer to start net $N_2O$ consumption. The expression of $N_2OR$ is found to be slower than the expression of the other denitrification enzymes (Otte et al. 1996). In that way $N_2O$ consumption would increase over time irrespective of the successive $NO_3^-$ or $N_2O$ additions and $O_2$ concentrations.

The observation that $O_2$ availability did not affect $N_2O$ production rates was surprising as $O_2$ concentration is regarded as an important regulator for denitrification (Knowles 1982). After a strong initial decrease in headspace oxygen, probably caused by slurry-atmosphere gas equilibration, a constant consumption of oxygen was observed. This may imply that our assumption of gas equilibrium was only true from 2.5 hours onwards. The calculated dissolved oxygen concentrations at 2.5 hours were 0, 0.3, 0.9 and 2.5% (for the 0, 1, 2 and 5 % treatments respectively). Several studies (e.g. Bergaust et al. 2008) used a similar range of dissolved oxygen concentrations values and observed a strong increase of $N_2O$ production at the higher $O_2$ concentrations. However, Takaya et al (2003) and Meyer et al. (1990) showed that levels of $N_2O$ production were respectively low and absent in the presence of $O_2$ presence. These studies, together with the observation in the current paper show that the effect of $O_2$ concentrations on net $N_2O$ production is ambiguous. Given the absence of an effect of oxygen in continuously shaken soil slurries, effects of oxygen in the sampling site are unlikely because of oxygen diffusion limitation under water saturated soil conditions.

All in all, we can conclude that (oxygen and nitrate) treatments in high- and low-flux sample lead to similar responses. The explanation for large differences in net $N_2O$ production between low and high-flux sample can not be related to $O_2$ or $NO_3^-$ concentrations. Impaired denitrification (and especially hampered $N_2O$ reduction during denitrification) appears to be responsible for the net $N_2O$ production. As the pattern of net $N_2O$ production in incubations and the field were highly comparable, the process for $N_2O$ emission can be explained by the same processes. Incomplete denitrification will therefore be responsible for $N_2O$ emission hotspots in the field.

The question remains why denitrification and $N_2O$ reduction are hampered in some but not in other samples. Low- and high-flux sample were always taken at the same time, so differences induced by weather or differences in experimental set up details can be excluded. Next to physical and biological factors, chemical factors can play a role in the $N_2O$ emission variability. Levels of electron donors or acceptors (besides $O_2$ and the nitrogen species) may vary among the different soil samples. These levels can influence denitrification and amounts of intermediates build up, but often do not have a great effect under natural conditions (Ambus 1993). A shortage of electron donor could induce a (temporal) build up of intermediates. In the batch incubation, all $NO_3^-$ was being reduced after repeated additions, indicating that a shortage of electron donors was not very likely. Differences in electron donor degradability which could lead to higher or lower $N_2O$ production rates (Pfenning and McMahon 1997) were not tested. Another effect of chemical origin is the pH value. The rate of denitrification under field conditions has been shown to be lower under acidic conditions than under neutral or alkaline conditions (Simek and Cooper 2002). The effect of acidity could result in both a positive effect on $N_2O$ emissions by increasing the $N_2O$:$N_2$ ratio (Simek and Cooper 2002) as well as a negative effect by decreasing total denitrification rates (Yamulki et al. 1997).

Another possible explanation is the microbial biodiversity in the studied soil system. Holtan-Hartwig et al. (2000) demonstrated that soil slurries from different origins exhibit different $N_2O$ reductase kinetic parameters. These differences are attributed to intrinsic differences in the (denitrifying) community composition of the soils, which are being related to differences in annual $N_2O$ emission fluxes. Cavigelli and Robertson (2001) also show that taxonomic differences between soil samples of different origin influences in situ $N_2O$ production. Differences in the denitrifying community composition, and associated differences in the nitrous oxide reductase kinetic parameters, will persist during short term incubations of the soil samples. Furthermore, Holtan-Hartwig et al. (2000) showed a shift in the kinetics of $N_2O$ production during incubations that are reminiscent of those caused by the repeated $NO_3^-$ additions in our study. These authors suggest that a shift in dominant (active) bacteria is the explanation, but *de novo* synthesis of $N_2O$ reducing enzyme could also explain this effect. The denitrifying community composition may thus be the key regulator of the variations in $N_2O$ production measured in the batch incubations and $N_2O$ emission measured in the field. It is important to note that, in the case of our study soil samples do not originate from geographically distinct sites, but are located within a few meters from each other. It is therefore probable that the denitrifying community compositions of the soils samples are more alike than in the studies mentioned above.

**Conclusion**

We conclude that denitrification is the process for $N_2O$ production and emission in the riparian buffer zone studied. The mechanism behind variability in $N_2O$ emission in the field is variability in $N_2O$:$N_2$ production ratio during denitrification. High $N_2O$ emission soils show impaired denitrification and high $N_2O$:$N_2$ ratios. Hotspots for $N_2O$ emission can therefore not be explained by higher $N_2O$ production or lower $N_2O$ consumption rates, but by the imbalance between these, i.e., a low $N_2O$ production accompanied by an even lower $N_2O$ consumption. The underlying factor for the variation in $N_2O$:$N_2$ production ratios remains obscure and object of further study as $O_2$ and $NO_3^-$ levels could not explain this variation.

**Acknowledgements**

The authors would like to acknowledge "Vereniging Natuurmonumenten" for allowing us to perform this research in Het Hazelbekke. This research was funded by the Centre for Wetland Ecology (CWE). The authors would like to thank John Hermans and Boran Kartal for assistance with analytical procedures. Furthermore, Joost Keuskamp is acknowledged for the calculations on oxygen diffusion.

# Chapter 4

## Decreased $N_2O$ reduction by low soil pH causes high $N_2O$ emissions

RN van den Heuvel, SE Bakker, MSM Jetten and MM Hefting

**Abstract**

Quantification of harmful $N_2O$ emissions from soils is essential for mitigation measures. An important $N_2O$ producing and reducing process in soils is denitrification, which shows deceased rates at low soil pH. No clear relationship between $N_2O$ emissions and soil pH has yet been established because also the relative contribution of $N_2O$ as the denitrification end product decreases with pH. Our aim was to show the net effect of soil pH on $N_2O$ production and emission and to answer the question: could soil pH be a tool to predict $N_2O$ emissions? Therefore experiments were designed to investigate the effects of pH on $NO_3^-$ reduction, $N_2O$ production and reduction and $N_2$ production in incubations with pH values set between 4 and 7. Furthermore field measurements of soil pH and $N_2O$ emissions were carried out. In incubations, $NO_3^-$ reduction and $N_2$ production rates increased with pH and net $N_2O$ production rate was highest at pH 5. $N_2O$:$N_2$ production ratio decreased exponentially with pH. $N_2O$ reduction appeared therefore more important than $N_2O$ production in explaining net $N_2O$ production rates. In the field, a negative exponential relationship with strong predictive value for soil pH against $N_2O$ emissions was observed. Soil pH could therefore be used as a predictive tool for average $N_2O$ emissions in the studied riparian ecosystem. The occurrence of small low pH spots in soils may explain the high spatial variability of $N_2O$ emissions and occurrence of local $N_2O$ emission hotspots. Future studies should focus on the mechanism behind small scale soil pH variability and the effect of manipulating the pH of organic rich soils where $N_2O$ emissions are predominantly regulated by denitrification activity.

**Introduction**

Nitrous oxide ($N_2O$) is a greenhouse gas (Kroeze 1994) with a high global warming potential and depletes stratospheric ozone (Crutzen 1970). Emission sources to the atmosphere should be quantified in order to mitigate emissions, but an accurate quantification is not present due to the lack of data (Stehfest & Bouwman 2006). Soils are an important source of $N_2O$ emissions and are believed to contribute approximately 65% of the total $N_2O$ emitted to the atmosphere (Seitzinger et al. 2000). Soil $N_2O$ emissions are hard to predict since these show high spatial and temporal variability, so that large uncertainties exist in the predictions (Bouwman et al. 1995). This variability is caused by the fact that several interacting nitrogen transforming processes in the soil influence $N_2O$ emissions (Jetten 2008), and as these processes are all influenced by environmental conditions, numerous factors influence $N_2O$ emissions. The frequent occurrence of hotspots and hot moments of denitrification and $N_2O$ emissions further complicates the predictability (McClain et al. 2003, Groffman et al. 2009).

Denitrification is the main $N_2O$ producing process in wet soils (Bremner 1997). Studies using pure cultures of denitrifiers grown in the laboratory show a strong effect of pH on denitrification with maximal rates at near neutral pH (Valera and Alexander 1961, Thomas et al. 1994, Thomsen et al. 1994). At the same time, the relative contribution of $N_2O$ production increases at lower pH (Thomsen et al. 1994). Recently, Saleh-Lakha et al. (2009) showed that expression levels of nirS and cnorB (genes for nitrite reductase and nitric oxide reductase, respectively) were severely impacted by pH 5 relative to pH values of 6 to 8. These phenomena can, however not directly be extrapolated to the soil environment where heterogeneous conditions prevail and microbial communities with numerous species interact and compete with each other. Simek and Cooper (2002) reviewed the present studies on the interaction between soil pH and denitrification. One of the conclusions was that pH is a master variable on denitrification in soils as it affects denitrification rates, denitrification end products and denitrifier community composition. Depending on the experimental set up and specific soil characteristics, contrasting conclusions can be drawn on the effect of pH on denitrification and $N_2O$ emissions. It is however, generally accepted that also in soils, denitrification rates are highest at near neutral pH (Simek and Cooper 2002), although adaptation to natural soil pH can occur (Parkin et al. 1985).

Low soil pH decreases denitrification rates, but simultaneously the contribution of $N_2O$ production to total nitrogenous gas production by denitrification is increased (Koskinen and Keeney 1982, Simek and Cooper 2002). The net effect of low pH on $N_2O$ emissions is therefore not straightforward. The soil environment will further complicate this relation as other environmental factors will influence denitrification rates. Furthermore, the

soil environment can trap the produced nitrogenous gasses and therewith create a window of opportunity for further reduction (Clough et al. 2005).

In the studies of Koskinen and Keeney (1982) and Parkin (1987) it was shown that the rate of organic C mineralization rather than pH controls the rate of denitrification in C-limited systems. However, in soils with high organic matter contents, organic electron donors are not the limiting factor for denitrification (Chapter 5, Hefting 2003). In previous studies of organic rich, water saturated soils, receiving constant high $NO_3^-$ influx (Hefting et al. 2006b), $N_2O$ emissions were shown to be caused by denitrification. Furthermore, oxygen and nitrate were shown not to be the drivers for $N_2O$ emission variability in this soil (Chapter 3). We hypothesized that pH could be a determining factor for $N_2O$ emissions in a water saturated, organic and nitrate-rich soil.

Our aim was to show the net relation between soil pH and $N_2O$ production and consumption in water-saturated soils and to answer the question: can soil pH value be a tool to predict $N_2O$ emissions? Therefore an experiment was set up to investigate the effects of pH on $NO_3^-$ reduction, $N_2O$ production and reduction and $N_2$ production. Furthermore field measurements of soil pH and $N_2O$ emissions were carried out.

**Methods**

*Experimental set up*

The short time effect of soil pH on $N_2O$ and $N_2$ production and $NO_3^-$ reduction was studied in incubations with pH values adjusted between 4 and 7. For this experiment, two soil samples were taken from the field; one with a relatively low pH value (pH 4.45) and one with a higher pH value (pH 5.25). These samples will be referred to as soil 4 and soil 5 respectively in this paper. The soil samples were diluted (1:1) with local surface water and stored at 4°C overnight until the experiment was performed.

The soil slurry was thoroughly homogenized using a commercial kitchen blender and sieved with a 2-mm sieve. From this slurry 19 ml was added to 60 ml glass bottles. A soil pH range between 4 and 7 (with intervals of 0.5 pH-unit for slurries from soil 4 and intervals of 1 pH-unit for slurries from soil 5) was created by adding a pH-buffer (1 ml of 1.20 mM $NaHCO_3$) and 0.5 M HCl or 0.5 M NaOH. A control treatment was supplied with 1 ml demineralised water instead of pH-buffer. All treatments were performed in duplicate. To obtain a final concentration of 0.5 mM $^{15}NO_3^-$, 1 ml of $^{15}N$-labeled 10 mM $NaNO_3$ (purity >99%, Cambridge Isotope Laboratories Inc., USA) solution was added to the bottles. The bottles were sealed with butyl rubber stoppers and aluminum caps. Headspaces of all bottles were changed (eight times) to a helium atmosphere at 1.5 bar. During the experiment all bottles were shaken at a horizontal shaker at 4°C.

*Analyses of $NO_3^-$, $NO_2^-$, $N_2O$ and $N_2$*

For 66 hours, every six hours 100 µl headspace gas was extracted from the bottles with a gas-tight needle and concentrations and isotopic composition of $N_2$ and $N_2O$ were determined, using a gas chromatograph mass spectrometer (Agilent 5975c MS coupled to Agilent 6890 GC, equipped with a Porapack Q column). At the start of the experiment and after 6, 12, 24, 36, 48 and 69 hours 1 ml of the slurry was sampled for $NO_3^-$ and $NO_2^-$ determination. $NO_3^-$ concentrations were determined using an auto-analyzer (SA-40, Skalar Analytical BV, The Netherlands) and $NO_2^-$ concentrations were determined colorimetrically by adding 0.9 ml ultrapure water, 1 ml 1% sulfanilic acid in 1 M HCl and 1 ml 0.1% naphtylethylene diaminedihydrochloride to 0.1 ml sample.

*Field $N_2O$ emission and soil pH data*

$N_2O$ emission and soil pH were measured in a forested riparian ecosystem ("Het Hazelbekke" in the Netherlands). Measurements took place within a maximum distance of 5 meters from the stream. Field measurements were performed in early spring 2008 and 2009. $N_2O$ emission measurements were performed in an area in the ecosystem with high $N_2O$ emission variability based on earlier measurements (Hefting et al. 2006a, Van den Heuvel et al. 2009). $N_2O$ emissions and pH were determined from ten clusters of seven soil spots, to obtain 70 measurements. $N_2O$ emissions were measured with a photo-acoustic infrared gas analyzer (type 1302, Bruel & Kjaer, Denmark). The gas analyzer was attached to a multipoint sampler (CBISS Ltd., England) that sampled seven flux-chambers (13 $cm^2$, height 25 cm) and one atmospheric reference sample simultaneously. Each spot was measured for at least 40 minutes, with 8-minute intervals. Soil samples were taken with a soil corer and stored cool (4$^oC$) overnight until further processing. The pH of the soil samples was determined with a pH meter (WTW, Germany) in the laboratory at the same day. Soil samples were weighed and diluted 1:1 with surface water from "Het Hazelbekke". The pH of the samples was determined after 10 minutes shaking at a horizontal shaker.

*Statistical analysis*

Statistical analyses were performed using SPSS 16.0 (2007, SPSS Inc., USA). Paired sample t-tests were used to evaluate differences between conversion rates ($NO_3^-$, $N_2O$ and $N_2$) of soil 4 and soil 5. $N_2O$:$N_2$ production ratio was ln-transformed before using a paired sample t-test for evaluating differences between soil 4 and soil 5. A non-parametric Kolmogorov-Smirnov test was used to test for significant differences between average values of $N_2O$ emissions of soil spots with a different pH values.

**Results**

*pH-dependent $N_2O$ and $N_2$ production*

The short term effect of pH on $N_2$ and net $N_2O$ production was studied in a series of slurry incubations over a pH range from 4 to 7. pH of the original soil sample was 5.25 for soil 5 and 4.45 for soil 4. $N_2O$ production rates were significantly ($p=0.001$) lower in soil 5 compared to soil 4. Net $N_2O$ production rate was highest at pH 5 (Fig. 1a and 1b). Net $N_2O$ production rate decreased with pH between values of 5 and 7. Soil 4 had a $N_2O$ production rate at pH 5 of 0.055 μmol N g $dw^{-1}$ $hr^{-1}$, while at pH 7 this rate was 59% lower. At values below pH 5, net $N_2O$ production of soil 4 was about 15% lower relative to the production at pH 5. Soil 5 had a net $N_2O$ production about 35% lower at pH 4 relative to pH 5.

$N_2O$ production rates were linear over the first period, followed by net $N_2O$ consumption (Fig 2a and 2b). The period of $N_2O$ production was dependent on pH. At pH 7, all $N_2O$ was consumed within 138 hours (Fig. 2b), while net consumption at pH 4 did not start before 138 hours and took over 400 hours to complete (Fig. 2a).

$N_2$ production showed a different pattern over time. The $N_2$ production started immediately at the beginning of the incubation and $N_2$ concentrations increased until 200 hours at pH 7 (Fig 2b). At pH 4, $N_2$ production was insignificant until net $N_2O$ reduction started, followed by an increase in $N_2$ production. The whole process took more than 400 hours to complete after the start of the incubation (Fig 2a). $N_2$ production rates increased with pH. At pH 4 $N_2$ production was close to the detection limit and was about 1.3% and 2.5% of the $N_2$ production at pH 7, for soil 5 and soil 4 respectively (Fig. 1). $N_2$ production rates were comparable between soil 5 and soil 4.

The above described production patterns resulted in a pH-dependent $N_2O$:$N_2$ production ratio in the first phase of the incubations of both soils. Soil 4 had a $N_2O$:$N_2$ production ratio of 67 at pH 4 and decreased exponentially with pH to 1.36 and 0.81 at pH 6.5 and 7 respectively, indicating that at pH 6.5 and pH 7 initial $N_2O$ and $N_2$ production rates were comparable (Fig. 3). For soil 5, a similar exponential decrease was observed in $N_2O$:$N_2$ production ratio against pH. The ratios, however, were more than 30% lower ($p=0.09$) in the soil with originally higher pH.

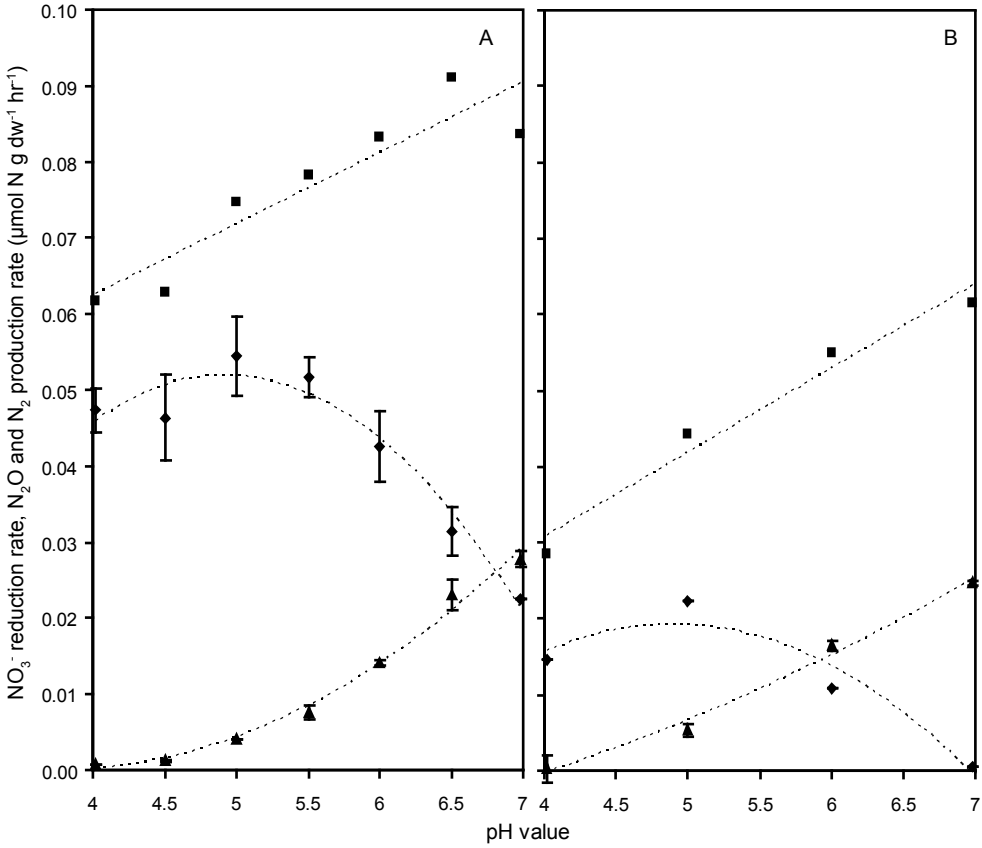



**Figure 1a and 1b: Effect of pH on $NO_3^-$ reduction and $N_2O$ and $N_2$ production in incubations of soil 4 (for explanation see text, 1a) and soil 5 (1b). $NO_3^-$ reduction rate (squares, µmol N g $dw^{-1}$ $hr^{-1}$), $N_2O$ production (diamonds, µmol N g $dw^{-1}$ $hr^{-1}$) and $N_2$ production (triangles, µmol N g $dw^{-1}$ $hr^{-1}$) are shown. Dotted lines show best linear fit (1a: $r^2$=0.84, 1b: $r^2$=0.97) for $NO_3^-$ and 2nd order polynomial fit for $N_2O$ (1a: $r^2$=0.95, 1b: $r^2$=0.92) and $N_2$ (1a and 1b: $r^2$=0.99). Error bars (for $N_2$ and $N_2O$ datapoints) show standard deviation.**

*pH dependent $NO_3^-$ reduction*

Not only $N_2O$ and $N_2$ production, but also $NO_3^-$ reduction was dependent on pH. $NO_3^-$ reduction was higher at higher pH values (Fig. 1a and 1b). $NO_3^-$ reduction rates were significantly (p=0.001) lower in soil 5 compared to soil 4. In the incubations with pH 5 to 7 (Fig. 2b) of soil 4, nitrate concentrations decreased linearly over time from 0.9 mM to below the detection limit (0.1 mM) within 70 hours. Nitrate concentrations in pH 4 and 4.5 incubations decreased to 0.2 mM over the course of 70 hours. Total N-gas ($N_2O$+$N_2$) production rates were 20 to 40 % lower than the measured $NO_3^-$ reduction rates in soil 4 (Fig. 1a). No significant $NO_2^-$ production was observed during the conversion of $NO_3^-$ to $N_2O$ and $N_2$. NO production was not determined.

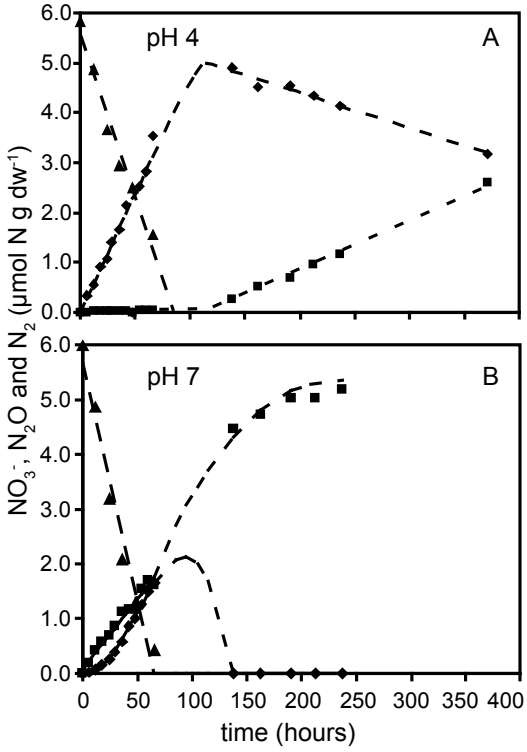



**Figure 2a and 2b: Nitrogen concentrations in incubations of soil 4 at pH 4 (2a) and pH 7 (2b) over time. $NO_3^-$, $N_2O$ and $N_2$ concentrations (µmol N g $dw^{-1}$ $hr^{-1}$, triangles, diamonds and squares respectively) are shown.**

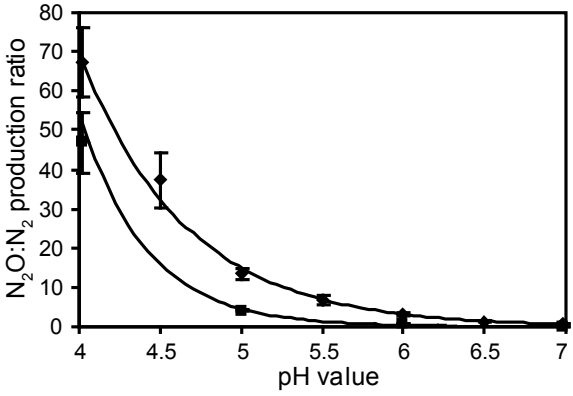



**Figure 3: Effect of pH (x-axis) on $N_2O$:$N_2$ production rate (y-axis). Trend lines shows best negative exponential fit for soil 4 (upper line, diamonds, $r^2$=0.99) and soil 5 (lower line, squares, $r^2$=0.99). Error bars show standard deviation.**

*pH dependent field $N_2O$ emission*

Field measurements showed a soil pH range of 3.9 to 6.6 in the sampling area. $N_2O$ emissions in the field ranged from -2.1 to 16.9 mg N-$N_2O$ $m^{-2}$ $day^{-1}$ with an average value of 1.8 mg N-$N_2O$ $m^{-2}$ $day^{-1}$. $N_2O$ emission values were not randomly distributed; soils spots with lower pH showed higher average $N_2O$ emissions, ranging from 4.44 mg N-$N_2O$ $m^{-2}$ $day^{-1}$ at soil spots with pH between 4 and 4.5 to 0.35 mg N-$N_2O$ $m^{-2}$ $day^{-1}$ for soils with pH between 6 and 6.5 (Fig. 4). In the field, 25% of the soil spots had a pH below 5; these soil spots were responsible for 77% of the total $N_2O$ emission. Predictive values of negative exponential relationships were 0.22 for soil pH against $N_2O$ emissions and 0.97 for average $N_2O$ emissions against 0.5 pH unit intervals.

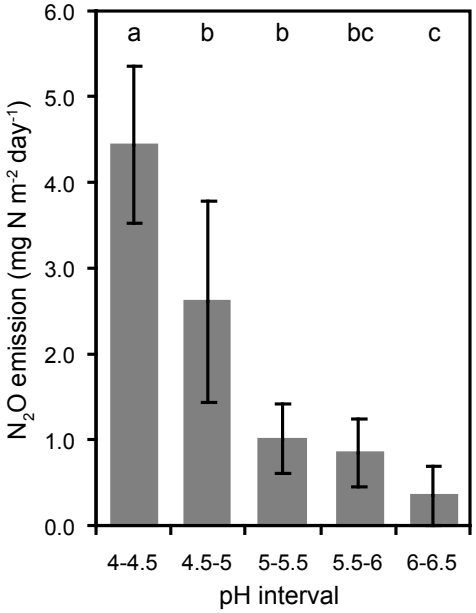



**Figure 4: Field $N_2O$ emission of soil spots with a range of soil pH values. Average $N_2O$ emissions (mg N $m^{-2}$ $day^{-1}$) against intervals of 0.5 pH units (error bars show standard error of the mean). Different letters represent significant different groups.**

**Discussion**

This research aimed at testing whether effects of pH on denitrification can explain relations between soil pH and $N_2O$ emissions. Therefore soil slurries were incubated at a range of pH between 4 and 7 and $N_2O$ emissions of soils with similar pH were evaluated in the field.

*Effect of pH on denitrification rates*

In the laboratory experiments with pH treatments over the range of 4 to 7, $NO_3^-$ reduction and $N_2$ production both significantly decreased at lower pH values. Denitrification rates in studies with comparable experimental set ups have shown a wide range of values, from 0.007 µmol N g $dw^{-1}$ $hr^{-1}$ (Enwall et al. 2005) to 0.6 µmol N g $dw^{-1}$ $hr^{-1}$ (Holtan-Hartwig et al. 2000). This range covers the rates found in our study (0.03-0.09 µmol N g $dw^{-1}$ $hr^{-1}$), indicating that the rates were not unusually high or low. Net $N_2O$ production rate appeared to have an optimum at pH 5. Furthermore, it was observed that at lower pH significant $N_2O$ reduction did not start until $NO_3^-$ was depleted, concurrent with the results of Blackmer and Bremner (1978) and very similar to the results reported by Thomsen et al. (1994) who used *Paracoccus denitrificans* pure culture. Although there are some differences regarding the results and set up between this study and the study of Thomsen et al. (1994), the resemblance is striking, indicating that when using highly homogenized soil slurries, results may be comparable to studies using pure cultures of denitrifying bacteria.

At lower pH values, total $N_2O$ production is decreased because of the decreased denitrification (defined as $NO_3^-$ reduction). However, $N_2O$ reduction to $N_2$ is also (and more significantly) decreased at lower pH, resulting in increased $N_2O$ product ratios; a well known phenomenon (Simek and Cooper 2002). In the present study, this resulted in maximum net $N_2O$ production rates at pH 5 and a prolonged period of high $N_2O$ concentrations in the incubations with lower pH values. $N_2O$ reduction appeared to be more important than $N_2O$ production in predicting net $N_2O$ production rates. Bandibas et al. (1994) however, showed a positive correlation between mean $N_2O$ emission and soil pH and no effect of pH on $N_2O$ emission was observed by Cuhel et al. (2010). This shows that although the pH effects on both denitrification and $N_2O$ product ratio are known, the effect on net $N_2O$ production can be different, depending on soil characteristics other than pH, including the denitrifying community.

The two types of soil (soil 4 and soil 5, low and higher pH respectively) showed comparable responses on the pH treatment. This implies that the pH treatment was overriding other parameters influencing denitrification in the studied soil. The absolute rates of $NO_3^-$ reduction and $N_2O$ production, however, were lower in the soil with field pH

5.25. Because pH and electron acceptor availability were known and electron donor availability was assumed not to be limiting (Chapter 5), we hypothesize that differences in denitrifier community were responsible for the higher activity of low pH soil. Cavigelli & Robertson (2001) and Holtan-Hartwig et al. (2000) also showed that denitrifier communities under controlled conditions can differ in their responses to environmental conditions (although pH was not controlled in the study of Holtan-Hartwig et al. (2000)). As it is generally believed that the size of the denitrifying community is smaller in acidic soils (Simek and Cooper 2002), we hypothesize in this study that pH acted as a short term control for denitrification rates in the incubations, while other environmental factors have acted as controls on the denitrifier community in the original soil samples.

*Relation of soil pH with $N_2O$ emissions*

$N_2O$ emissions were comparable to emissions found in studies of other riparian buffer ecosystems (Dhondt et al. 2004, Teiter and Mander 2005) and recent studies in the same area (Van den Heuvel et al. 2009, Chapter 3). Like in many other studies (e.g. Brumme and Beese 1992, Hefting et al. 2006a, Van den Heuvel et al. 2009) a high variability of $N_2O$ emissions was observed. This was especially true for spots with lower pH values, where almost the full range of $N_2O$ emissions was covered. A strong increase in average $N_2O$ emissions was observed at soils with lower pH values. These results resemble those of Weslien et al. (2009) where seven soil spots were measured and where soil pH had a strong correlation with $N_2O$ emissions. As shown in our study, low pH values decrease both $N_2O$ production and reduction. The observed negative exponential relation between soil pH and $N_2O$ emission shows great similarity with the $N_2O$:$N_2$ ratio observed in the batch incubations. This suggests that $N_2O$ reduction not only determines net $N_2O$ production, but is also one of the determining parameters for soil $N_2O$ emissions. It is important to note that $N_2O$ emission is not only determined by net $N_2O$ production but also by the movement of $N_2O$ through the soil and by gas exchange between soil and atmosphere (Clough et al. 2005). The diffusion barrier may be the explanation for the fact that $N_2O$ emissions were not highest at pH 5 (where the highest production is observed) but at lower pH values. As long as $NO_3^-$ is present, no significant $N_2O$ reduction is observed below pH 4 and 4.5, so all produced $N_2O$ will eventually be emitted to the atmosphere. At pH 5, both $N_2O$ production as well as $N_2O$ reduction are increased relative to pH 4 and if the produced $N_2O$ is either trapped in the soil or transported to another soil layer, the potential for reduction to $N_2$ increases (Clough et al. 2005).

*Soil pH as predictive tool for $N_2O$ emissions and $N_2O$ emission hotspots?*

Could pH be used as a predictive tool for $N_2O$ emissions? A high variability of $N_2O$ emissions was observed, especially at spots with low soil pH. On a single sample basis, soil pH had a low predictive value for $N_2O$ emissions, probably because of this high variability. However, intervals of pH units showed strong predictive power in order to predict average $N_2O$ emissions. Part of the variation in the field may be explained by the fact that next to decreased $N_2O$ reduction, sufficiently high denitrification rates and gas exchange with the atmosphere are prerequisites which have to be met for high $N_2O$ emissions to occur. If one of the latter prerequisites is not met, soil spots with low pH and therewith low $N_2O$ reduction will not emit high amounts of $N_2O$. However, if these prerequisites are met, high $N_2O$ emissions will occur and in that way, soil pH value could be a valuable predictive tool for average $N_2O$ emissions.

The exponential relationship between soil pH and $N_2O$ emissions can be an explanation for the phenomenon of $N_2O$ emission hotspots which were reported and subject of research in several studies (Hefting et al. 2006a, Groffman et al. 2009, Van den Heuvel et al. 2009). Several mechanisms have been shown to cause $N_2O$ emission hotspots in other ecosystems (Groffman et al. 2009); we argue that in the ecosystem in our study, low soil pH spots cause $N_2O$ emission hotspots. In soils with spatial and temporal variability in pH, a period of high denitrification establishing an enlarged and activated denitrifier community followed by a period of low pH with low $N_2O$ reduction capacity could create extreme $N_2O$ emission hotspots.

*Consequences for future research*

Soil pH was shown to be a key factor in variability of $N_2O$ emission. The soil exhibited pH values from lower than 4 to higher than 6.5 within only a few meters distance; a phenomenon also reported by Yang et al. (1995) who showed that high random variation exists even at the centimetre scale. The mechanism behind this variability in soil pH has not been studied in the current research, but the alkalizing and acidifying effects of denitrification and nitrification, respectively, may play a role. A better understanding of the underlying mechanisms of pH variability and predictability would further improve our ability to forecast and model $N_2O$ emissions and other pH affected soil processes. Furthermore, the strong increase of $N_2O$ emissions at low pH spots raises the idea that if we are able to increase the soil pH of such an ecosystem, we may be able to drastically decrease the amount of $N_2O$ emitted to the atmosphere. The use of liming and therewith increasing the soil pH as a measure to mitigate $N_2O$ emissions has been studied in a limited number of natural ecosystems. In temperate forest soils, effects of liming on $N_2O$ emissions differed from a decrease of more than 70% (Brumme and Beese 1992) to no significant

change (Klemedtson et al. 1997) and an increase of more than 60% (Butterbach-Bahl et al. 1997). Hence care should be taken to generalize results between soil types and ecosystems. Future studies should be undertaken to investigate the effect of raising the soil pH in organic rich soils where $N_2O$ emissions are caused by denitrification.

**Conclusion**

From the results presented in this paper, it can be concluded that the negative effect of low pH on $NO_3^-$ reduction and especially $N_2O$ reduction leads to increased $N_2O$ production. Although many factors affect $N_2O$ emissions, the pH effect on $N_2O$ reduction leads to increased $N_2O$ emissions in the studied riparian buffer system. In this riparian ecosystem, soil pH value can not be used as a predictive tool for $N_2O$ emissions of single samples, but soil pH value has strong predictive power for average $N_2O$ emissions of a sufficient amount of samples. Furthermore, soil pH appears to be a likely causal factor for $N_2O$ emission hotspot behavior.

**Acknowledgements**

The authors would like to thank Gerrit Rouwenhorst for analytical assistance and "Vereniging Natuurmonumenten" for allowing us to perform this research in "Het Hazelbekke". The authors would like to thank Jos Verhoeven, Boran Kartal and Philippe Van Cappellen for advice and valuable discussions. This research was funded by the Centre for Wetland Ecology (CWE).

# Chapter 5

## Soil denitrification at pH 4 by a *Rhodanobacter* dominated community

RN van den Heuvel, E van der Biezen, MSM Jetten, MM Hefting, and B Kartal

**Abstract**

Soil denitrification is a major source of nitrous oxide emission that causes ozone depletion and global warming. Low soil pH influences the relative amount of $N_2O$ produced and consumed by denitrification. Furthermore, denitrification is strongly inhibited in pure cultures of denitrifiying microorganisms below pH 5. Soils however, have been shown to denitrify at pH values as low as pH 3. Here we used a continuous bioreactor to investigate the possibility of significant denitrification at low pH under controlled conditions with soil microorganisms and naturally available electron donors. Significant $NO_3^-$ and $N_2O$ reduction were observed for three months without the addition of any external electron donor. Batch incubations with the enriched biomass showed that low pH as well as low electron donor availability promoted the relative abundance of $N_2O$ as denitrification end product. Molecular analysis of the enriched biomass revealed that a *Rhodanobacter*-like bacterium dominated the community in 16S rRNA gene libraries as well as in FISH-microscopy during the highest denitrification activity in the reactor. We conclude that denitrification at pH 4 with natural electron donors is possible and that a *Rhodanobacter* species may be one of the microorganisms involved in acidic denitrification in soils.

**Introduction**

Already in 1946, it was suggested that soils could be a significant source of atmospheric nitrous oxide ($N_2O$) (Adel 1946) which is a strong greenhouse gas and depletes stratospheric ozone (Crutzen 1970). In the last decades, substantial progress has been made identifying the sources and mechanisms of nitrous oxide emission in natural and agricultural soils. Nitrous oxide was identified as a free, obligatory intermediate in denitrification (Knowles 1982), which has been shown to be an important source for nitrous oxide emissions from many soils (Webster and Hopkins 1996a). Several environmental factors have been observed to negatively affect soil denitrification rates and to simultaneously increase the $N_2O$:$N_2$ ratio (Van Cleemput 1998) and thereby possibly increasing soil $N_2O$ emissions (Chapter 4).

In addition to oxygen and nitrate, pH is one of the key parameters affecting denitrification rates in the environment (Knowles 1982, Simek and Cooper 2002). In pure culture studies it was shown that low (as well as high) pH hampers denitrification and generally denitrification was observed to be insignificant below pH 5 (Valera and Alexander 1961). In soils and experimental soil slurries, however, significant denitrification has been reported to occur at pH values below 4 (e.g. Muller et al. 1980, Baeseman et al. 2006) with strongly increased $N_2O$ emissions from low pH soils (Chapter 4). This apparent contradiction between pure cultures and soil incubations may be explained by the fact that the conditions used in pure culture studies differ significantly from the *in situ* conditions in soils and soil slurries. The main differences between pure culture studies and soils are the degree of known and controlled parameters and the interactions between soil microorganisms. Furthermore, the majority of microbial diversity can not be isolated using traditional culturing techniques and are therefore quite obscure (Rappe and Giovannoni 2003).

Another complicating factor in studying soil environments is the existence of microscale chemical patchiness (e.g. with deviating pH values). Alldredge and Cohen (1987) showed a pH gradient of almost one pH unit over a range of 2 mm. Soils may contain sediment or organic matter particles up to centimeters in size that could foster heterogeneity in environmental conditions, thereby creating favorable and less favorable spots for denitrification. For example, Parkin (1987) showed that an 80 mg organic matter particle was responsible for 85 % of the denitrification of a 98 g soil core. These examples show that average soil conditions must always be judged critically, as studied processes may occur under non-average circumstances. It is thus at present unknown whether the reported denitrification at low pH is a significant process in soils. It could be occurring either in small pH-neutral patches or be restricted to only a short time period not yielding enough energy for the denitrifiers to survive and grow.

pH not only affects the denitrification rate and the $N_2O$:$N_2$ product ratio, but also the denitrifying soil community composition (Wallenstein et al. 2006). If microorganisms that are able to denitrify at low pH would exist, low pH soils with available electron acceptors and donors would provide a niche for these microorganisms. Parkin et al. (1985) showed that slurries originating from pH 4 soils had maximum denitrification rates at values around pH 4, implying that the apparent denitrifying community was adapted to low pH. Unfortunately, no characterization of that denitrifying soil community has been made. In studies where microbial communities were characterized, correlations with denitrification parameters were either unclear or absent (Philippot and Hallin 2005). It is therefore unknown how conditions favorable for denitrification at low pH would affect quantity and diversity of species and furthermore it is unknown if species with the ability to denitrify at lower pH do exist.

In order to study the possibility of acidic denitrification, the *in situ* niche should be mimicked in the laboratory (Kartal and Strous 2008) including pH, electron donors and micronutrients. Despite several studies, the effects of indigenous organic electron donors in soils on denitrification are relatively unknown. Organic carbon was predicted to be important in creating a favorable niche for denitrifiers (Tiedje et al. 1982) leading to enhanced denitrification rates (Baudoin et al. 2009). Not only total denitrification rates but also the end-product ($N_2O$ or $N_2$) can be affected by the type of organic carbon (Dendooven et al. 1996, Mathieu et al. 2006). Soils contain a high variety of organic carbon components, which may or may not be utilized by denitrifying bacteria, however, enrichment studies to date mostly use glucose, methanol, acetate or other short-chain sugars at artificially high concentrations as organic electron donors resulting in well-known fast growing denitrifiers like *Paracoccus denitrificans* and *Pseudomonas aeruginosa* (Blaszczyk 1983). To prevent the enrichment of well-known fast growing denitrifiers, only naturally available electron donors were used in this study to enrich environmentally relevant denitrifiers.

Pure culture studies and field studies both have their advantages and disadvantages. To avoid the artificial conditions of pure cultures and to create a field-like environment in the laboratory we used a bioreactor to investigate the possibility of significant denitrification at low pH under controlled conditions with soil microorganisms and naturally available electron donors. Using a complementary array of methods (enrichment, activity tests, clone libraries, FISH and isotopic labeling) we showed that denitrification at low pH with natural electron donors is possible and that a *Rhodanobacter* species was preferentially enriched at in the bioreactor.

**Methods**

*Reactor operation*

A Sequencing Batch Reactor (SBR with a working volume of 4.5 L was inoculated with 0.5 kg of soil slurry (1:8 soil:surface water) originating from a riparian buffer zone (described in Hefting and De Klein 1998). Each cycle of the SBR consisted of 11 hours of filling, followed by 45 minutes of settling and 15 minutes of drawing of the supernatant. The SBR was filled with 0.25 L tap water containing 5mM $NO_3^-$ and (during phase II and III) soil extract each cycle at a flow rate of 0.174 ml $min^{-1}$. Soil extract was obtained by diluting soil with local surface water (1:1), followed by vigorous blending using a commercial kitchen blender and finally autoclaving the liquid phase of the soil slurry. The SBR and medium vessel were flushed continuously with Argon/$CO_2$ (95/5%, 25 ml $min^{-1}$) to maintain anoxic conditions. The pH was controlled by automated addition of HCl or $NaHCO_3$ when necessary. The SBR was operated at room and stirred at 200 rpm.

*pH incubation experiments*

Effect of pH on $N_2O$ and $N_2$ production was investigated by incubating reactor material at various pH values. 60 ml bottles were filled with 30 ml reactor material and capped with butyl-rubber stoppers. 5 mM $NO_3^-$ (GR grade, Merck, Germany) or $^{15}NO_3^-$ (>99%, Cambridge Isotope Laboratories Inc., USA) was added and pH was set using a 691 pH meter (Metrohm, Switzerland) and addition of NaOH or HCl to the desired pH value. Headspace gas was replaced (ten times) by alternately applying under-pressure and He (in pH 5, 6 and 7 batches) or 90%/10% He/$CO_2$ mixture (pH 2, 3 and 4 incubations). The bottles were incubated at room temperature and were shaken continuously at 200 rpm. pH was measured again at the end of the incubations.

*Analytical Methods*

$N_2O$ concentrations in the off gas of the bioreactor or in the headspace of the batch incubations were determined with a gas chromatographer (Agilent 6890) equipped with a porapack Q column and an electron capture detector (ECD). Isotopic composition of $N_2$ and $N_2O$ were determined with a gas chromatographer (Agilent 6890) coupled to a quadruple inert mass spectrometer (Agilent 5975c). $NO_3^-$ concentrations were determined colorimetrically as described previously (Kartal et al. 2006).

*Clone libraries*

Reactor material was sampled at the start of the reactor and the end of each phase. DNA was isolated using Powersoil DNA isolation kit (MoBio, USA) according to the manufacturer's instructions. Isolated DNA was amplified with bacterial primer set 616F-

630R using GoTag Mastermix (Promega, USA) followed by cloning in *Escherichia coli* with the pGEM-T Easy cloning vector (Promega, USA). Plasmid-DNA was isolated using Genejet plasmid miniprep kit (Fermentas, Canada). Subsequent sequencing was performed by the DNA Diagnostics Center of Radboud University Medical Center using M13 forward and reverse primers. A BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to check for related sequences in GenBank.

Rhodanobacter thiooxydans *incubations*

*Rhodanobacter thiooxydans* (Lee et al. 2007, DSMZ 18863$^T$) pure culture cells were obtained from DSMZ (Braunschweig, Germany) and checked for growth and $NO_3^-$ and $N_2O$ reducing ability on R2A medium. $^{15}NO_3^-$ was added to the incubations (pH 4 and 7) and concentration of 15N-labeled $N_2O$ and $N_2$ were determined.

*Fluorescence in situ hybridization (FISH)*

Reactor material samples were taken each month and chemically fixed, followed by hybridizations with fluorescently labeled oligonucleotide probes as described previously (Schmid et al. 2003). Hybridized and DAPI-stained cells were counted in representative pictures from monthly samples. A specific probe was designed based on the consensus 16s-rRNA sequence. Running a probe-check (Loy et al. 2008) against the SILVA and RDP II databases resulted in 3 distinct hits without mismatches; *Rhodanobacter thiooxydans*, *Rhodanobacter lindaniclasticus* and an uncultured gamma proteo bacterial species. Allowing for 1 or 2 mismatches resulted in several hits, all in the *Xanthomonadaceae* family. The (Cy3 labeled) probe was purchased from Thermo Fisher Scientific (Ulm, Germany), tested on *Rhodanobacter thiooxydans* pure culture and showed specific hybridization at 30% formamide concentration.

**Results**

*$NO_3^-$ and $N_2O$ reduction in the denitrifying reactor*

Soil slurry, collected from a riparian ecosystem with $N_2O$ production at pH 4 (Chapter 4), was incubated in a bioreactor that was operated as a Sequencing Batch Reactor (SBR; Irvine and Busch 1979) previously established as a robust technique for the enrichment of slow growing microorganisms (Strous et al. 1998) and fed with 5mM $NO_3^-$ containing influent. Three distinct phases could be distinguished in the operation of the reactor (Fig 1):

Phase I: Increasing $NO_3^-$ reducing activity on endogenous natural electron donors at pH 4 for three months (day 0 to 91);

Phase II: Decreasing $NO_3^-$ reducing activity despite supplied natural electron donors at pH 4 for seven months (day 92 to 300);

Phase III: Increasing $NO_3^-$ reducing activity on supplied natural electron donors at pH 6 for one month (day 301 to 329).

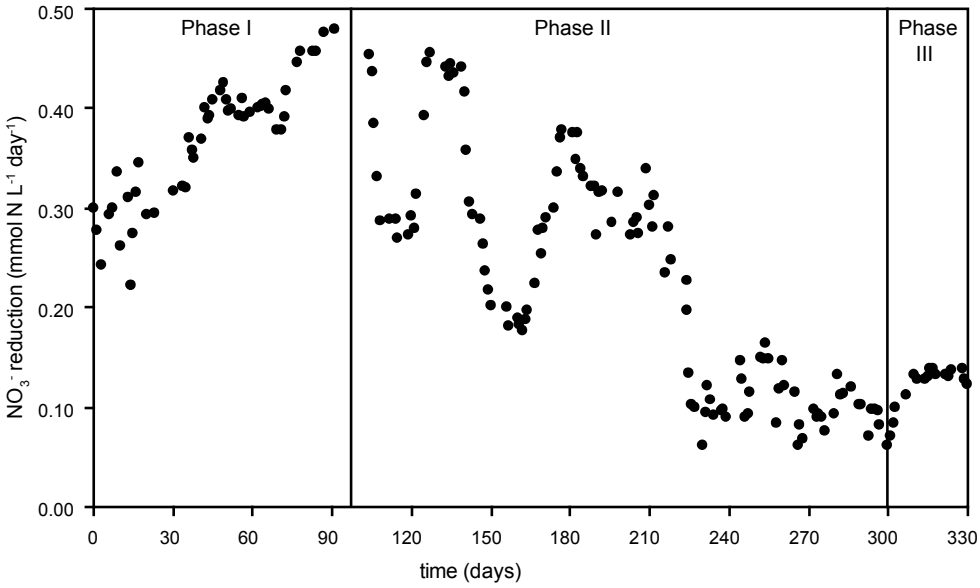



**Figure 1: $NO_3^-$ reduction (in mmol N $L^{-1}$ $day^-$) in a denitrifying bioreactor at pH 4 over time. During phase I no electron donors were supplied, during phase II naturally available electron donors were supplied, at the start of phase III, the pH was increased to 6.**

Nitrate reduction was observed immediately after the start of the reactor. During phase I, $NO_3^-$ reduction increased from 0.25 mmol $L^{-1}$ $day^{-1}$ to almost 0.50 mmol $L^{-1}$ $day^{-1}$ (Fig 1). During phase II, slow but gradual decrease in $NO_3^-$ reduction was observed to values below 0.10 mmol $L^{-1}$ $day^{-1}$. During phase II, short periods of increased activity (up

to 0.45 mmol $L^{-1}$ $day^{-1}$), were observed due to changes in and additions (soil extract) to the reactor influent. After the pH was increased from 4 to 6, $NO_3^-$ reduction increased approximately 20 %, marking the start of phase III. During phase III $NO_3^-$ reduction increased again above 0.10 mmol $L^{-1}$ $day^{-1}$ and a further increase in nitrate reduction activity over time was observed (Fig 2).

During the increase in $NO_3^-$ reduction in phase I, a decrease in $N_2O$ production was observed. No more than 10 % of the reduced $NO_3^-$ was recovered as $N_2O$ at the transition from phase I to phase II. During phase II, $N_2O$ was the main product of the $NO_3^-$ reduction and accounted on average for 90% of the reduced $NO_3^-$ (data not shown) and approximately for 100% at the end of phase II. At the transition to phase III, $N_2O$ production decreased within one day from 0.12 mmol $N_2O$-N $L^{-1}$ $day^{-1}$ to 0.07 mmol $N_2O$-N $L^{-1}$ $day^{-1}$. Up to 40 % of the $NO_3^-$ was converted to $N_2$ in phase III (Fig 2).

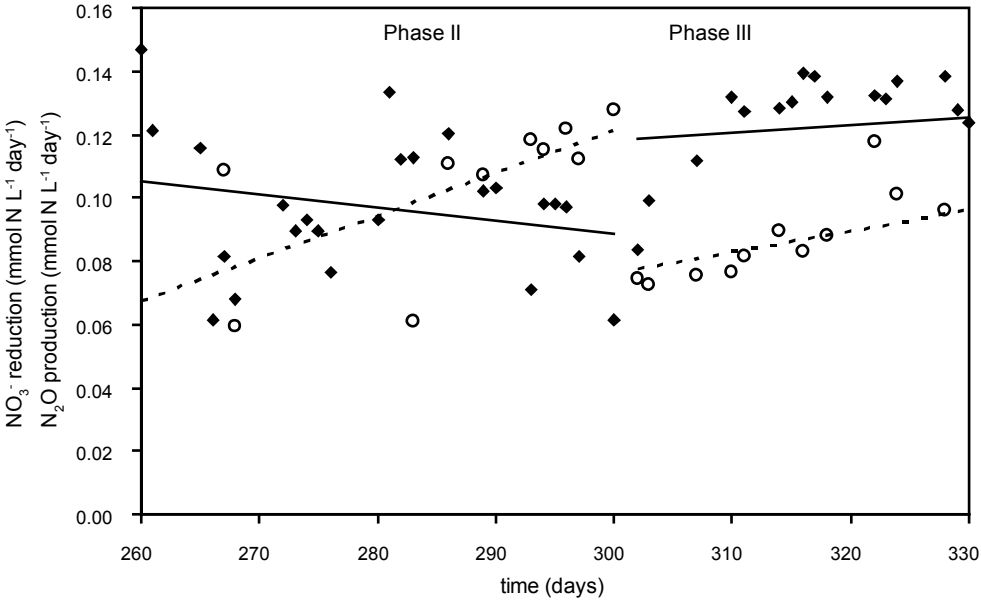
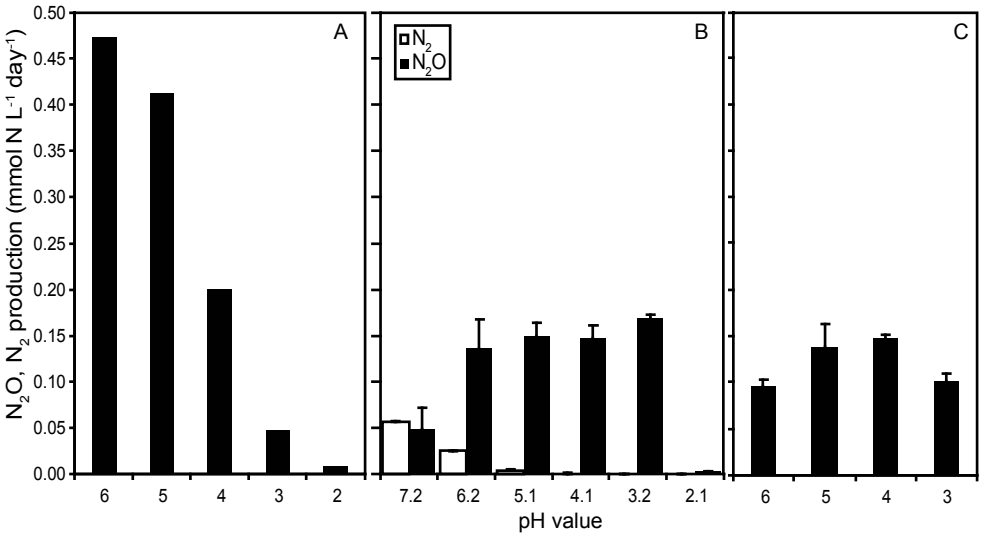



**Figure 2: $NO_3^-$ reduction and $N_2O$ production in the denitrifying bioreactor before and after a switch in pH. $NO_3^-$ reduction (black diamonds, solid trendlines, in mmol $L^{-1}$ $day^{-1}$) and $N_2O$ production (open circles, dotted trendlines, in mmol N $L^{-1}$ $day^{-1}$) over time. The pH of the reactor was increased from 4.0 to 6.0 on day 301.**

*pH batch incubations*

Additionally, $N_2O$ production was measured in a series of batch incubations with a range of pH values (3-7) in each of the three phases of the reactor. In phase I, $N_2O$ production increased with increasing pH (Fig 3a). Incubations performed with material harvested in phases II and III showed another pattern; at pH 6 and 7 $N_2O$ production rates

were lower than $N_2O$ production rates at pH 3, 4 and 5. Measurement of $N_2$ production showed that the decrease in net $N_2O$ production was attributable to reduction of $N_2O$ to $N_2$ at pH 6 and 7 (Fig 3b). Furthermore, significant $N_2O$ production was observed at pH 3 in all phases. In phase II, denitrification and $N_2O$ production rate at pH 3 were even higher than those at pH 4 to 7 (Fig 3b). Batch incubations of autoclaved samples showed that no significant $N_2O$ or $N_2$ was formed via chemo-denitrification in the range of pH 3 to 7 (data not shown).




**Figures 3a, 3b, 3c: Effect of pH on $N_2O$ production in batch incubations in the three phases of operation of the reactor (a: day 52, b: day 204, c: day 324). $N_2O$ production (y-axis, grey bars, mmol N $L^{-1}$ $day^{-1}$) in incubations with a range of pH values (x-axis). White bars in figure b show $N_2$ production (mmol N $L^{-1}$ $day^{-1}$). Error bars show standard deviation.**

*Electron donors*

The start of phase II was marked by a strong decrease in $NO_3^-$ reducing activity, most likely caused by depletion of endogenous electron donors. Addition of the original soil slurry to a batch incubation of the reactor material enhanced $NO_3^-$ reduction and $N_2O$ production by more than 70 % relative to the control treatment. Additions of (autoclaved) soil on day 119 (of reactor operation) to the reactor and addition of soil extract from day 163 on led to immediate short-term increases in $NO_3^-$ reduction, but could not prevent an overall decrease in $NO_3^-$ reduction rate over time (Fig. 1).

*Microbial diversity in the bioreactor*

16S rRNA clone libraries were constructed from samples of the original soil slurry and from samples of the reactor material taken at the end of each phase. The clone library constructed at the end of phase I showed that about 70% of the retrieved 16S rRNA gene fragments were highly similar to various species within the (gamma proteobacterial) *Rhodanobacter* genus. The consensus sequence showed the highest similarity (99%) to *Rhodanobacter thiooxydans* (Lee et al. 2007). Clone libraries constructed at phase II and III showed relatively lower abundance of *Rhodanobacter* sp. sequences (Fig 4).

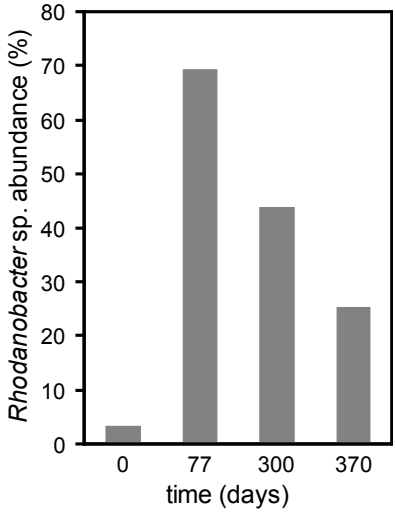



**Figure 4: Relative abundance of *Rhodanobacter*-like bacteria in the bioreactor over time. Percentage of *Rhodanobacter*-like bacteria in 16S rRNA gene clone libraries of reactor material. Total number of clones was 31, 42, 85 and 60 respectively.**

Hybridizations with an oligonucleotide probe (Rhodano1) based on the consensus 16S rRNA of the enriched species showed that more than 60 % of the DAPI cells count hybridized with the Rhodano1-probe in the first period of phase II (Fig 5a), after which a gradual decrease in dominance was observed during phase II (Fig 5b)

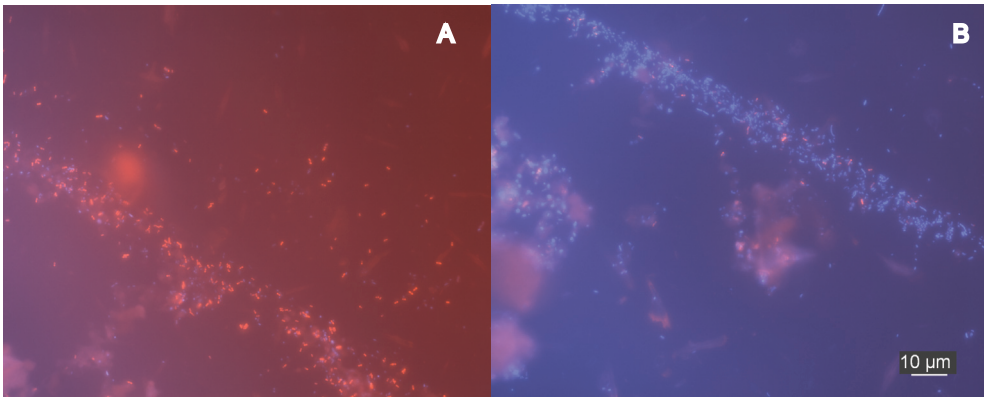



**Figure 5a and 5b: Abundance of *Rhodanobacter*-like bacteria in the bioreactor. Fluorescent in situ hybridization pictures of two samples of the reactor (a: day 134, early phase II, b: day 233, late phase II). Rhodano1 probe hybridized cells in red, DAPI-stained cells in blue.**

*Denitrifying activity of* Rhodanobacter thiooxydans

In order to test the ability of denitrification at low pH in known *Rhodanobacter* species, *Rhodanobacter thiooxydans* (DSMZ 18863$^T$) was grown on R2A media, and tested for denitrification. Cells showed growth over a pH range of 4 to 7. Furthermore, $NO_3^-$ as well as $N_2O$ reduction was observed both at pH 7 and pH 4.

**Discussion**

Soil slurry from a soil with denitrification capacity at low pH values was used as an inoculum for a continuously stirred sequencing batch reactor (SBR). $NO_3^-$ reduction and $N_2O$ production were observed at pH 4 in that reactor for almost a year. Aggregates in the bioreactor were not larger than 1 mm, the reactor material was highly homogenous and the pH was stable at pH 4, therefore pH was uniform within the SBR. We can therefore conclude that denitrification occurred at pH 4 in the entire reactor. Furthermore, significant denitrification was observed at pH 3 in batch incubations. This suggests that denitrification can be performed by soil microorganisms at pH 3 as was previously reported (Baeseman et al. 2006).

$N_2O$ was the main product of denitrification for most of the time during operation of the reactor. Similarly, our earlier studies using soil slurry incubations at pH 4 found $N_2O$ as the primary or sole end product of denitrification (Chapter 4). Interestingly, $N_2O$ production in the reactor strongly decreased over time in phase I (between day 35 to 90) during the highest $NO_3^-$ reducing activity, indicating an increase in $N_2O$ reduction. This increased $N_2O$ reduction could have been due to two possible events: first, the $N_2O$ reducing microbial community adapted to the conditions present, including low pH or

second, a community capable of reducing $N_2O$ at low pH was enriched. FISH analysis and clone libraries of this period showed that a *Rhodanobacter*-like bacterium constituted up to 70 % of the bacterial community. Furthermore, the closely related type strain showed $N_2O$ reduction capability at pH 4. Therefore, we believe that the enrichment of this genus was responsible for the increased $N_2O$ reduction, however it can not be excluded that other ways of adaptation were present as well.

Batch incubations with biomass from the SBR during phase I of the reactor material at various pH values showed higher $N_2O$ production at higher pH, which was most likely reflecting higher denitrification rates at higher pH values. Recent studies (Chapter 4, Cuhel et al. 2010) as well as the batch incubations with biomass from phase II showed that the $N_2O$:$N_2$ ratio increases at decreasing pH. Batch incubations during phase II and III showed that denitrification and $N_2O$ production rates were comparable over a pH range of 3 to 6 indicating that electron donor availability rather than pH was the main determining factor of $N_2O$:$N_2$ ratio during these phases. Incubations at pH 3 showed an increase in total $N_2O$ production in the phase II and III incubations, relative to the phase I incubation This may be explained as an adaptation of the microbial community to lower pH, and, more specifically, by enrichment of the *Rhodanobacter*-like bacterium, capable of denitrification at pH 3. However, at the higher pH ranges (5-6), $N_2O$ production was decreased relative to the phase I batch incubation, again suggesting that electron donor availability rather than pH was the strongest limiting factor at that time.

For more than three months, no addition of electron donors was necessary while denitrification rate was increasing. Many studies on different soil ecosystems showed that soil denitrification can be carbon limited (e.g. Burford and Bremner 1975, Swerts et al. 1996), but as $NO_3^-$ concentrations in the reactor were higher than under natural soil conditions (Chapter 4), denitrification does not seem to be limited by organic carbon in this soil. After 3 months, as the (easily biodegradable) electron donors decreased, denitrification rate decreased was also reduced, probably due to electron donor limitation. Enhancement of denitrification rates (by more than 70%) in batch incubations by additions of autoclaved natural electron donors supported this hypothesis. Addition of soil extract to the reactor could not prevent the denitrifying activity and relative numbers of *Rhodanobacter* sp. to decrease. Probably the electron donors supplied with the soil extract did not contain sufficient quantity or quality needed for *Rhodanobacter* sp. to grow and denitrify at high rates. As mentioned earlier, the observed increase of $N_2O$ production in phase II was probably due to electron donor limitation, similar to a decreased $N_2O$:$N_2$ production ratio in soil cores amended with organic carbon found by Weier et al. (1993). The electron donor limitation was likely also the reason for the relatively small effect of pH on total denitrification in the batch incubations of phase II and III. The observation that the $N_2O$:$N_2$

ratio increased both at low pH (in the phase II batch incubation) and at low electron donor availability is in good agreement with the findings of Van Cleemput (1998) that limiting conditions for denitrification are favorable for the formation of $N_2O$ rather than $N_2$.

As expected, in the original soil sample a relatively low abundance of *Rhodanobacter* sp. was observed due to the highly heterogeneous conditions in time and the multitude of microbial niches in soils. In general, presence and abundance of denitrifying bacteria and denitrification genes are poor predictors of denitrification rates (Cheneby et al. 1998; Philipot et al. 2009). Still, it has been shown that the denitrifying community is a factor in controlling denitrification rates (Cavigelli and Robertson 2001). Considering the enrichment of a single genus up to 70 % in phase I, it is highly likely that the conditions in the reactor, suited for denitrification at pH 4 with *in situ* electron donors, selected for the *Rhodanobacter* genus. Most of the seven described members of the *Rhodanobacter* genus were isolated from (acidic) soils (Nalin et al. 1999, De Clercq et al. 2006, Weon et al. 2007, An et al. 2009), which is in agreement with the present observation that *Rhodanobacter* sp. is a strong competitor in an acidic and anaerobic soil-like niche. Therefore, despite the limited initial abundance, *Rhodanobacter* sp. may be responsible for the majority of the denitrifying activity in low pH soils.

**Conclusion**

This study showed that (soil) denitrification also thrives at low pH. At pH 4, denitrification was observed at conditions comparable to the original soil ecosystem with respect to electron donors and acceptors by the enriched *Rhodanobacter* sp. This suggests that it is highly likely that indeed denitrification does occur in soils with pH values as low as 4 (Tiedje et al. 1982, Chapter 4) or even below 3 (Baeseman et al. 2006) for prolonged periods of time. It suggests that pure cultures of denitrifiers are capable of denitrification at lower pH than previously assumed.

**Acknowledgements**

The authors would like to acknowledge “Vereniging Natuurmonumenten” for allowing us to take soil samples in “Het Hazelbekke”. This research was funded by the Centre for Wetland Ecology (CWE). The authors would like to thank Baolan Hu, Katinka van de Pas-Schoonen, Katharina Ettwig and Wouter Maalcke for assistance in analytical procedures and maintenance of the reactor. Jos Verhoeven and Markus Schmid are acknowledged for valuable discussions.

# Chapter 6

## Summary, integration and perspectives

Nitrous oxide ($N_2O$) is a greenhouse gas with a global warming potential of 296 $CO_2$ equivalents and is involved in the depletion of the ozone layer. Studies on the sources of emission in the 1980's and 1990's (see e.g. Kroeze et al. 1999) were an important step towards understanding of emission mechanisms and ultimately the design of emission reduction measures. Through these studies it was revealed that natural and agricultural soils are important sources of $N_2O$ emissions and are responsible for about 50 % of the total $N_2O$ emitted to the atmosphere (Seitzinger et al. 2000). Nitrification (Bremner and Blackmer 1978), denitrification (Delwiche and Bryan 1976) and possibly nitrifier denitrification (Wrage et al. 2001) were shown to be processes responsible for $N_2O$ production and emission in soils. Remarkably, $N_2O$ emission patterns from soils often show typical hotspot behaviour at different spatial scales. Hotspots are soil spots which show a disproportionately high emission relative to the surrounding area. Riparian buffer zones (as a whole) are often hotspot ecosystems with high $N_2O$ emission in the landscape (Groffman et al. 2000). Within riparian buffer zones a hotspot pattern of $N_2O$ emission has been observed (Hefting et al. 2006a). These hotspots can be responsible for a large fraction of the $N_2O$ emission from the entire ecosystem or landscape, while only covering a small fraction of the area (Chapter 2). Integration of hotspots in mechanistic models is a big challenge (Groffman et al. 2009) and impossible without knowing the underlying mechanisms and the influencing biotic and biotic characteristics.

This study aimed at finding the underlying mechanisms and required conditions for the occurrence of $N_2O$ emission hotspot behavior within a riparian buffer zone. The study especially focussed on processes and conditions which are distinct between hotspots and non-hotspots. Therefore, nitrogen transformations were studied to identify the responsible $N_2O$ producing process(es). Based on previous studies, the environmental factors oxygen, pH and nitrate were expected to affect soil $N_2O$ emissions. Therefore, the effects of oxygen and nitrate were studied under controlled conditions in the laboratory to unravel their relation to hotspot behaviour. The effect of pH was studied in more detail both in batch and continuous culture experiments. Furthermore, it was attempted to characterize hot spots in terms of distribution, spatial scale, temporal scale and involved microorganisms.

**Denitrification is the source of $N_2O$ production and emission**

Environmental conditions in the studied riparian zone are favourable for denitrification: the soil is characterized by a high organic matter content (10-20%) providing sufficient electron donors. The constant $NO_3^-$ influx (> 1mM) provides electron acceptors and the water-saturated soil (up to 100% water filled pore space) close to the stream ensures the anoxic conditions that are required for denitrification. The riparian buffer zone as a whole, turned out to be a $N_2O$ emission hotspot in the landscape (Hefting et al. 2006a) and was therefore selected as a study site.

Slurry incubations of hotspot and non-hotspot soils were performed under various controlled conditions. The results of the incubation experiments described in chapter 2 clearly showed that $N_2O$ was predominantly produced by denitrification rather than by nitrification. But as $N_2O$ production was similar for soil slurries originating from hotspot or non-hotspot soil for all conditions, the relevance of denitrification on hotspots in the field was not demonstrated. In chapter 3, however, it is revealed that slurry incubations of hotspot soils showed high net $N_2O$ production, whereas incubations of non-hotspot soils showed less net $N_2O$ production. This indicates that the process responsible for field $N_2O$ emission is also producing $N_2O$ in slurry incubations under controlled conditions. In most incubations the conditions were anoxic and $NO_3^-$ or $N_2O$ was supplied, creating a favourable environment for denitrification. Furthermore, an experiment with additions of $^{15}N$-labelled $NO_3^-$ showed that the produced $N_2O$ originated from $NO_3^-$ and was further converted to $N_2$ in both hotspot and non-hotspot soils (data not shown). Slurry incubations with additions of $^{15}N$ labelled $NH_4^+$ showed no $N_2O$ formation originating from ammonium oxidation. No significant ammonium oxidation was observed under either aerobic or anaerobic conditions, suggesting that potential activity for nitrification and anammox is generally low in the studied soils. Moreover, no significant dissimilatory nitrate reduction to ammonium was observed. These results clearly demonstrated that denitrification is the responsible process for $N_2O$ emission from the studied riparian buffer zone soil.

The strong similarity between patterns of $N_2O$ emission in the field and $N_2O$ production in the shaken slurry incubations originating from different field spots indicate that variability in soil-atmosphere exchange parameters appear not to be important in controlling $N_2O$ emission variability. In contrast to that, differences in net $N_2O$ production appear to drive field $N_2O$ emission variability.

**Decreased $N_2O$ reduction rather than increased production causes increased emissions**

After determining that denitrification was the main process responsible for $N_2O$ emission, the question arose whether high net $N_2O$ production rates were either due to increased $N_2O$ production rates or to decreased $N_2O$ reduction. Slurry incubations of

hotspot soils unexpectedly showed lower potential denitrification rates (measured as total nitrate reduced or nitrogen-gases produced) compared to non-hotspot soils. At the same time more net $N_2O$ was produced by hotspot soils. Total denitrification rate thus was not predictive of net $N_2O$ production rate. The results from the slurry incubations in chapter 3 showed the same pattern more convincingly. Furthermore, incubations with additions of $NO_3^-$ and $N_2O$ clarified that the imbalance between $N_2O$ production and $N_2O$ reduction was a much better predictor of net $N_2O$ production than denitrification rate. These results implied that incoherencies between $N_2O$ production and reduction were mainly due to decreased $N_2O$ reduction, leading to increased net $N_2O$ production. Net production in these incubations showed qualitative similarity with field $N_2O$ emission implying that not only in incubations but also in soils, the imbalance between $N_2O$ production and reduction determined rates of $N_2O$ production and emission.

**Low pH increases the imbalance between $N_2O$ production and reduction in incubations**

The results in Chapter 3 showed that higher concentrations of $O_2$ and $NO_3^-$ increased the imbalance between $N_2O$ production and consumption in slurry incubations. However, it is also shown that $O_2$ and $NO_3^-$ concentrations cannot explain differences in net $N_2O$ production between hotspot and non-hotspot soils (chapter 3). It was observed that the hotspot soils (used for the slurry incubations described in chapter 3) generally had a lower pH value than the non-hotspot soils (data not shown). This observation and comparable information in the review of Simek and Cooper (2002) gave rise to the idea that pH could also be affecting the imbalance between $N_2O$ production and reduction. Hence, an experiment was set up, where the pH of the incubated soil slurries was altered. The results of chapter 4 showed a strong pH effect on both $N_2O$ production and reduction. A negative exponential relation was found between pH and $N_2O$:$N_2$ ratio (a measure for the imbalance). At pH values below 5 virtually no $N_2O$ was reduced to $N_2$ as long as $NO_3^-$ was present, similar to observations of Thomas et al. (1994) on pure cultures of *Paracoccus denitrificans*, indicating that the studied soil behaved like a denitrifying culture. These results were confirmed in chapter 5 where the $N_2O$ and $N_2$ production in batch incubations of an enrichment culture over a range of pH values was studied. Furthermore, the ratio of produced $N_2O$ to the amount of $NO_3^-$ reduction by the enrichment culture showed an immediate drop after increasing the ambient pH from 4 to 6 (chapter 5). These results led to the hypothesis that the low soil pH in hotspot soils could, in a comparable way as found in the slurry incubations, be responsible for an increased net $N_2O$ production and ultimately, $N_2O$ emission.

**Hotspot $N_2O$ emission from acidic soil spots**

Field measurements of soil pH and $N_2O$ emissions were undertaken to test the hypothesis that low soil pH could lead to $N_2O$ emission hotspots. A strong negative exponential relation was found between average soil $N_2O$ emissions and pH (Chapter 4). A high variability in $N_2O$ emissions was is found, similar to many earlier studies (e.g. Perdomo et al. 2009, Dinsmore et al. 2009, Yao et al. 2009, this thesis Chapter 2). Remarkably, this variability was only observed from soil spots with low pH values. In chapters 4 and 5 it was shown that at higher pH, $N_2O$ production and reduction were rather balanced resulting in low net $N_2O$ productions. The higher pH will thus prevent high $N_2O$ emissions independent of denitrification rate in high pH soil spots. In soil spots with low pH value, however, $N_2O$ production rates were increased relative to $N_2O$ reduction rates. The occurrence of high variability in $N_2O$ emissions at low pH can be explained by differences in denitrification rates; soils with low denitrification rates will emit low amounts of $N_2O$, whereas soils with high denitrification rates will produce hotspots of $N_2O$ emission.

**Source of soil pH variability**

The source of pH variability has not been investigated in the studied riparian ecosystem. It was observed that low pH spots were far less abundant than spots with higher pH value (data not shown). The average pH value of soil in the riparian zone studied is around 5.5 to 6. Spots with values below 5 are an exception in this particular zone. In order to investigate the source of soil pH variability, the focus should therefore be on the mechanism producing more protons and causing lower soil pH as this is the anomaly in the soil.

One of the obvious candidate processes for producing low soil pH is nitrification, since nitrifying bacteria and archaea produce two protons per ammonium consumed. Furthermore nitrification produces nitrate (and nitrite), which is required for denitrification. In chapter 3 it was, however, observed that the water-saturated soils had a low nitrification potential, indicating that nitrification was not the most likely candidate for creating acidic soil spots in this riparian buffer zone.

Another possible mechanism is upwelling of local acidic groundwater at certain spots or the lacked of upwelling of the more regional pH-neutral groundwater (Hefting et al. 2006b), both leading to local low pH spots. Upwelling of groundwater is however mostly related to much larger spatial scales than that of the hotspots found (Keller et al. 1988).

A third possibility is local acidification by plant root exudates. Roots excrete organic acids (Jones 1998). These organic acids can acidify the soil on a very local scale

and simultaneously provide organic electron donors for denitrification (Henry et al. 2008). In view of the obtained results on the underlying mechanism and spatial scale of $N_2O$ emission hotspots, the last explanation is the most plausible, however, no measurements have been undertaken to confirm this hypothesis.

**Possibility of soil denitrification in soils with pH below 5**

In chapter 4 it was described that significant denitrification was observed in soil slurries with pH values as low as pH 4, which is remarkable. Although soil denitrification at such low pH values was reported previously (Simek and Cooper 2002), pure culture studies of denitrifiers always indicated that denitrification is optimal around neutral pH (Valera and Alexander 1961, Thomsen et al. 1994). Denitrifying activity of cultured denitrifying bacteria strongly decreases with decreasing pH and is generally absent below pH 5 (e.g. Thomas et al. 1994). This gave rise to the idea that acidic soil denitrification might be happening inside soil aggregates with a pH more neutral than the bulk pH and possibly only for short periods of time. Chapter 5 describes a long term bioreactor experiment where the possibility of acidic denitrification was investigated. At pH 4, long term $NO_3^-$ reduction and $N_2O$ production were observed. Furthermore, a *Rhodanobacter*-like species was enriched and the closest related pure culture strain available (*Rhodanobacter thiooxydans* DSMZ $18863^T$) was found to be capable of acidic denitrification. Simultaneous $NO_3^-$ and $N_2O$ reduction at pH 4 was possible whenever ample electron donors were present. It was shown that the soil harbors an excess amount of electron donors relative to the natural availability of $NO_3^-$ as electron acceptor. Together, these results proved that denitrification can occur at pH 4 by endogenous soil microorganisms using naturally available electron donors and nitrate and provide evidence that *in situ* denitrification in low pH soils is highly likely.

**Mechanism of the effect of pH on $N_2O$ reduction (at $NO_3^-$ availability)**

Batch incubations with the enriched biomass from the bioreactor showed that low pH as well as low electron donor availability promoted the relative abundance of $N_2O$ as denitrification end product. The way in which low pH decreased $N_2O$ reduction has not been studied in this project, but two hypotheses can be formulated based on observations in other publications.

First of all, pH can have a direct effect on the $N_2O$ reduction at the transcriptional or enzyme level. In other words; pH could affect the expression or the activity of the (periplasmic) $N_2O$ reductase (NosZ). It has been shown that nitrite and nitric oxide reductase gene expressions are substantially decreased at low pH (Saleh-Lakha et al 2009). A similar effect on NosZ may exist, but no report on the effect of pH on NosZ expression

could be found. Sato et al. (1999) showed that purified $N_2O$ reductase of *Rhodobacter sphaeroides*, had an optimum activity at pH around 9 and less than 20% of the activity was left at pH 6. Coyle et al. (1985) showed that activation of nitrous oxide reductase of *Pseudomonas perfectomarina* was strongly enhanced by pre-treatment with buffers with higher pH. Also in this case, optimum values were around 9 and below pH 6 less than 1 % of the $N_2O$ reductase activity was measured. Whether the same pattern would be observed for $N_2O$ reductase activity of other denitrifying microorganisms is unknown. Furthermore, it is unclear whether the purified enzyme reacts *in vitro* the same way as it does *in situ*. The results described in chapter 3 and unpublished results from pilot studies showed that $N_2O$ reduction is not decreased at low pH values if $NO_3^-$ is not present. This indicates that a direct effect of pH on expression or activity of $N_2O$ reductase is not the most likely explanation for the observed overall effect of pH on net $N_2O$ production, as a direct effect of pH on $N_2O$ reductase would likely be independent of $NO_3^-$ availability.

Second, pH affects the availability of organic electron donors. At low pH, organic acids that can serve as electron donors may become insoluble (Piccolo 2002). Simultaneously chapter 5 and several other studies (Weier et al. 1993, Dendooven. et al 1996) have shown that at low availability of organic electron donors $NO_3^-$ reduction to $N_2O$ is favored over $N_2O$ reduction to $N_2$. Thus, a low pH may lead to a shortage of organic electron donors and thus to decreased $N_2O$ reduction rates. Chapters 4 and 5 of this thesis and Firestone et al. (1980) have shown that $NO_3^-$ reduction is favored over $N_2O$ reduction at low pH, which could be caused by a decreased availability of electron donors. However, chapter 5 showed increasing $N_2O$ reduction and enrichment of *Rhodanobacter* sp. despite a decreasing concentration of organic electron donors, indicating that in the studied soil a lack of electron donor (by low pH) is unlikely. The underlying mechanism of the effect of pH on $N_2O$ therefore remains speculative.

**Importance of other environmental factors**

Integration of the results discussed so far shows that low soil pH can be considered as the main factor causing $N_2O$ emission hotspots. In explaining and predicting $N_2O$ emission hotspots, other factors should however not be overlooked because of their potential impact on total $N_2O$ production (and consumption) in hotspots and non-hotspots. As denitrification is the process producing the majority of the $N_2O$, it has to operate at a sufficiently high rate for hotspots to occur. Electron donors, electron acceptors and denitrifying organisms should therefore be present simultaneously.

Absence of oxygen promotes denitrification as most denitrifiers are facultative anaerobes (Knowles 1982). The apparent water saturation of the soil ensures oxygen limited conditions and moreover, chapter 3 showed that the soil oxygen concentration is

probably not important in controlling the $N_2O$ emission hotspot behaviour in the studied soil. Differences in bioavailability and type of electron donors may cause differences in denitrification rates and $N_2O$ production (Pfenning and McMahon 1997), but electron donors seem to be abundantly present in the studied soil (Chapter 5). $NO_3^-$ availability is required for denitrification to occur, but Chapter 2 and 3 showed that $NO_3^-$ concentrations did not influence denitrification rates. Higher temperatures will promote denitrification rates (Holtan-Hartwig et al. 2002), but spatial differences in temperatures were assumed to be negligible in the study area, and therefore supposedly not relevant. Although the aforementioned environmental factors are important requirements for denitrification, the present conditions in the buffer zone soil seem to be ideal for acidity induced hotspot emissions.

Fluctuations of environmental conditions will influence denitrification rates in several ways. $N_2O$ reduction is the last step in the cascade of the reduction processes of denitrification. It has been shown that $N_2O$ reductase activity can lag behind the activity of other denitrification enzymes (Otte et al. 1996) although Baumann et al. (1996) showed that induction of the nitrous oxide reductase gene can be immediate for other denitrifying bacteria. Denitrifiers will adapt to changes in environmental factors by means of adjusting expression levels of catabolic proteins (Baumann et al. 1996). If $N_2O$ reductase lags behind the other reductases (Otte et al. 1996), a disturbance event will lead to relatively low $N_2O$ reduction and therewith to an increased $N_2O$ product ratio. Figure 7 in Chapter 2 showed a strong increase of $N_2O$ emission after (careful) accessing of the area by the investigator in the current study. This observation may be explained by the aforementioned disturbance effect. Restrained permission to access such ecosystems is therefore advisable.

**Denitrifying community**

It is generally believed that denitrifying bacteria are present in all soils, but not to the same extent. A higher abundance of denitrifiers will likely lead to increased denitrification rates (Cheneby et al. 2009). Furthermore, differences in species composition of the soil will possibly lead to differences in denitrification rates and product ratios (Philippot et al. 2009), although the relation is still poorly understood (Philippot and Hallin 2005). Several bacteria do not possess all genes of the denitrification reductases (especially NosZ is often lacking (Zumft 1997)), which means that these lack the ability of one or more of the reduction steps. Furthermore, species/communities vary in their responses to environmental factors (Holtan-Hartwig et al. 2000, Cavigelli and Robertson 2001). As stated before, studies on pure culture denitrifiers showed that denitrification and $N_2O$ reduction were absent at low pH in several denitrifiers (e.g. Valera and Alexander 1961, Thomsen et al. 1994), the present study showed that *Rhodanobacter thiooxydans* reduced

$NO_3^-$ and $N_2O$ simultaneously at pH 4. Environmental factors change denitrifier community composition and at the same time activity of the denitrifier community activity (Wallenstein et al. 2006). Interactions between factors (including pH), processes and denitrifying community composition are numerous. Despite these interactions, a strong relation between soil pH and $N_2O$ emission was observed (chapter 4), indicating that soil pH is a master variable for $N_2O$ emission variability in soils.

**Temporal and spatial scale of $N_2O$ emission hotspots**

The **distribution of $N_2O$ emission hotspots** was shown not to be random over the ecosystem (Chapter 2). The majority of the high emission spots were found in the zone no more than five meters from the stream. In earlier studies, high emission spots were also measured up to ten meters from the stream (Hefting et al. 2006a). It was shown that $NO_3^-$ rich groundwater interacted with the organic rich topsoil layer. In the current study (5 to 6 years later) a lower water table level was observed (approximately 10 cm at the field border), implying that the $NO_3^-$ rich groundwater enters the organic layer closer to the stream. As shown in Chapter 3, denitrification was the dominant process contributing to the high $N_2O$ emissions and the zone where $NO_3^-$ rich groundwater entered the organic soil will be the zone where the denitrification occurs. The observation of the distribution of high emission sites thus coincided with the theoretically favourable zone for high $N_2O$ emissions.

The **spatial scale of $N_2O$ emission hotspots** (as described in chapter 2) showed that hotspot behaviour was present at spatial scales from square centimetres to square metres. The highest emissions were found at the smallest spatial scale, and it was observed that these were always located in environments of elevated $N_2O$ emissions. Above, it is stated that a multitude of conditions are required for denitrification and these all influence denitrification rates, after which low pH creates $N_2O$ emission hotspots. Each additional factor moving in the favourable direction will likely increase denitrification rate. At higher denitrification rates, pH or other factors increasing the $N_2O$:$N_2$ product ratio, will have a higher effect on $N_2O$ emissions. The occasional occurrence of low soil pH in the study site leads to emission hotspots at spots with high denitrification rates, whereas $N_2O$ emissions will only be slightly enhanced in spots with low denitrification rates.

The **temporal scale of $N_2O$ emission hotspots** has been given only slight attention in this research. Hotspot $N_2O$ emission was observed for a limited number of days (data not shown). The cause of decreasing emissions over time was not investigated, but several hypotheses can be put forward: First of all; denitrification could be decreased (e.g. by shortage of $NO_3^-$ or to high levels of $O_2$). A second hypothesis could be that the pH of the spot increased (e.g. by denitrification, or by an increase of the pH of the groundwater

entering that particular soil spot). Furthermore it was shown in Chapter 5 that the denitrifying community composition can adapt to low pH and increase its ability to reduce $N_2O$ over time. This may have happened in a similar way in the low pH hotspot soil.

**Extrapolation of the mechanism behind $N_2O$ emission hotspots to other ecosystems**

This research was carried out in one ecosystem. As described above, the key factors for $N_2O$ emission hotspot existence are pH variability and denitrification occurrence. The latter is secured by high organic matter content of the soil, constant $NO_3^-$ influx, permanent high soil water content and presence of denitrifying organisms. The presence of a similar combination of factors in other ecosystems will likely lead to the same $N_2O$ emission behavior. Organic matter content, nitrate concentrations and soil water content are relatively easy to measure. Little is known, however, about the bacteria capable of denitrifying in acidic soils. Chapter 5 showed one example (*Rhodanobacter* sp.), still it is unknown how important this single species is in Hazelbekke, let alone in other ecosystems. The assumption that "everything is everywhere, but the environment selects" (Baas Becking 1934) suggests that micro-organisms capable of denitrification at low pH (whether or not a *Rhodanobacter* species) will be present in the above described system. If that is the case, organic wetland soils with nitrate input will show $N_2O$ emission hotspots at acidic soil spots. Systems experiencing other conditions will likely not respond to variability in acidity in the same way. However, other parameters than pH may also bring about hotspot behaviour: if a system experiences favourable conditions for denitrification, but one variable is increasing the imbalance between $N_2O$ production and consumption, $N_2O$ emission hotspots may develop. Weier et al. (1993) showed that C source concentrations, water content and nitrate concentrations affected $N_2O$:$N_2$ product ratios from soil cores. Simultaneously, Thomas et al. (1994) showed that oxygen, nitrate, pH and different carbon sources all affected $N_2O$:$N_2$ ratios. In riparian buffer zone Het Hazelbekke and probably also in similar ecosystems, low pH leads to $N_2O$ emission hotspots, but the studies of Thomas et al. (1994) and Weier et al (1993), together with the above explained concept, suggest that in different ecosystem types, other conditions may lead to $N_2O$ emission hotspots.

**Possibility to use hotspots in $N_2O$ emission models?**

According to Groffman et al. (2009) there is a wealth of data illustrating the importance of hotspots, and approaches for including them in experimental designs, but the importance of hotspots is not accounted for on a routine basis. From a large number of ecosystems, quite some soil characteristics are known or can be measured or deduced rather easily to a certain level of detail. These characteristics are already being used in $N_2O$

emission prediction models like DNDC (Li et al. 1992) and DeNit (Reth et al. 2005). From these characteristics together with the above described concept (favourable conditions for denitrification but one condition increasing the $N_2O$:$N_2$ ratio) can be used to predict the occurrence of $N_2O$ emission hotspots. The spatial pattern of hotspots within the ecosystem will not influence average ecosystem emissions and is therefore of lesser importance. However, the occurrence of hotspots will significantly affect average ecosystem emissions (chapter 2) and is therefore important to be modelled. Integration of hotspot occurrence in prediction models will improve accuracy of these models in predicting average ecosystem emissions.

**Implications for creation of buffer strips**

Recently, the European Union decided to grant almost 18 Million Euros for the Netherlands' Rural Development Strategy (RDP2). In RDP2 it is formulated that "emissions of greenhouse gases from Dutch agriculture will have to decline further" and on the same page it is stated that no further initiatives are required on this point apart from the dissemination of information about cost-effective mitigation measures for nitrous oxide and methane emissions (Regie-bureau POP 2009). Part of the granted money will be allocated to construct buffer strips along water bodies bordering agricultural areas. Several studies (e.g. Groffman et al.2000, Hefting et al. 2006a) showed that such buffer strips might be emitting considerable amounts of $N_2O$. In order to let emissions of nitrous oxide in agricultural areas indeed decline further, it is important that the newly constructed buffer strips will not be sources of high $N_2O$ emissions. When creating new buffer strips, care should be taken to avoid conditions leading to high $N_2O$ emissions and $N_2O$ emission hotspots.

**Management options**

Increasing average pH, would probably lead to a decrease in abundance of acidic soil spots. However, changing the pH of the soil will result in a multitude of effects possibly interacting with each other. In the discussion of chapter 4 it is described that effects of liming on $N_2O$ emissions have been studied and the results varied from strong increases (Butterbach-Bahl et al. 1997) to strong decreases (Brumme and Beese 1992).

Lowering of the water table will probably result in decreased denitrification activity, $N_2O$ emission hotspot occurrence and total ecosystem $N_2O$ emissions. The nitrogen removal buffering function will, however, be lost and $N_2O$ emissions may occur elsewhere.

Increasing the water level ("rewetting") and measures to increase water retention, as is done in many Dutch ecosystems including Het Hazelbekke as a restoration measure (Provincie

Overijssel 2009) to obtain more natural levels, will probably enhance the buffering function of the system. The distribution of $N_2O$ emission hotspots would probably shift upward from the stream, as the $NO_3^-$ rich groundwater will enter the organic rich topsoil layer further from the stream, but it is unlikely that this will affect total $N_2O$ emissions from the ecosystem.

Decreased nitrogen loadings will result in decreased total denitrification and $N_2O$ emissions (Hefting 2006a). The occurrence of hotspots (spots with disproportionately high $N_2O$ emissions relative to the surrounding area) will probably not decrease, but as the rate of $N_2O$ emission of both the hotspots and the surrounding area will be reduced, ecosystem $N_2O$ emissions will be decreased. If the buffering function of riparian zones should be retained, the best option to reduce $N_2O$ emissions is to reduce $NO_3^-$ loadings to these ecosystems and to make $NO_3^-$ influx less variable in time and space. In that case, most of the $NO_3^-$ entering the buffer zone will then be removed with minor $N_2O$ emissions (Hefting et al. 2006a). No management options specifically aiming at reducing $N_2O$ emission hotspot occurrence can be given yet. Future research should therefore aim at finding the source of pH variability in soils, in order to generate more mitigation measures.

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## Samenvatting

In dit proefschrift wordt een onderzoek beschreven dat zich richtte op het verklaren van het voorkomen van hotspots van lachgas uitstoot. Het begrijpen en verklaren van de verantwoordelijke mechanismen van de uitstoot van dit schadelijke gas is een eerste stap in het reduceren van deze uitstoot.

### Introductie

$N_2O$ (lachgas) is een van de gassen in de dampkring. Mede door toedoen van menselijk handelen is de concentratie van dit gas in de atmosfeer met ongeveer 20% gestegen in de afgelopen anderhalve eeuw. Er zijn twee schadelijke effecten bekend van $N_2O$: het eerste is het feit dat afbraakproducten van $N_2O$ ozon afbreken en op die manier de ozonlaag dunner maken. Daarnaast is $N_2O$ een zeer sterk broeikasgas. $N_2O$ is ongeveer 300 keer sterker dan $CO_2$ als broeikasgas.

Er bestaan verschillende bronnen van $N_2O$, enkele zijn natuurlijk terwijl anderen een humane oorsprong hebben. In landbouwgebieden en gebieden die beïnvloed worden door de (bemesting van de) landbouw wordt relatief veel $N_2O$ uitgestoten in vergelijking met de oorspronkelijke natuurlijke situatie. Voor een belangrijk deel wordt dit veroorzaakt door de hoeveelheid stikstof die via bemesting in de bodem komt.

Stikstof kan in verschillende verbindingen voorkomen in de bodem. De belangrijkste verbindingen zijn ammonium ($NH_4^+$) en nitraat ($NO_3^-$). Deze stikstofverbindingen worden continu door planten en micro-organismen gebruikt en zijn derhalve niet stabiel in de bodem. Micro-organismen gebruiken stikstofverbindingen niet alleen voor hun bouw maar ook voor hun energievoorziening. Twee van deze energieleverende processen genereren niet alleen energie maar onder bepaalde omstandigheden ook $N_2O$. Het eerste is de omzetting van $NH_4^+$ naar $NO_3^-$ en wordt nitrificatie genoemd. $N_2O$ wordt hierbij als bijproduct gevormd. Denitrificatie is het andere proces en hierbij wordt $NO_3^-$ omgezet naar $N_2$, het onschadelijke stikstofgas waarmee ongeveer 80% van de atmosfeer is gevuld. In dit proces wordt $N_2O$ eerst geproduceerd en vervolgens geconsumeerd (zie hoofdstuk 1, fig. 2).

Zoals genoemd kunnen deze processen naast het eindproduct ook $N_2O$ produceren. Er zijn vele factoren die beïnvloeden in welke mate nitrificatie en denitrificatie $N_2O$ produceren. De fysische factoren zijn onder andere de temperatuur en de mate van gasdiffusie door de bodem, hetgeen met name wordt bepaald door bodemstructuur, vochtgehalte en verschil in luchtdruk tussen bodem en atmosfeer. De chemische factoren kunnen in drie categorieën ingedeeld worden: elektron donoren, elektron acceptoren en de zuurgraad van de bodem. Elektron donoren zijn met name belangrijk bij denitrificatie. De hoeveelheid en vorm van elektron donoren kunnen de relatieve en absolute hoeveelheid van geproduceerd $N_2O$ beïnvloeden. In de bodem zijn organische (koolstof)verbindingen de

belangrijkste elektron donoren. Nitraat en nitriet ($NO_2^-$) zijn elektron acceptoren welke noodzakelijk zijn voor denitrificatie. Deze verbindingen kunnen echter ook $N_2O$ consumptie remmen. Zuurstof ($O_2$) is een andere belangrijke elektron acceptor en remt zowel productie als consumptie van $N_2O$ in de denitrificatie. Daarnaast veroorzaakt ook een zuurdere bodem een hogere relatieve $N_2O$ productie van denitrificatie en nitrificatie, maar door een remmend effect op de processnelheid van nitrificatie en denitrificatie is de absolute productie moeilijk voorspelbaar. Verder beïnvloedt de zuurgraad (pH) de samenstelling van de microbiële gemeenschap. Deze biologische factor is van belang voor het verloop van de stikstofomzettingen en de hoeveelheid $N_2O$ die daarbij geproduceerd wordt. Vanwege de vele factoren die elkaar en $N_2O$ uitstoot beïnvloeden is het van groot belang om een fundamenteel inzicht te hebben in de belangrijkste processen die zorgen voor $N_2O$ uitstoot.

Bodems met relatief veel stikstofverbindingen kunnen belangrijke bronnen van $N_2O$ uitstoot zijn. Voor dit proefschrift is onderzoek gedaan in een beekbegeleidende bufferstrook gekenmerkt door een vochtige organische bodem begroeid met Elzen en een ondergroei van met name Brandnetels en Bramen. Deze bufferstrook ('t Hazelbekke) liet in eerder onderzoek zien relatief veel $N_2O$ uit te stoten vanwege de hoge concentratie nitraat in het grondwater, afkomstig van de naastgelegen landbouw. In voorgaande onderzoeken bleek dat de uitstoot van $N_2O$ niet gelijkmatig is verdeeld binnen een ecosysteem. Vaak worden zogenaamde hotspots van $N_2O$ uitstoot gezien, terwijl de rest van het bodemoppervlak relatief weinig $N_2O$ uitstoot. Deze hotspots kunnen een aanzienlijk deel van de totale hoeveelheid van de $N_2O$ van een bepaald gebied uitstoten, maar tot nog toe was onbekend hoe deze hotspots werden veroorzaakt.

In dit proefschrift is daarom uitgezocht welk mechanisme verantwoordelijk is voor deze hotspots. Daarvoor is met name gekeken naar de verschillen tussen deze hotspots en de rest van de bodem (non-hotspots). Specifiek werden de volgende vragen getracht te beantwoorden:

- Wat is de maat van een hotspot?
- Is de verdeling van hotspots binnen een ecosysteem willekeurig?
- Wordt de relatief hoge $N_2O$ uitstoot in hotspots veroorzaakt door verhoogde diffusie, verhoogde $N_2O$ productie of verlaagde $N_2O$ consumptie?
- Welk stikstofomzettend proces is verantwoordelijk voor de relatief hoge $N_2O$ productie?
- Welke factoren zijn verantwoordelijk voor de verhoogde $N_2O$ uitstoot?
- Kan denitrificatie bestaan in een bodem met een zuurgraad van 4?
- Welke micro-organismen zijn verantwoordelijk voor de $N_2O$ productie in hotspots?

**Denitrificatie als bron van $N_2O$ productie en uitstoot**

De bodem van het onderzochte ecosysteem wordt gekenmerkt door een hoog organisch stof gehalte, een hoog vochtgehalte en een constante aanvoer van nitraat in het grondwater. Deze drie kenmerken geven optimale omstandigheden weer voor denitrificatie. Het bodemmateriaal werd in water verdund in afgesloten flesjes geïncubeerd onder gecontroleerde omstandigheden in het laboratorium en daaruit bleek dan ook dat denitrificatie de belangrijkste bron van $N_2O$ productie was (hoofdstuk 2). Verder bleek dat wanneer hotspot-bodems geïncubeerd werden, er veel meer $N_2O$ werd geproduceerd, dan wanneer er non-hotspot-bodems geïncubeerd werden (Hoofdstuk 3). Wanneer er toevoegingen van stabiele isotopen van nitraat en ammonium gedaan werden, bleek nog duidelijker dat vrijwel alle geproduceerde $N_2O$ afkomstig was van denitrificatie. Zowel onder zuurstofrijke als zuurstofarme condities trad nauwelijks oxidatie van ammonium op. Voorgenoemde resultaten en het feit dat bodemincubaties hetzelfde $N_2O$ productiepatroon lieten zien, maken het zeer aannemelijk dat de $N_2O$ uitstoot in het ecosysteem wordt veroorzaakt door denitrificatie.

**Verlaagde $N_2O$ consumptie door lage pH verantwoordelijk voor netto $N_2O$ productie**

Hoewel denitrificatie als verantwoordelijk $N_2O$-producerend proces aangewezen kon worden, was hiermee niet de vraag beantwoord of hotspots worden veroorzaakt door hogere productie of lagere consumptie van $N_2O$. In incubaties bleek dat de snelheid van denitrificatie geen voorspellende waarde had met betrekking tot de $N_2O$ productie. Een relatief lage consumptie ten opzichte van de productie bleek meer voorspellende waarde te hebben (Hoofdstuk 3). Een relatief lage consumptie van $N_2O$ zou dus een hoge $N_2O$ uitstoot kunnen verklaren. Met behulp van incubaties werd achterhaald dat niet zuurstof of nitraat aangewezen konden worden als verantwoordelijke factoren die de onbalans tussen $N_2O$ productie en consumptie in hotspot bodems konden verklaren (Hoofdstuk 3). De pH, daarentegen, bleek juist wel als verantwoordelijke factor aangewezen kunnen worden (Hoofdstuk 4). De pH bleek namelijk altijd lager te zijn in hotspot-bodems en wanneer in incubaties de pH van de bodem verlaagd werd, ging de $N_2O$ productie direct omhoog. Welk onderliggend mechanisme er voor kan zorgen dat de pH zo'n sterke invloed heeft op het eindproduct van de denitrificatie is nog niet bekend.

**Hotspot $N_2O$ uitstoot door zure bodems**

In het lab was dus bewezen dat de pH een sturende rol kan hebben in de $N_2O$ productie. In de bodem zijn er echter vele factoren die van belang zijn in het voorspellen van de $N_2O$ uitstoot. Uitgebreide metingen van pH en $N_2O$ uitstoot in het veld moesten daarom uitsluitsel geven of ook daar de pH een sturende rol kan hebben. Alhoewel er heel veel

variatie in de $N_2O$ uitstoot in het veld werd geobserveerd, was er toch een duidelijk patroon zichtbaar van hogere $N_2O$ uitstoot bij lagere bodem pH (Hoofdstuk 4). Bij een hogere pH werd nooit een hoge uitstoot gezien. Bij een hogere pH zijn de $N_2O$ productie en consumptie in balans (Hoofdstuk 3), maar in bodems met een lagere pH is de uitstoot afhankelijk van de denitrificatie snelheid. Wanneer (bij lage pH) de denitrificatiesnelheid laag is zal de $N_2O$ uitstoot logischerwijs ook laag zal zijn omdat er weinig $N_2O$ wordt geproduceerd. Wanneer (bij lage pH) de denitrificatie snelheid echter hoog is, zal de $N_2O$ uitstoot groot zijn door de onbalans tussen productie en consumptie. De hoge mate van variabiliteit van de pH van de bodem was verrassend. De tijd ontbrak helaas om de oorzaak van deze variabiliteit goed te kunnen onderzoeken.

**Denitrificatie bij pH 4 mogelijk door bestaande bacteriën?**

De waarneming dat de $N_2O$ uitstoot het hoogst is door bodems met een pH van tussen de 4 en 5 is opmerkelijk te noemen. Denitrificatie in de bodem wordt namelijk uitgevoerd door bacteriën. Tot op heden voeren deze bacteriën in laboratoria de denitrificatie het beste uit onder neutrale pH waarden (pH 6-8). Bij pH waarden onder de 5 wordt over het algemeen geen of zeer weinig denitrificatie waargenomen. Om te onderzoeken of (bodem) denitrificatie al dan niet mogelijk was, werd een bioreactor gebruikt waarmee de natuurlijke omgeving gesimuleerd werd (Hoofdstuk 5). Door middel van het vergelijken van de dna sequenties van de aanwezige bacteriën met die van bekende bacteriën werd duidelijk dat een bepaalde bacterie van het genus *Rhodanobacter* zich vermeerderde in deze bioreactor. In incubaties bleek deze bacterie ook in staat om te denitrificeren bij een lage pH. Verder werden de al in de bodem aanwezige organische verbindingen gebruikt om te denitrificeren. Deze resultaten tezamen geven voldoende aanleiding om te kunnen concluderen dat er wel degelijk denitrificatie in de bodem bij pH 4 wordt uitgevoerd. De genoemde *Rhodanobacter* bacterie zou een belangrijke rol kunnen spelen in de $N_2O$ hotspots, maar wellicht zijn er nog meer soorten bacteriën die kunnen denitrificeren bij een lage pH.

**Distributie, maat en tijdsbestek van hotspots**

Aan de hand van de resultaten in latere hoofdstukken zijn de distributie, schaal en tijdsbestek waaraan in hoofdstuk 2 aandacht is besteed te verklaren. De meeste hotspots lagen dicht bij de beek. De bodem in deze zone verschilt met name in vochtgehalte van de andere zones. Deze vochtige zone, met een organische bodem waar nog nitraat in het grondwater aanwezig is, is optimaal voor een hoge denitrificatiesnelheid. Een onbalans in productie en consumptie door een lage pH kan daar een hotspot veroorzaken.

De ruimtelijke schaal, oftewel de maat van een hotspot kan niet eenduidig genoemd worden. Hotspots bleken op alle onderzochte schalen aanwezig. Het beste is dat te

verklaren doordat een hotspot wordt veroorzaakt door een lage pH en een hoge denitrificatie snelheid. Deze snelheid wordt beïnvloed door vele factoren, welke allemaal weer op verschillende ruimtelijke schalen variabel zijn.

Het tijdsbestek van hotspots is n iet uitvoerig onderzocht. De waarnemingen die gedaan zijn, wezen erop dat hotspots enkele dagen kunnen bestaan en daarna weer uitdoven. Het uitdoven zou veroorzaakt kunnen worden door een verhoging in pH of een verlaging van de denitrificatiesnelheid. Een andere hypothese is dat de bacteriën zich onderwijl hebben aangepast en daarom de $N_2O$ sneller consumeren.

**Concept geldig voor andere ecosystemen?**

De resultaten die uit dit onderzoek naar voren zijn gekomen zijn geldig voor het onderzochte ecosysteem. Het is van belang om te weten of het beschreven concept (lage pH + hoge denitrificatie = $N_2O$ hotspots) ook opgaat voor andere ecosystemen. Tot nog toe is dat niet onderzocht. Echter, gesteld kan worden dat in ecosystemen met vergelijkbare omstandigheden (organische vochtige bodem met nitraataanvoer en pH variabiliteit) dit concept naar alle waarschijnlijkheid ook zal gelden. Daarnaast is het waarschijnlijk dat hotspots ook veroorzaakt kunnen worden ook een andere factor die de balans tussen $N_2O$ productie en consumptie verstoort in combinatie met een hoge denitrificatiesnelheid. Deze kennis zou gebruikt kunnen worden in modellen die de totale $N_2O$ uitstoot van ecosystemen proberen te voorspellen.

**Mogelijkheden voor $N_2O$ reductie**

Op dit moment bestaan er overheidsplannen om het aantal beekbegeleidende bufferstroken flink uit te breiden in Nederland. Het huidige, maar ook voorgaand onderzoek heeft laten zien dat deze bufferstroken aanzienlijke hoeveelheden schadelijk $N_2O$ kunnen produceren. Bij het ontwerpen en aanleggen zal nagedacht moeten worden over hoe deze uitstoot zo veel mogelijk te vermijden. Specifieke handvatten om $N_2O$ uitstoot te reduceren door middel van het reduceren van hotspots kunnen helaas niet direct uit het huidige onderzoek gedestilleerd worden. Alhoewel het ontstaan van hotspots niet voorkomen zal worden door het verlagen van nitraatconcentraties (afkomstig van bemesting) in het grondwater, zal dit echter vrijwel zeker de totale hoeveelheid $N_2O$ uitstoot verminderen vanwege de verminderde totale denitrificatie.

## Dankwoord

Klaar! Dat voelt wel heel erg lekker. Dat ging natuurlijk niet zomaar en ook niet zonder hulp en ondersteuning. Voor die ondersteuning in de afgelopen 4 en half jaar wil een aantal mensen bedanken.

Allereerst mijn begeleidingsteam. Hoewel het voltallige begeleidingsteam vrijwel nooit bij elkaar is geweest vanwege overvolle agenda's waren allen altijd van goede wil om met elkaar (en mij) samen te werken. Ik wil jullie bedanken voor het feit dat jullie altijd met mij geprobeerd hebben de lijn in het onderzoek vast te houden en bereid waren over de grenzen van jullie eigen vakgebied heen te kijken.

Meer specifiek wil ik Mike bedanken voor de interesse en het vertrouwen in mijn werk, terwijl dat soms erg ver van je eigen onderzoeksonderwerpen lag. De maandagochtend begeleidingsoverleggen waren altijd een goed moment om vooruit en achteruit te kijken en vervolgens weer wat concreter aan de slag te gaan. Daarnaast kan ik zeggen dat ik jouw immer strategische manier van besturen en onderzoek doen als voorbeeld zal nemen in mijn toekomstige carrière.

Jos, jou wil ik vooral bedanken voor de steun op de momenten dat ik er niet meer zo'n zin in had. Dat vertrouwen heeft me aan het werk gehouden en is van belang geweest om dit boekje af te maken.

Mariet, dit onderzoek heeft zich in de eerste periode in de schaduw van die van jouw gelopen. Soms had ik het daar moeilijk mee, maar tegelijkertijd heeft dat grote voordelen gehad. Typerend voor jouw persoon is dat je zowel inhoudelijk en persoonlijk veel begeleidingstijd en –aandacht gestopt hebt in mij en het onderwerp. Ik ben je daar zeer erkentelijk voor. Hoewel ik je aan het begin soms wel streng vond, ben ik over het geheel heel erg blij dat jij voorgenoemde rollen op je genomen hebt. Zonder jou was er inhoudelijk geen project geweest en weet ik niet wat ik ervan gebakken zou hebben.

Boran, ook jij was vaak streng voor me, nog veel strenger dan Mariet, maar gelukkig werd jij pas aan het einde van mijn project co-promotor en toen ik daar wel mee om gaan. Je hebt een uitzonderlijk persoonlijke manier van begeleiden, dat voelt heel warm en geeft motivatie. Daar kunnen je huidige en toekomstige AiO's van profiteren. (oeps, in het Nederlands… nou ja, dat lukt wel, toch?).

Nico, ook jij hebt me op een heel persoonlijke manier begeleid. Daarnaast was je lange tijd mijn link met Nijmegen op de momenten dat ik daar niet veel kwam. Je vertrek naar een bedrijf kwam niet onverwacht, mede door je zakelijke manier van onderzoek doen. Dank voor al je aandacht en interesse, ook nadat je vertrokken was.

Philippe, wat mij betreft hebben wij helaas te weinig tijd met elkaar doorgebracht. Je zeer wetenschappelijke manier van werken, inhoudelijke kennis, maar bovenal je kennis van mijn onderwerp (ook al hadden we elkaar maanden niet gezien) wekte veel gezag op. Dank voor de momenten dat je erbij kon zijn.

Markus, helaas is van de plannen aan het begin van het project jouw onderwerp er karig af gekomen. In de laatste fase van mijn project (toen je ondersteuning wel nuttig geweest had kunnen zijn) was je vertrokken. Onze discussies voor die tijd hadden me op dat moment gelukkig al voldoende inhoudelijke kennis opgeleverd om het ook zonder jou te kunnen doen, dank daarvoor.

Ik moet hier zeker ook Vereniging Natuurmonumenten bedanken. Ondanks het feit dat jullie niet direct het nut van ons onderzoek inzagen hebben jullie ons toch toestemming

verleend om onderzoek te doen in het Hazelbekke. Wie weet hoe de resultaten eruit zagen als we in een ander gebied hadden moeten werken?

Het aantal malen veldwerk dat ik gedaan heb in de 3 jaar dat ik in het Hazelbekke heb rondgelopen zou zeker aanzienlijk minder geweest zijn als mevrouw Visser daar niet had gewoond. Mevrouw Visser, enorm bedankt voor uw immer vanzelfsprekende Twentse gastvrijheid. Dank zij u waren de veldwerkdagen ook leuk op de momenten dat het weer en/of de resultaten niet mee zaten.

Het weer en de resultaten zaten niet altijd mee. Daar kunnen Paula en Elise van mee praten. Paula, jij was de eerste student die ik begeleid heb en ik heb er veel van geleerd. Ik hoop jij ook. Elise, jij kwam juist bij mij in het laatste moment van mijn project. Dat heeft ertoe geleid dat ik al goed wist wat ik wilde. Jij hebt dat onderwerp heel goed opgepakt en samen hebben we een project uitgevoerd wat tot leuke resultaten en hopelijk een mooi artikel heeft geleid.

Veel geleerd heb ik ook van de studenten die een scriptie hebben geschreven onder mijn begeleiding. Anne, Floor en Marinde, stuk voor stuk hebben jullie heel goed scripties geschreven en de (door mij aangedragen onderwerpen) helder en overzichtelijk beschreven. Dat heeft mij weer geholpen met het schrijven van enkele stukken van mijn proefschrift.

Om hier al mijn collega's van beide vakgroepen persoonlijk te bedanken zou zeker enkele pagina's in beslag nemen en daarmee de drukkosten aanzienlijk verhogen. Gezien mijn calvinistische aard doe ik dat dus maar niet. Collega's en ex-collega's: bedankt voor de interesse in mijn onderwerp, maar natuurlijk vooral bedankt voor de persoonlijke aandacht! Zelfs al mijn (ex)-kamergenoten persoonlijk bedanken gaat niet, omdat ik zeker mensen zou vergeten. Goed, voor Victor en Edu en Judith en Bas wil ik nog een uitzondering maken; met jullie heb ik veel tijd doorgebracht op mijn kamer in Utrecht. Met jullie was het gezellig (te gezellig voor mijn spanningsboog). Daarnaast heb ik ook op de kamer gezeten met Alice, Suzanne en Yuki in Utrecht. Het aantal kamergenoten in Nijmegen is echt ontelbaar, hetgeen wel leidde tot meer tijd doorbrengen op het lab, dank daarvoor :-).

Twee andere collega's wil ik nader bedanken omdat zij bij mijn promotie mijn paranimfen zullen zijn. Judith en Katharina, jullie projecten zijn ongeveer tegelijk gestart en geëindigd met dat van mij, daarom snapten we wederzijds wat er in ons om ging. Jullie waren speciale collega's voor mij, ik hoop dat jullie bij mijn promotie van buiten net zo stralen als van binnen.

Verder nog een persoonlijk dankwoord aan mijn ouders voor hun interesse en vertrouwen. Ik mag niet nalaten mijn vader specifiek te bedanken omdat hij zowel inhoudelijk bij het proefschrift als bij de uitvoering van het veldwerk een bijdrage heeft geleverd. Er zijn vast niet veel promovendi die dat kunnen zeggen.

Op persoonlijk vlak nog een bedankje aan mijn loopvrienden omdat het lopen (met jullie) ervoor gezorgd heeft dat het stressniveau soms wat gereduceerd werd en de nodige geestelijke ontspanning bracht.

Als laatste mijn meisjes; Susan, jij snapte inhoudelijk en projectmatig waar ik mee bezig was. Daarnaast wist je vaak ook nog hoe ik er emotioneel mee bezig was (alhoewel ik dat redelijk goed kan verbergen). Dank je voor precies de juiste mix van mij wijzen op mijn verantwoordelijkheden en mij vrij laten in mijn eigen keuzes. Laura; ik denk niet dat je veel positiefs hebt bijgedragen aan mijn proefschrift, maar je bent wel lief.

## Curriculum Vitae

Ik werd geboren op 9 mei 1982 in Voorthuizen. Daar volgde ik met groot genoegen de basisschool (de Koningin Wilhelmina school) alhoewel de meesters en juffen vonden dat ik wat te veel droomde en daardoor mijn werk niet op tijd af had. Daarna volgde ik het voorbereidend wetenschappelijk onderwijs aan het Johannes Fontanus College. Met belangstelling in exacte maar ook breed oriënterende vakken volgde ik een vakkenpakket met daarin Geschiedenis, Aardrijkskunde, Wiskunde B, Natuurkunde, het verplichte Engels en Nederlands en mijn favoriete vak, Biologie.

Geïnteresseerd in diergedrag begon ik in het jaar 2000 aan de studie Biologie aan de Universiteit Utrecht. Al snel vond ik de ecologie veel interessanter. Mijn stages heb ik dan ook gevolgd bij de vakgroepen Landschapsecologie (Universiteit Utrecht) en Plant Ecology (Smithsonian Environmental Research Center, Edgewater, USA). Tijdens die stages heb ik voornamelijk gekeken naar wat er in de bodem gebeurde en aanwezig was.

De complexiteit van de bodem interesseerde me dermate dat ik heel verheugd was een promotieproject te mogen invullen (binnen het Centre for Wetland Ecology) gericht op die bodemcomplexiteit (lees de voorgaande pagina's voor een inhoudelijke beschrijving). In dit project is gebruik gemaakt van de expertise en faciliteiten van de vakgroepen Microbiologie (Radboud Universiteit Nijmegen) en (voorheen) Landschapsecologie (Universiteit Utrecht) hetgeen geleid heeft tot dit proefschrift.

Na deze (relatief korte) wetenschappelijke carrière is het nu tijd voor iets heel anders.