N_2O reduction in soils

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Summary

N_2O is a known greenhouse gas that increased by 16% over the last 200 years. The main sources are native and agricultural soils where predominantly soil bacteria perform nitrification and denitrification with N_2O as side and intermediate product, respectively. A complete denitrification, at which N_2O is reduced to N_2, is assumed to be the main elimination or sink process of N_2O in the soils, beside dissolution in water. N_2O sinks were first observed and measured in field studies. For a better understanding of the N_2O sink processes laboratory studies with defined conditions were carried out, most of them under anaerobic conditions and airtight closed systems. We studied N_2O sink processes in an experimental set up that had a continuous gas flow through soil samples to avoid a limitation of substrate gas concentrations over the experiment time. This set up allowed us to keep temperature and gas concentrations of N_2O and O_2 constant or selective to change them at discretion during the experiments. The concentrations of N_2O and CO_2 at the inlet and at the outlet of the incubation vessels could be measured continuously with a gas chromatograph. Therefore, we could determine the N_2O consumption and the CO_2 emission throughout the experiments.

Our overall objective was to determine the ability and capacity of different soils to consume N_2O at low oxygen concentrations and to determine the influence of certain parameters on N_2O consumption and its rate.

A change in the ratio of ^{15}N to ^{14}N as well as in the ratio of ^{18}O to ^{16}O in the N_2O molecule was observed with coexistent raise of the N_2O concentration in the atmosphere. This observation gives reason to believe that one of the N_2O production or consumption processes might be the reason for the change in the fractionation factor. Our first objective was to check to what extend the N_2O consumption could influence the isotopic signature in the remaining N_2O molecules. Therefore, we determined the N_2O reduction rate, the reduction rate constant and synchronously the isotopic signature of N and O in the remaining N_2O that left the sample. We observed that with a decreasing reaction rate constant the fractionation factors for N and O increased and vice versa. We could as well determine the ratio of the stable isotopes N / O that lay mostly between 2 and 4 with an average around 2.4, which is in agreement with other observations. We could conclude that the fractionation factors of the stable isotopes N and O depend on the N_2O reduction rate constant and that the ratio of the enrichment factors for the stable isotopes N and O is constant. This ratio can help to detect N_2O consumption if reduction is the dominating process in the turnover of N_2O.

The predominant faith of N_2O in soils is either the emission to the atmosphere, the dissimilatory reduction to N_2 or the dissolution in water. However, other types of N_2O sink were observed, for example N_2O fixation with following transforming to NH_3. Hence, our second objective was to test, if these observations and a possible incorporation of $N\text{-}N_2O$ into soil organic matter are of ecological relevance in soils. We approached this problem by using

labelled $^{15}N_2O$ as the only nitrogen source for four different soils over several days in our flow through set-up. We measured N_2O consumption continuously and $\delta^{15}N$ of soil organic matter before and after the experiment. The results revealed an average of the ^{15}N enrichment in the soil organic matter of about 0.019%. Therefore, we could conclude that assimilatory reduction of N_2O plays a negligible role as a N_2O sink in soils, at least for our tested soils.

Although denitrification and therefore N_2O consumption is known to occur under anaerobic conditions there are observations of N_2O consumption at low O_2 or aerobic conditions. This could indicate that N_2O consumption is a more widespread and important process in soil as assumed. Our third objective of this thesis was to study the ecological relevance of N_2O consumption in view of the total respiration in soils. Thereby, N_2O and total respiration rates and the Michaelis-Menten Constant (k_m) values for N_2O consumption were determined at different temperatures and oxygen concentrations. The received k_m values were between 1.8 – 10.4 ppm in soil gas phase (0.045 – 0.26 μM in soil solution) at all applied temperature and oxygen concentrations. This range fits with k_m values of other observations and suggests that there might be a common range of k_m values for N_2O consumption in soils. In contrast, the k_m values determined for pure cultures of N_2O reducing bacteria were about 9 times larger (2.4 – 7.5 μM in soil solution, converted: 96 -300 ppm). This result might point to a higher affinity of the N_2O reducing enzyme in soils than in pure cultures. The ratio of N_2O to total respiration gave us the amount of electrons that were transmitted to a N_2O molecule instead to O_2. This was for our soils up to 1.25%. Our observations showed that N_2O and aerobic respiration could occur simultaneously probably in different microhabitats within the soil.

Only 1% of the microbes in soils have the ability to produce the enzyme N_2O reductase that reduces N_2O to N_2. The production of the enzyme is controlled by environmental influences like oxygen concentration and the concentration of the denitrification intermediate products. Our objective for this part was to find out to what extent N_2O treatments could influence the microbial community and the N_2O reducing bacteria. We used two different DNA fingerprinting methods, RISA (ribosomal RNA intergenic spacer region analysis) and DGGE (denaturation gradient gel electrophorese) on four different soils, which showed N_2O consumption. Through this method, we could conclude that all tested soils have strong differences in their microbial community. The treatment of the soils caused a shift in the microbial community, but it was not clear which of the parameter the high temperature, low oxygen, and/or high N_2O concentration, had the most effect on the microbial community.

We could prove that all soils we tested have the ability to reduce N_2O to N_2 at low oxygen concentrations. The potential of this process depends highly on the N_2O and O_2 concentrations, temperature, and aggregate sizes in the soil. Altogether, we enhanced our knowledge about the N_2O consumption process and could conclude that this process is of ecological importance in soils.

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Chapter 1

Introduction

One of the most important greenhouse gases beside carbon dioxide (CO_2) and methane (CH_4) is nitrous oxide (N_2O). At present, it accounts for 6% of the total amount of the green house gases in the atmosphere. However, it has a 320 stronger global warming potential (GWP) than CO_2 and remains in the atmosphere for approximately 114 years before removal, mainly by destruction in the stratosphere (IPCC, 2006). It also catalyses the destruction of the stratospheric ozone (Crutzen, 1970; Crutzen and Ehhalt, 1977; Crutzen, 1981; Bouwman, 1998). The atmospheric concentration of N_2O has risen about 16%, from about 270 ppb during the pre-industrial era to 319 ppb in 2005 (IPCC, 2006). It is currently increasing by approximately 0.3% per year and may significantly affect the global climate over the next 100 years (IPCC, 2001). The sources are mainly biomass and fossil fuel burning, raising cattle, some industrial activities, including nylon manufacture and agricultural cultivation behaviour as fertilization with industrial fertilizer (IPCC, 2006). Although the sources are diverse and poorly quantified, the upward trend appears to result from fuel combustion and applied fertilizers (Bowman, 1990). However, natural sources like oceans and especially native soils emit a large portion (55%) of N_2O (IPCC, 2006). The main sources for N_2O are agricultural and native soils, in which predominantly nitrification and denitrification are responsible for the production of N_2O. Both processes are part of the nitrogen cycle (Fig. 1.1), which is one of the essential nutrient cycles in terrestrial ecosystems.

During nitrification in general ammonia-oxidising bacteria (for example *Nitrosomonas*) oxidise ammonia (NH_4^+) to nitrite (NO_2^-) (Eq. 1) and in a second step nitrite-oxidising bacteria (for example *Nitrobacter*) oxidise nitrite to nitrate (NO_3^-) (Eq. 2). In both steps of the nitrification, N_2O can be released as a side product (Firestone and Davidson, 1989; Bremner, 1997). Either it can be formed by spontaneous disintegration of nitrogen hydroxide (NOH), an unstable, enzyme-bound intermediate during the first step of nitrification (Hynes and Knowles, 1984) or NO_2^- can be reduced to N_2O by nitrifier denitrification (Wrage et al., 2001). Nitrifiers use the released energy for living.

$$NH_4^+ + O_2 \rightarrow NO_2^- + 4H^+ + 2e^- \quad E = 272 \text{ kJ/mol} \qquad (1)$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad E = 70 \text{ kJ/mol} \qquad (2)$$

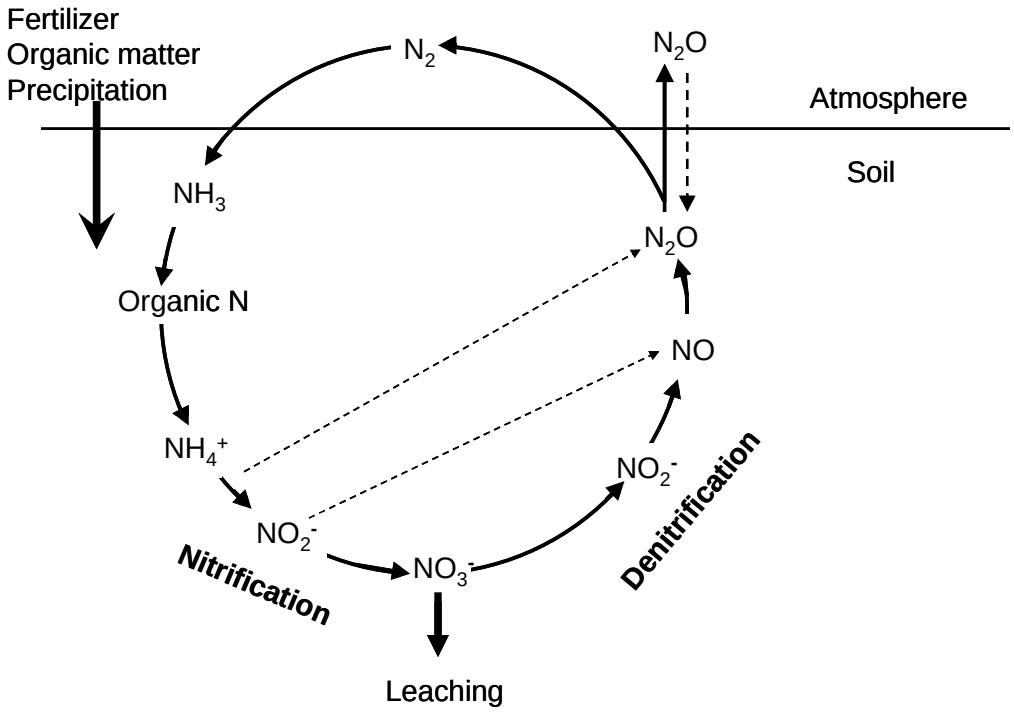

Fig. 1.1 Nitrogen cycle

During denitrification, mainly denitrifying bacteria reduce nitrate or nitrite to nitric oxide (NO), nitrous oxide (N_2O) or to molecular nitrogen (N_2) in dissimilatory reactions (Eq. 3) (Payne, 1981; Firestone and Davidson, 1989; Bremner, 1997). Denitrifiers gain their energy from the oxidation of a reductive (Schlegel, 1992).

$$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2 \qquad (3)$$

The regulation of NO and N_2O production and consumption by nitrification and denitrification is depicted in the "Hole-in-the-pipe" model (Fig. 1.2).

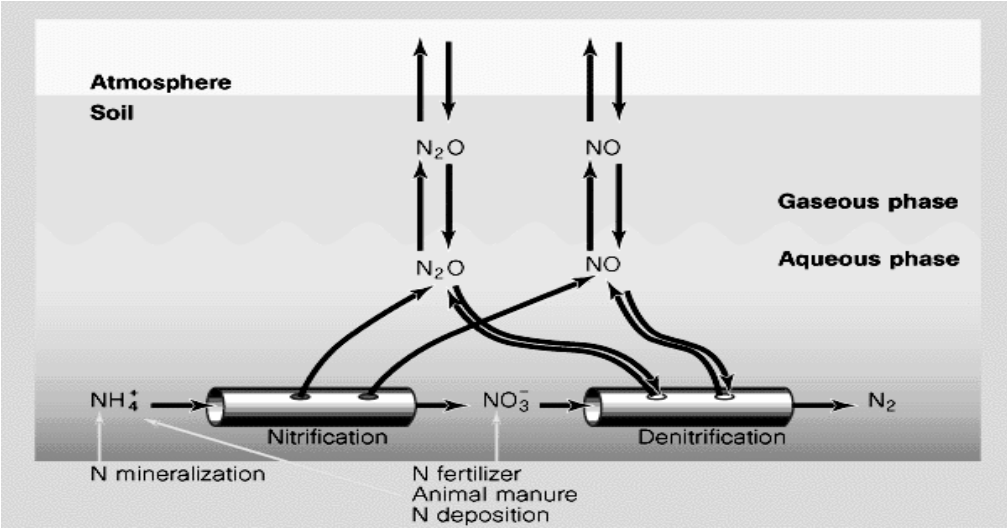

Fig. 1.2 "Hole-in-the-pipe" model (Davidson, 1991 adopted by Bouwman, 1998)

Recent studies found that various microorganisms belonging not only to bacteria but also to Eukarya and Archaea could be involved in the processes of denitrification and nitrification. Even some of the fungi are able to produce N_2O and N_2 (Shoun et al., 1992; Hayatsu et al., 2008).

Nitrification and denitrification depend on many different parameters, some of them with opposing effects in both processes. High oxygen concentrations in the soil usually inhibit the denitrification, but promote nitrification and vice versa (Cavigelli and Robertson, 2001; Laverman et al., 2001). The water-filled pore space (WFPS) in soils regulate these processes in a similar way (Freney et al., 1978; Bandibas et al., 1994). Additional variables influencing nitrification and denitrification are, for example, the availability of organic carbon compounds (Thompson, 1989; Laverman et al., 2001), nitrogen compounds (Blackmer and Bremner, 1976; Mosier and Kroeze, 2000; Harrison and Webb, 2001; Wlodarczyk et al., 2005), soil pH (Blackmer and Bremner, 1976; Stevens and Laughlin, 1998) and temperature (Thompson, 1989; Holtan-Hartwig et al., 2002).

Compared to the dimension of N_2O emission, N_2O sinks are small. In the stratosphere, photo dissociation and reaction with electronically excited oxygen atoms (O (^{1}D)) are the main sink (90%) for atmospheric N_2O (Volk et al., 1997; Lal and Sheel, 2000). In the soil the main N_2O sinks are assumed to be the complete denitrification to N_2 and N_2O dissolution in water (Chapuis-Lardy et al., 2007). More than 30 years ago, N_2O uptake was observed in field studies, but these data were rejected as analytical errors (Chapuis-Lardy et al., 2007). Nowadays sinks and reduction processes of N_2O are of interest in view to find a solution regarding the N_2O emission. Various studies in fields and laboratories were conducted to achieve a better understanding of the N_2O consumption processes. Field studies were conducted to estimate the portion of N_2O uptake from the atmosphere into soils (Ryden, 1981; Neftel et al., 2000; reviewed in Chapuis-Lardy et al., 2007). The potential of the N_2O consumption in soils was determined in laboratory studies with different techniques, like isotopic techniques (Clough et al., 1999; Groenigen et al., 2005a; Groenigen et al., 2005b), gas chromatography (Holtan-Hartwig et al., 2000; Holtan-Hartwig et al., 2002) or molecular biology (Kristjansson and Hollocher, 1980; Snyder and Hollocher, 1987; Teraguchi and Hollocher, 1989; SooHoo and Hollocher, 1991). The measuring of the isotopic signatures of the produced N_2O in deeper soil and the remaining N_2O on its upward diffusion through the soil revealed that a great part of N_2O is reduced in the soil before it emits into the atmosphere (Clough et al., 1999; Groenigen et al., 2005b). Through measuring the gas concentration of soil samples with gas chromatography, it was possible to estimate the influence of various parameters on N_2O consumption. Blackmer and Bremner (1978), Stevens et al. (1998) and Simek et al. (2002) observed that with decreasing soil pH the potential of N_2O consumption decreases. Another important parameter for N_2O consumption is the ratio of water-filled pore space (WFPS). If the WFPS rate increases above 80%, N_2O is consumed due to oxygen

limitation (Veldkamp et al., 1998). Using molecular biology techniques the involved enzyme (N_2O reductase) and the corresponding gene (nosZ) for the N_2O reduction processes could be detected (for example Zumft and Matsubara, 1982; Riester et al., 1989). If the enzyme N_2O reductase predominantly controls the N_2O reduction process, we can hypothesise that N_2O reductase is a biological process with first order kinetics. Meaning that the reduction rate of N_2O is proportional to the N_2O concentration (Plante and Parton, 2007).

Although nowadays the knowledge about N_2O consumption in soils is increasing, there are still uncertainties about the capacity and quality of this process. For example, new doubts of the global N_2O budget emerged because of isotopic anomaly in N_2O (Lal and Sheel, 2000). The sources of the enrichment of the N and O isotopes in N_2O can be new atmospheric N_2O sources and sinks in the atmosphere (McElroy and Jones, 1996) or N_2O production and consumption processes in the soil (Tilsner et al., 2003).

1.1 Scope and aims

This PhD thesis concentrates on improving the knowledge about N_2O consumption in soils. Our overall hypothesis is that a great portion of the produced N_2O will be consumed within the soil. The general objective was to determine the ability and potential of N_2O consumption in different soils at low oxygen concentrations. We also wanted to find out to what extent parameters like N_2O and O_2 concentrations, temperature, and soil aggregate sizes influence N_2O consumption. Further, we want to prove the hypothesis that N_2O reductase is a biological process with first order kinetics. Different mechanisms of N_2O consumption were investigated regarding their relative importance in a natural environment by measuring N_2O consumption within soil samples in a laboratory. The soils remained for the examinations as natural as possible, meaning that the soil samples were placed in the experimental set-up directly after their collection from the field without any other disturbance except the breaking and sieving to aggregates. Experiments were conducted in a continuous flow-through incubation set-up in which it was possible to control the environment of soil samples regarding temperature, N_2O and O_2 concentration. The concentrations of N_2O and CO_2 were continuously measured with a gas chromatograph throughout the experiments. This thesis is structured into four sections.

The first section (Chapter 2) concentrates on the measurement of fractionation factors of N and O isotopes during N_2O reduction. The background here is the measurement of changes in the isotopic composition of N_2O with the simultaneous increase of N_2O in the atmosphere. The detection of the cause for the isotopic signature changes in the atmospheric N_2O molecule might help to understand the global N_2O budget. A separation of net flux into gross consumption and gross production rates was necessary. With this separation, it was possible to determine gross consumption when gross production was low. The changes of N_2O

concentrations allowed the separation of net N_2O fluxes. Thus, the fractionation factors could be definitely assigned to N_2O consumption. Our objective was to find out if these fractionation factors are constant. The experiments were conducted with various O_2 and N_2O concentrations to obtain a range of N_2O reduction rates and reaction rate constants. At the same time, the fractionation factors of the stable isotope N and O of the remaining N_2O and their ratios (N / O) were determined.

The second part (Chapter 3) of this thesis is concerned with the faith of N_2O-N during the consumption process. Apart from anaerobic denitrification, other processes have been found to be able to consume N_2O. These are N_2O reduction by nitrogenase (Jensen and Burris, 1986) and direct N_2O uptake by an N_2O-fixing organism with following transformation of NH_3 (Yamazaki et al., 1987). However these processes have been studied in microbiogical model systems, and their importance on N_2O consumption in the natural environment is unknown. To find out the ecological relevance of any N_2O assimilation into the soil biomass we used labelled $^{15}N_2O$ in a nitrogen free environment and determined the ratio of ^{15}N in the soils before and after the experiments.

N_2O consumption was observed under conditions with low oxygen or suboxic concentration, sometimes even under aerobic conditions in the field and in the lab (Zumft, 1997; Takaya et al., 2003). The third section (Chapter 4) of this study deals with the ecological relevance of N_2O respiration to total soil respiration in suboxic soils. We determined the N_2O respiration rates, the total respiration rates, and their k_m values at various temperatures and oxygen concentrations. The ratio of N_2O to total respiration shall give us an idea about the ecological relevance of N_2O respiration in soils. Additionally we determined the effect of the soil aggregate size on the potential of N_2O consumption.

In the last section (Chapter 5) we describe a molecular approach to learn more about the microbiological community in soils that showed the ability to reduce N_2O. The objective here was to test if the soil microbiological communities show differences through N_2O treatment and if similarities can be found in the composition of microbial community between the soils. We compared four different soils due to their microbial community and to their bacteria that have the genetic code for the enzyme N_2O reductase (nosZ) with and without N_2O treatment.

Chapter 2

Fractionation factors for stable isotopes of N and O during N_2O reduction in soil depend on reaction rate constant

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2.1 Abstract

Nitrous oxide (N_2O) is a major greenhouse gas that is mainly produced but also reduced by microorganisms in soils. We determined factors for N and O isotope fractionation during the reduction of N_2O to N_2 in soil in a flow-through incubation experiment. The absolute value of the fractionation factors decreased with increasing reaction rate constant. Reaction rates constants ranged from 1.7 10^{-4} s^{-1} to 4.5 10^{-3} s^{-1}. The minimum, maximum and median of the observed fractionation factors were for N -36.0 ‰, -1.0 ‰ and -9.3 ‰ and for O -74.0 ‰, -6.9 ‰ and -26.3 ‰, respectively. The ratio of O isotope fractionation to N isotope fractionation was 2.4 ± 0.3 and independent from reaction rate constants. This leads us to conclude that fractionation factors are variables while their ratio in this particular reaction might be a constant.

2.2 Introduction

Atmospheric concentrations of nitrous oxide (N_2O) increased since the beginning of the industrialisation by about 13 % (IPCC, 2001). At the same time, its isotopic composition changed in $\delta^{15}N$ by -2 ‰ and in $\delta^{18}O$ by -1.2 ‰ (Röckmann et al., 2003). Numerous studies have reported stable isotope signatures of N_2O produced in soil and emitted to the atmosphere. So, the hope has been expressed that these isotope signatures will help closing the global N_2O budget (Alberts et al., 1994; Wada and Ueda, 1996; Webster and Hopkins, 1996; Barford et al., 1999; Pérez et al., 2001; Groenigen et al., 2005b). However, N_2O can be produced in soil from different substrates through different processes, summarised as nitrification and denitrification. Nitrous oxide can also be consumed by reduction to N_2. The isotopic signature of N_2O emitted from soil is usually the result of a combination of substrate signatures, production processes and consumption (Tilsner et al., 2003). Further, fractionation

by some of these processes is possibly rate-dependent (Bryan et al., 1983). Modelling and interpretation of isotopic signals is only possible, if fractionation factors of each step in the production and consumption processes of N_2O are well defined. N_2O consumption is an important process in nature and was observed in field and laboratory experiments (Clough et al., 1999; Pérez et al., 2001; Bol et al., 2003; Wrage et al., 2004; Groenigen et al., 2005b). Recent studies indicate that consumption of N_2O in soil might be more important than assumed so far (Wrage et al., 2004; Chapuis-Lardy et al., 2007; Conen and Neftel, 2007).

Here, we concentrate on the isotopic fractionation during N_2O consumption commonly observed under low O_2 concentrations, where large proportions of N_2O are reduced to N_2. For this purpose, we exposed soil samples to varying concentrations of O_2 in order to induce a range of N_2O reduction rates and possibly reaction rate constants, following the approach of Barford et al. (1999). We also altered N_2O concentrations to enable separation of net flux into gross consumption and gross production rates. Fractionation factors during N_2O reduction can only be estimated when the consumption rate of N_2O within a sample is much larger than the production rate. Otherwise, the observation would be substantially affected by N_2O produced from nitrification and denitrification with unknown isotopic signatures.

2.3 Material and Methods

Three samples were obtained between May and July 2006 from the top 10 cm of a soil (Gleysol above limestone) under mixed forest near Basel, Switzerland (47° 28' N, 7° 42' E, 476 m a.s.l). The soil organic carbon concentration was 5.1 %, nitrogen concentration 0.47 %, and pH (in $CaCl_2$) was about 7.1. Soil gravimetric moisture content ranged from 30.8 % to 48.2 %. Each sample was broken down to aggregates < 6.3 mm on the day of collection and about 200 g (fresh) of it was immediately transferred to an incubation vessel (diameter: 6.5 cm, height: 12.5 cm) made of glass.

In this vessel, it was exposed for seven days to a stream of changing concentrations of O_2 and N_2O in N_2. Measurements of stable isotopes started at the end of day 2 of the incubation, after N_2O flux had reached a steady state. On the following days, concentrations of O_2 were changed every 24 hours (stepwise increase from 0.2, 0.5, 1.0, 1.5 % and again to 0.2 %), allowing 4 hours after each change for equilibration before measurement. During the remaining 20 hours, N_2O concentrations were increased every 5 hours (0.1 to 3.0 µmol mol^{-1}), allowing at least two hours after each change for equilibration. The range of O_2 and N_2O concentrations we applied in our experiment match with the lower range of concentrations which were observed in a coastal forest studied by Yu et al. (2006).

The fully automated experimental set-up consisted of the three gas streams N_2 (>99.999 %), synthetic air (20 % O_2, 80 % N_2) and N_2O (100 ppm in N_2) which were controlled by mass flow controllers and flowing together to a single stream. This stream (150 ml min^{-1}) was split

into a first stream (80 ml min^{-1}) for use as drying gas in a Nafion® sample dryer (MD™ Series Gas dryer, Perma Pure LLC.; Toms River, N.J., U.S.A.) and into a second stream going to a humidifier (gas-washing bottle). The gas stream leaving the humidifier was split into two equal streams (35 ml min^{-1}), one by-passing the sample vessel and going directly to a 4-port selection valve (Valco Instruments Co. Inc.; Houston, Texas, U.S.A.), and one entering the sampling vessel at the bottom and leaving it at the top before going to a different position on the same selection valve. This valve sent alternatingly every 20 minutes one of the two streams through the sample dryer to a 6-port valve (Valco Instruments Co. Inc.; Houston, Texas, U.S.A.) equipped with a 2 ml sampling loop. Samples were analysed for N_2O concentration by gaschromatography (SRI-8610C, SRI Instruments, Torrance, CA, U.S.A.) with electron capture detector. Concentrations of O_2 were regularly controlled with an O_2 sensor (CheckMate9900, PBI-Dansensor A/S; Ringsted, Denmark).

Net N_2O flux was calculated from the difference in N_2O concentrations between the inlet and the outlet of the incubation vessel divided by the flow rate. We assume that N_2O reduction in the range of concentrations applied is a first order process, following the first order rate law:

$$-\frac{\Delta[S_t]}{\Delta[t]} = k[S_t]_0 \qquad (1)$$

To determine k, Equation 1 can be written as:

$$k = -\left(\ln\frac{[S_t]}{[S_0 + S_P]}\right)\frac{1}{t} \qquad (2)$$

S_0 and S_t are N_2O concentrations [µmol mol^{-1}] entering and leaving the incubation vessel, respectively. S_P is the concentration increase resulting from a small N_2O production in the soil within the vessel [µmol mol^{-1}]. t is the mean residence time of the gas in the incubation vessel [s] and k is the reaction rate constant [s^{-1}].

The reaction rate indicates the quantity of substrate turned over per unit of time and is dependent on substrate concentration, whereas the reaction rate constant indicates the speed of the reaction, independent from substrate concentration. Thus, the reaction rate constant is a qualitative parameter of a process.

The overflow of the sample loop was taken immediately after GC injections for stable isotope analysis. This was directed to a trap, 20 cm 1/8" stainless steel tube, cooled with liquid nitrogen. CO_2 was filtered before the trap with soda lime (Fluka, Buchs, Switzerland). At liquid nitrogen temperature, N_2O is trapped while most N_2 and O_2 pass the trap. The sampling time was chosen such that roughly the same amount of N_2O (0.11 - 0.22 µg) was obtained for the isotope measurements independent from the applied concentration. N_2O was measured by Continuous Flow GC/MS on a Finnigan MAT 253 mass spectrometer (Thermo Finnigan MAT GmbH, Bremen, Germany). Separation of N_2O from remnant N_2 and O_2 was performed

on a Porabond Q capillary column. Isotope values are reported relative to the isotope ratio of the inlet stream.

We calculate the fractionation factor of the reduction reaction for this Rayleigh type experiment.

$$\frac{R_S}{R_0} = f^{(\alpha - 1)} \tag{3}$$

R_0 and R_S are the isotope ratios $^{15}N/^{14}N$ of the nitrous oxide entering the incubation vessel and the remaining nitrous oxide fraction f leaving the incubation vessel. Since we report our δ-values for the outlet concentrations versus the δ-values of the inlet concentrations equation 3 can be rewritten as:

$$\delta_S + 1 = f^{(\alpha - 1)} \tag{4}$$

$$\ln(\delta_S + 1) = (\alpha - 1)\ln f \tag{5}$$

Further on we report the fractionation factor as $\varepsilon = \alpha - 1$ in ‰.

2.4 Results

The mean residence time in the incubation vessel is about eight minutes. During that time, the soil consumed between 11 and 53 % of the available N_2O including the small amounts of N_2O produced by the soil. The mean fraction of all substrate (N_2O) concentrations consumed depended on the O_2 concentration in the gas mixture (Table 2.1). With increasing O_2 concentrations, the fraction of N_2O consumed decreased. After 6 days, the O_2 concentration was changed to 0.2 % again, which was followed by an increase of the consumed fraction of N_2O, reaching almost the same level again as four days earlier.

We assume N_2O consumption to be a first order process within the range of applied N_2O concentrations. Further, we assume N_2O production to be independent from N_2O concentration in the incubation vessel. These assumptions imply, that N_2O consumption approaches zero with decreasing N_2O concentrations in the incubation vessel, while N_2O production remains unchanged. Thus, a linear regression fitted to net N_2O flux over N_2O concentration indicates soil N_2O production at zero N_2O concentration (Fig. 2.1). Production of N_2O determined this way was small in all three samples. Nevertheless, at low N_2O concentrations, where gross consumption was small, soil production became a relevant contribution to net flux. We therefore decided arbitrarily to consider gross production negligible as long as its value did not exceed 13.5 % of gross consumption. Data not meeting

this requirement was excluded from further analysis. In the remaining data, soil production was on average equivalent to about 1.2 % of gross consumption.

Table 2.1: Fraction of N_2O consumed during an eight minute passage through 200 g of soil aggregates at different O_2 concentrations. Mean values and standard deviations (SD) were calculated for samples of the same O_2 concentration averaging over all N_2O concentrations for the particular O_2 concentration.

Day since field sampling	Amount of samples	O_2 conc. (%)	Fraction consumed	
			Mean	SD
3	9	0.2	0.54	0.24
4	7	0.5	0.34	0.09
5	3	1.0	0.16	0.01
6	6	1.5	0.13	0.04
7	4	0.2	0.46	0.18

Reaction rate constants of N_2O reduction were independent from N_2O concentrations but depended on O_2 concentrations. They were largest at 0.2 % O_2, on average half as large at 0.5 % O_2, and about four times smaller at 1.0 % or 1.5 % O_2. Fractionation factors for N ranged from -36.0 ‰ to -1.0 ‰ for reaction rate constants between 1.7 10^{-4} s^{-1} and 4.5 10^{-3} s^{-1}. Fractionation factors with the largest absolute value were observed for the smallest reaction rate constants and vice versa (Fig. 2.2). The median of the observed fractionation factor for N was -9.3 ‰ (1 standard error = 1.3 ‰). Each value represents the mean of 2 to 20 replicate measurements at one particular N_2O und O_2 concentration for one sample. A regression fitted through all data followed a function with the equation $y = -0.12\ x^{-0.57}$ ($r^2 = 0.49$, $p < 0.001$).

Fractionation factors for O ranged from -74.0 ‰ to -6.5 ‰ and were similarly dependent on reaction rate constants as fractionation factors for N (Fig. 2.3). The median of the observed fractionation factors for O was -26.3 ‰ (1 standard error = 2.6 ‰). A regression fitted through all data followed a function with the equation $y = -1.50x^{-0.38}$ ($r^2 = 0.47$, $p < 0.001$).

Fractionation factors for O were on the whole by a factor of 2.4 ± 0.3 larger than fractionation factors for N (Fig. 2.4). A linear regression forced through the origin suggested a significant correlation ($p < 0.001$) between the fractionation factors for O and N. The ratio of the fractionation factor for O to the fractionation factor for N was always larger than 2 and in only 3 out of 29 cases significantly larger than 4 (Fig. 2.4).

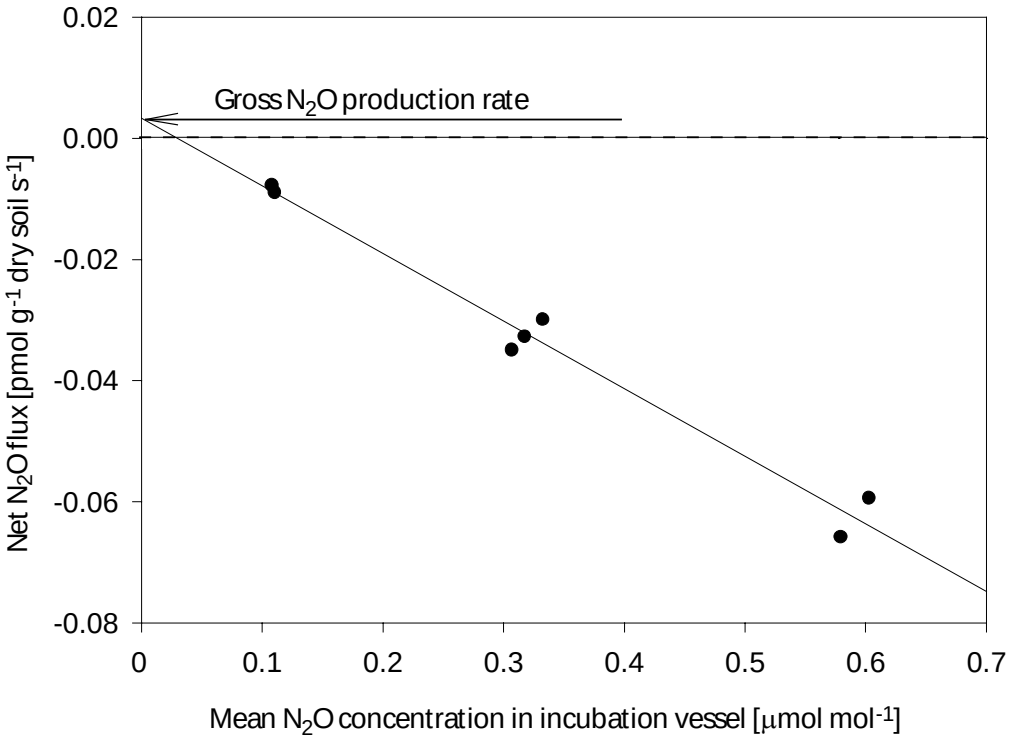

Fig 2.1: Example of net N_2O fluxes as a function of N_2O concentrations in the incubation vessel. Gross N_2O production rate is close to net N_2O flux at zero N_2O concentration.

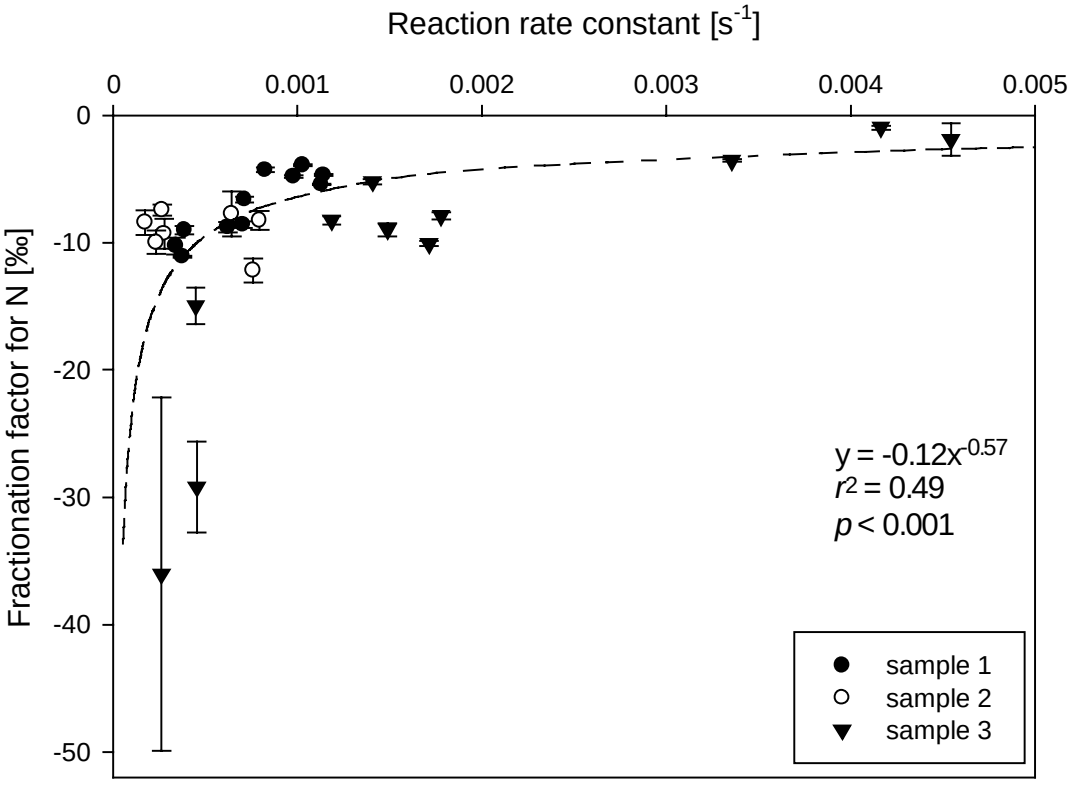

Fig. 2.2: Fractionation factor for N as a function of reaction rate constant. Error bars indicate one standard error. Each point represents the mean of 2-20 replicate measurements at one particular combination of N_2O and O_2 concentration. The median is -9.3 ‰ (1 standard error = 1.3 ‰).

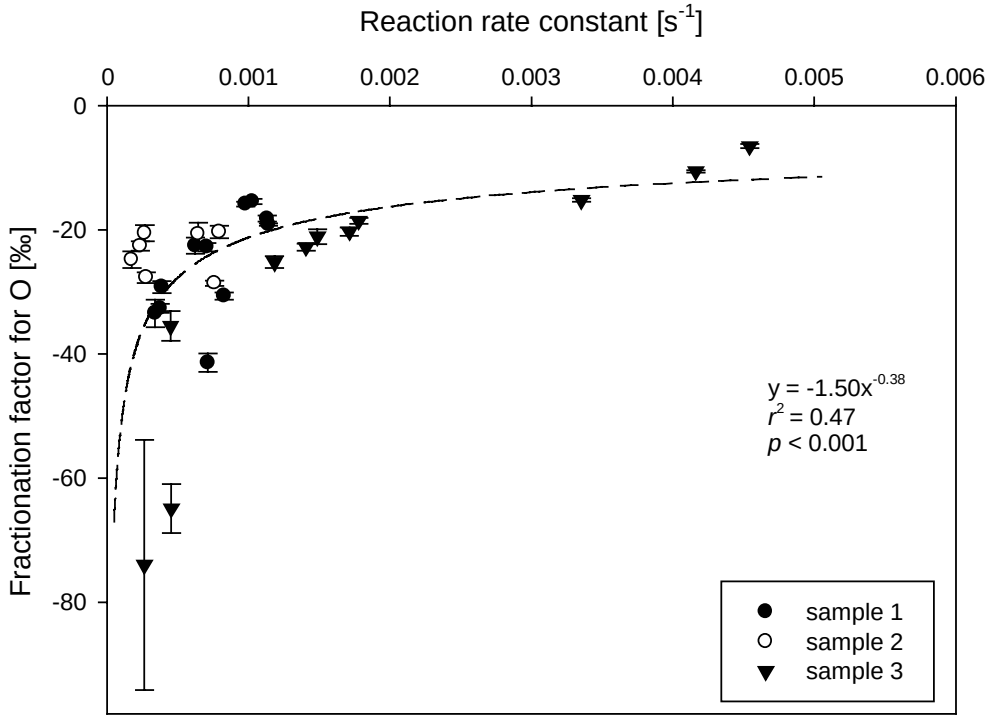

Fig. 2.3: Fractionation factor for O as a function of reaction rate constant. Error bars indicate one standard error. Each point represents the mean of 2-20 replicate measurements at one particular combination of N_2O and O_2 concentration. The median is -26.3 ‰ (1 standard error = 2.6 ‰).

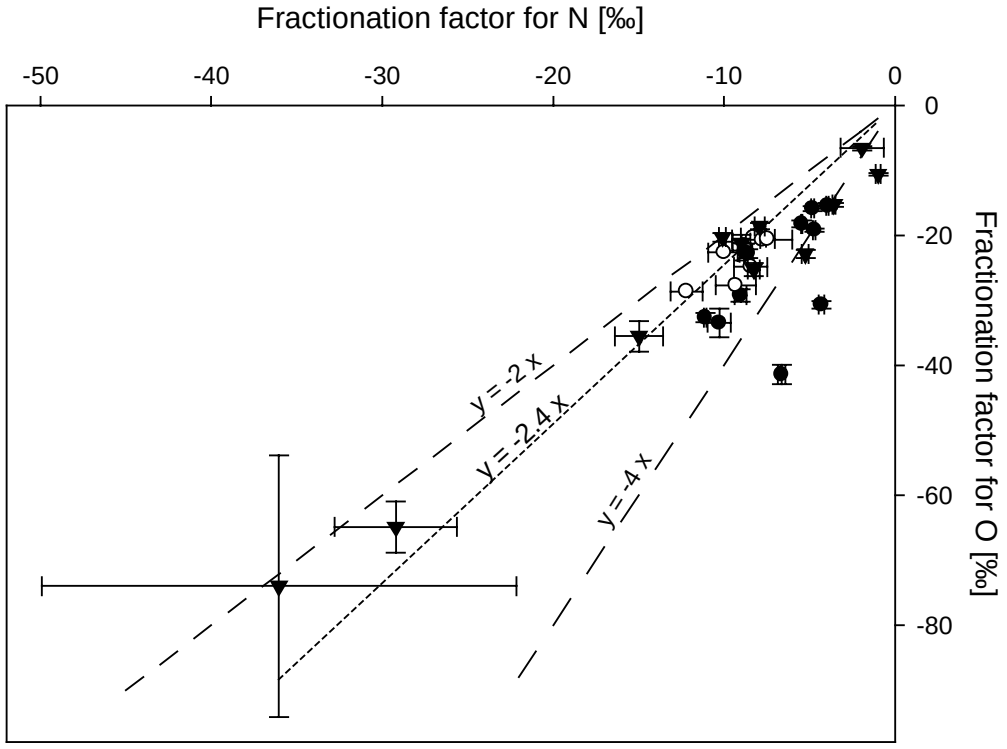

Fig. 2.4: Fractionation factors for O against fractionation factors for N. Error bars indicate one standard error. Each symbol represents a different soil sample. A linear regression, forced through the origin, and its function is indicated. Auxiliary lines for y = -2 x and y = -4 x are shown.

2.5 Discussions

With different O_2 concentrations we were able to produce a range of reaction rate constants in the process of N_2O reduction to N_2 ranging from 1.7 10^{-4} s^{-1} to 4.5 10^{-3} s^{-1}, similar to the range observed in the field at 5 cm depth on a comparable soil type in central Switzerland (6 10^{-4} s^{-1} to 4 10^{-3} s^{-1}) (Neftel et al., 2000). Net N_2O flux at zero N_2O concentrations at the inlet of the incubation vessel indicated N_2O soil production (Fig. 3.1). Ideally, there should have been no soil production to exactly determine fractionation of N and O during N_2O reduction to N_2. Such a condition is unlikely to occur in natural soils, where mineralisation continuously produces substrate for N_2O production and where a large proportion of the microbial population is able to produce N_2O. Our decision to arbitrarily consider gross production negligible as long as its value did not exceed 13.5 % of gross consumption has probably resulted in only a slight underestimation of fractionation factors for N and O, since the data fulfilling this selection criteria exhibited on average a soil production which was equivalent to 1.2 % of the gross consumption. Given the scatter in our data caused by other factors, we consider it unwarranted to attempt correction, which itself could only be based on inexact assumptions.

We found a median fractionation factor for N of -9.3 ± 1.3 ‰ which is within the range of fractionation factors of -6.3 to -9.8 ‰ found in experiments with Siberian soil from larch and birch forests (Menyailo, 2006; Menyailo and Hungate, 2006). These and our median fractionation factor are slightly smaller than the 12.9 ± 2.6 ‰ determined for *Paracoccus denitrificans* in a steady-state reactor (Barford et al., 1999). In another study with a pure culture of *Pseudomonas denitrificans* and soils, the fractionation factor for N was also larger for the pure culture (10.9 ‰) than for the soil (1.0 to 9.2 ‰) (Ostrom et al., 2007). One interpretation could be that the studied pure cultures had a slower rate of reaction than the average of the N_2O reducing population in the soils. More importantly, our fractionation factor decreased with increasing reaction rate constant, whereas Barford et al. (1999) concluded that the fractionation factor for $\delta^{15}N$ was constant over a range of O_2 concentrations. However, O_2 concentrations in our study were much higher and reaction rate constant might not have changed within the range of O_2 concentrations in the mentioned study. The difference in our findings to those of Barford et al. (1999) could also result from the fact that we were studying natural soil with a variety of denitrifying organisms and not one single denitrifying species. Differences in fractionation factors between species may occur (Casciotti et al., 2003). Hence, shifts in the relative contribution of different organisms to overall N_2O reduction with changing O_2 concentrations could explain our finding. The observation of the extent of isotopic fraction could be also explained through the different oxic and anoxic zones in the soil matrix and/or through the implied varying oxygen concentration throughout the experiment. At conditions with a relatively oxic soil matrix the scale of anoxic zones is limited. The length of the diffusion pathway and thus diffusion time

for N_2O to reach these anoxic zones where active N_2O reductase enzymes occur increased. The latter might well explain the simultaneous increase in isotopic fractionation. Isotope fractionation not being constant has also been observed for denitrification of NO_2^- (Bryan et al., 1983).

Reported ranges of fractionation factors for O during N_2O reduction range from -12.6 ‰ to -24.9 ‰ and from -2.7 to -24.5 ‰ for soils (Menyailo and Hungate, 2006; Ostrom et al., 2007). For pure culture a value of -24.8 ‰ was reported (Ostrom et al., 2007). The maximum values of these ranges are close to our median value of -26.3 ± 2.6 ‰. Again, we found a significant correlation of the fractionation factor for O with the reaction rate constant in the N_2O reducing process. Thus, we propose to consider the fractionation factors for N and O as variables. From our point of view, differences in reported fractionation factors for the biological reduction of N_2O might be the result of differences in the reaction rates constant as well of changing physical environment such as the diffusion pathways for N_2O.

The dependence of N and O fractionation factors on the rate of N_2O reduction makes it difficult to use isotopic shifts in N_2O alone to gain quantitative insights into the importance of N_2O consumption in a particular soil system. A constant ratio of the fractionation factors for O and N may at least qualitatively allow the detection of N_2O consumption in soils and other environments. The ratio found in our study (2.4 ± 0.3) was the same as found independently for two other soils from Siberia and one from the USA (2.5) (Menyailo and Hungate, 2006; Ostrom et al., 2007). This supports the hypothesis of the ratio of fractionation factors for O and N during biological N_2O reduction being constant. If so, it might be helpful to identify the process of N_2O reduction also in other environments. For example, decreasing N_2O concentrations in suboxic marine environments were found to coincide with a simultaneous enrichment in ^{18}O and ^{15}N with a ratio between 2 and 4 (Yoshinari et al., 1997). We could now interpret this as an indication for N_2O reduction being the dominant process of N_2O turnover in these waters.

The main sink for N_2O in the atmosphere is photochemical destruction in the stratosphere. For this process the fractionation factors for $\delta^{18}O$ and $\delta^{15}N$ are about equal (McLinden et al., 2003). In contrast we and others found that the biological reduction of N_2O appears to discriminate much stronger against $\delta^{18}O$ (Menyailo and Hungate, 2006; Ostrom et al., 2007). This difference may allow to better quantify the relative importance of either sink in the global N_2O budget.

2.6 Conclusion

Our findings lead us to conclude that the magnitude of stable isotope fractionation factors in the biological reduction of N_2O in soils, and possibly also in aquatic environments, are subject to the rate constant at which this process occurs. Observations of stable isotope enrichments

in the natural environment can therefore only give an indication of the magnitude of N_2O reduction, as a rate constant has usually to be assumed. However, the ratio of the enrichment factors for the stable N and O isotopes can help to qualitatively detect N2O reduction if reduction the dominating process in the turn-over of N_2O.

2.7 Acknowledgements

This study was supported by the Swiss State Secretariat for Education and Research through grant Nr. C04.0254 to COST Action 856, the University of Bern, and the Swiss National Science Foundation. We thank two anonymous reviewers for their helpful comments.

Chapter 3

The fate of N_2O consumed in soils

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3.1 Abstract

Soils are capable to consume N_2O. It is generally assumed that consumption occurs exclusively via respiratory reduction to N_2 by denitrifying organisms (i.e. complete denitrification). Yet, we are not aware of any verification of this assumption. Some N_2O may be assimilatorily reduced to NH_3. Reduction of N_2O to NH_3 is thermodynamically advantageous compared to the reduction of N_2. Is this an ecologically relevant process? To find out, we treated four contrasting soil samples in a flow-through incubation experiment with a mixture of labelled (98 %) $^{15}N_2O$ (0.5-4 ppm) and O_2 (0.2-0.4 %) in He. We measured N_2O consumption by GC-ECD continuously and $\delta^{15}N$ of soil organic matter before and after an 11 to 29 day incubation period. Any $^{15}N_2O$ assimilatorily reduced would have resulted in the enrichment of soil organic matter with ^{15}N, whereas dissimilatorily reduced $^{15}N_2O$ would not have left a trace. None of the soils showed a change in $\delta^{15}N$ that was statistically different from zero. A maximum of 0.27 % (s.e. ± 0.19 %) of consumed $^{15}N_2O$ may have been retained as ^{15}N in soil organic matter in one sample. On average, ^{15}N enrichment of soil organic matter during the incubation may have corresponded to a retention of 0.019 % (s.e. ± 0.14 %; n = 4) of the $^{15}N_2O$ consumed by the soils. We conclude that assimilatory reduction of N_2O plays, if at all, only a negligible role in the consumption of N_2O in soils.

3.2 Introduction

Nitrous oxide (N_2O) is produced in soils during the processes of nitrification and denitrification (Firestone et al., 1980). Since industrialisation, the global atmospheric N_2O concentration increased from about 270 ppb in 1800 to 319 ppb in 2005 (IPCC, 2006). Currently, more than one third of all N_2O emissions are of anthropogenic origin and primarily due to agriculture (IPCC, 2006). However, soils can also act as a sink for N_2O (reviewed in Chapuis-Lardy et al., 2007). In general, it is implicitly assumed that complete denitrification (reduction of N_2O to N_2) is the only process responsible for observed sink activity. Once produced by a soil organism, a molecule of N_2O is presumed to take one of the three known routes (Ostrom et al., 2007) (Fig. 3.1): (1) complete denitrification to N_2 within the cell prior

to its escape into the gas phase (reviewed in Zumft, 1997); (2) escape from the cell into the gas phase of soil and potentially to the atmosphere; or (3) complete denitrification to N_2 upon re-entering a cell capable to reduce N_2O (e.g. Neftel et al., 2000; Clough et al., 2005). To the best of our knowledge other pathways have not been considered in natural soil so far. Here, we hypothesise a fourth pathway of assimilatory reduction to NH_3 may be responsible for some of observed N_2O consumption in soil (Fig. 3.1).

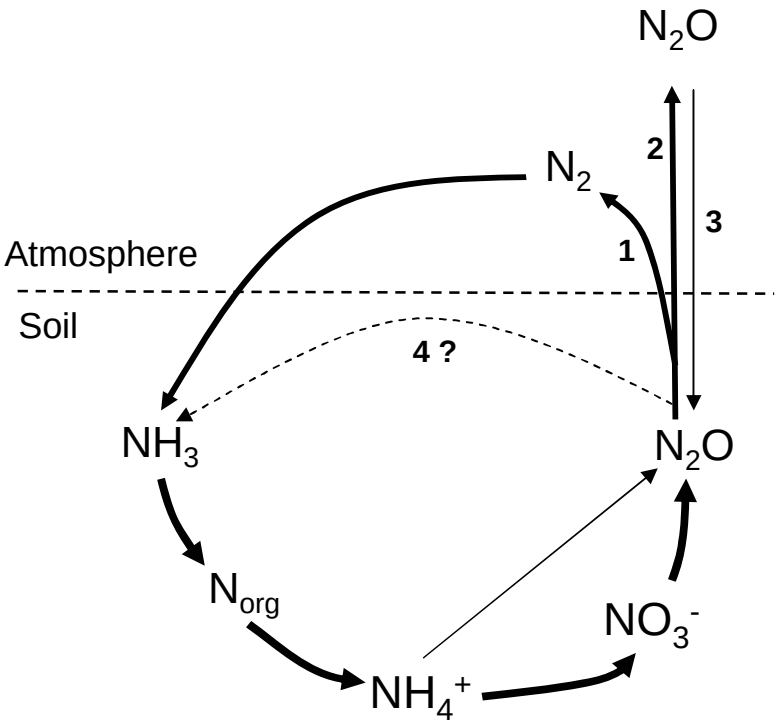

Fig. 3.1: Origin and possible fate of N_2O in soil. (1) Complete denitrification to N_2 before escape from the cell; (2) escape from cell; (3) re-entering a cell and subsequent reduction to N_2, or (4) assimilatory reduction to NH_3. The ecological relevance of pathway (4) in natural soil is unknown and the focus of this study.

The only enzyme known to reduce N_2O to NH_3 is nitrogenase. First evidence was provided by Mozen and Burris (1954). Later studies confirmed that N_2O can be a substrate for nitrogenase which reduces N_2O to N_2 with subsequent reduction of N_2 to NH_3 (Hoch et al., 1960; Hardy and Knight, 1966; Jensen and Burris, 1986). However, one year later, reduction of N_2O to N_2 was already questioned by Yamazaki et al. (1987). They concluded from the stable isotope kinetics during N_2O fixation by *Azotobacter vinelandii* that N_2O fixation by nitrogenase must be an apparent one-step reaction transforming N_2O directly into NH_3, without the intermediary N_2. This conclusion was supported by a re-interpretation of the original results of Jensen and Burris (1986) by Burgess and Lowe (1996). The re-interpretation suggested there may be an additional pathway from N_2O to NH_3 which does not involve N_2 as an intermediary. Enzyme kinetics indicate a low affinity of N_2O to nitrogenase. A k_m value of 24 kPa for purified component proteins from *Klebsiella pneumoniae* has been determined by

Jensen and Burris (1986). Yet, (apparent) substrate affinity can vary substantially between organisms and possibly methods. For N_2O consumption by anoxic soil incubations and denitrifiers, for example, k_m values differing by a factor of 2000 have been reported (Conrad, 1996). Even so, we would not expect nitrogenase to substantially contribute to N_2O consumption within the range of naturally occurring N_2O concentrations. Yet, other, unknown processes may account for some of the observed N_2O consumption in soil. Discovery of new processes and responsible organisms continues to our days (e.g. Strous et al., 1999). Strong support for the possible existence of assimilatory reduction of N_2O to NH_3 comes from thermodynamical considerations. Shestakov and Shilov (2001) concluded after the theoretical study of model reactions involving N_2O that a direct reduction of N_2O to NH_3 would be possible and thermodynamically advantageous to a reduction of N_2. The dissociation energy for the N-N bond in N_2O is only half that of the N_2 molecule (Herzberg, 1966). Our objective was to clarify whether such a reduction of N_2O is an ecologically relevant process occurring in soil.

3.3 Material and Methods

Samples were obtained from the upper 10 cm of soil at three locations in Switzerland and one location in South-West Germany. The first site, A, is located at Zurich-Reckenholz (47°26'N, 8°32'E at 491 m a.m.s.l.), the second site, B, is located in central Switzerland (47°17'N, 7°44'E at 450 m a.m.s.l.; for more details see Flechard et al., 2005). Both sites have soil types classified as cambisol and are experimental grassland sites of the Research Station ART (Agroscope Reckenholz-Tänikon), Switzerland. The third site, C, is located close to Basel (47°28'N, 7°42'E at 476 m a.m.s.l.) in a mixed deciduous forest on pseudogley over limestone; and the fourth site, D, is an open pine (*Pinus spp.*) forest on a peat bog in the Black Forest located 70 km north of Basel (47°52'N, 8°06'E at 975 m a.m.s.l.).

The soils of these sites range in texture from sandy loam to clay loam and organic, in pH from 2.9 to 7.0, in the C:N ratio from 9.4 to 28.8 and in the soil moisture from 23.0 to 93.6 % (Table 3.1). Fresh samples were broken into aggregates of ≤ 6.2 mm within 5 hours after collection from the field sites and 200 g were placed into an incubation vessel (glass, 415 cm^3) at 20°C for a 24 hours pre-incubation period. To minimise entry of atmospheric N_2 into the sample environment, we placed the incubation vessel during the entire experiment in an aluminium coated foil bag (volume about 2 litres), which was continuously flushed with He (200 ml min^{-1}). During the incubation, the samples were exposed to a mixture of labelled (98 %) $^{15}N_2O$ (0.5-4 ppm) and O_2 (0.2-0.4 %) in He (with a purity of 99.9999 %). Labelled $^{15}N_2O$ had been produced by the following thermal method. An amount of 0.1 g fully (98 %) labelled NH_4NO_3 (Cambridge Isotope Laboratories, Inc., Andover, USA) was gently heated with 5 ml 6M HNO_3 and 0.05g NaCl. The arising $^{15}N_2O$ was collected in a syringe. Later it was transferred with a stream of He into a 10 litre He bottle filled to 1.1 bar pressure and

topped up with He to 6 bar. Close to 100 % labelling of N_2O with ^{15}N was confirmed by mass spectrometry (T. Blunier, personal communication).

Table 3.1: Summary of soil sample properties and the results of the incubation experiments with ^{15}N labelled N_2O.

Sample	*A*	*B*	*C*	*D*
Ecosystem	Grassland	Grassland	Forest	Forest
Soil type	Cambisol	Cambisol	Pseudogley	Histosol
Texture	Sandy loam	Clay loam	Loam	Organic
C:N ratio	11.2	9.4	14.6	28.8
pH in 0.01 M $CaCl_2$	6.1	5.7	7.0	2.9
Moisture [%]	23.0	33.9	33.3	93.6
dry weight of sample [g]	154	132.2	133.4	12.8
Initial mass of ^{15}N in sample [mg]	1.02	1.55	1.91	0.82
Duration of incubation [days]	11	20	12	29
$^{15}N_2O$ offered to sample [mg ^{15}N]	1.23	1.86	0.81	1.84
$^{15}N_2O$ consumed [mg ^{15}N]	0.21	0.18	0.29	0.20
Shift in $\delta^{15}N$ during incubation [‰]	0.12	-0.23	-0.09	0.66

In the incubation experiment, a gas stream of the labelled $^{15}N_2O$, O_2 and He was mixed together and monitored by mass-flow controllers. It passed a humidifier and was divided into two equal streams with flow rates around 30 ml min^{-1}. One of the gas streams passed through the incubation vessel containing the soil sample before entering a 6-port selection valve (Valco Instruments Co. Inc.; Houston, Texas, U.S.A.). The second gas stream arrived directly at the selection valve. This valve selected alternating one of the gas streams and sent it through a Nafion® dryer (MD™ Series Gas dryer, Perma Pure LLC.; Toms River, N.J.,U.S.A.) to a 2 ml sample loop on an injection valve (10-port selection valve, Valco Instruments Co. Inc.; Houston, Texas, U.S.A.) for 5 minutes, while the other gas stream was vented to the atmosphere. Concentrations of N_2O were measured by GC-ECD (SRI8610C Gas Chromotograph; SRI Instruments Inc.; Las Vegas, N.V., U.S.A.). By varying the concentration of N_2O in the gas mixture, we were able to determine rates of gross production and gross consumption of N_2O from a linear regression fitted through the measured net N_2O fluxes against N_2O concentrations (Vieten et al., 2007). The $\delta^{15}N$ of soil organic matter (SOM) was measured (on three or more sub-samples) before and after an 11 to 29 day incubation period (Table 3.1) on the Flash Elemental Analyser (Thermo Finnigan; Milano, Italy) connected to a CF-IR-MS (DELTA^{plus}XP; Thermo Finnigan MAT; Bremen, Germany).

3.4 Results and Discussions

During the incubation period of 11 to 29 days, between 0.81 and 1.86 mg of fully labelled $^{15}N_2O$ were flowing through each soil sample, containing a background of 0.82 mg to 1.91 mg of ^{15}N in organic matter (Table 3.1). During this time, 0.18 mg to 0.29 mg of the offered $^{15}N_2O$ was consumed (Table 3.1). Figure 3.2 presents the fraction of consumed $^{15}N_2O$-N that might have been retained as part of the soil organic matter. This fraction was between -0.20 % (±0.11 %) and 0.27 % (±0.19 %) with an average across all four soils of 0.019 % (±0.12 %). None of the measured values were significantly different from zero ($p > 0.05$). Thus, we can consider them as measurement noise. Still, their presentation is useful insofar as it serves to illustrate the sensitivity of our method. This sensitivity was about one order of magnitude larger than that of the classical assay for N_2 fixation by acetylene reduction, as for example described by Weaver and Danso (1994). This sensitivity was brought about by the long duration of the incubation, the large atom fraction of ^{15}N in the consumed N_2O (>0.98) and the high rates of N_2O consumption by the soil samples. During the incubation period, an equivalent of 11.6 % to 24.4 % of ^{15}N initially present in the soil samples was consumed as $^{15}N_2O$ (Table 3.1).

No significant N_2O production by the soil samples themselves was detected during these experiments. Therefore, $^{15}N_2O$ would have been the principal source for potential N_2O assimilation. During our experiments, we incubated the soils with He (purity of 99.9999 %) plus small amounts of O_2 and $^{15}N_2O$. Since we flushed the aluminium-coated foil bag that was around the incubation vessel continuously with He, we reduced the potential influx of atmospheric N_2 into the sample. We did not measure N_2 concentrations in the sample air but estimate that it has been in the order of ppm rather than per mil. Compared to the natural environment, the competitive advantage of N_2O (here: 0.5 - 4 ppm) relative to N_2 to be assimilated had been shifted by orders of magnitude in favour of N_2O. Thus, the likelihood for soil organisms to reduce N_2O instead of N_2 to NH_3 was substantially increased. Maybe, we should underline here that it was not our objective to measure nitrogenase activity but to find out whether some proportion of N_2O consumed in soil is assimilated, by whatever reduction pathway possible. Our method of choice ($^{15}N_2O$ labelling) would be unable to tell us anything specific about the nature of the pathway. We still chose this method because detection of ^{15}N in tissues of biological systems exposed to labelled N gas is the only direct, unequivocal method for demonstrating that its assimilation occurred (Weaver and Danso, 1994).

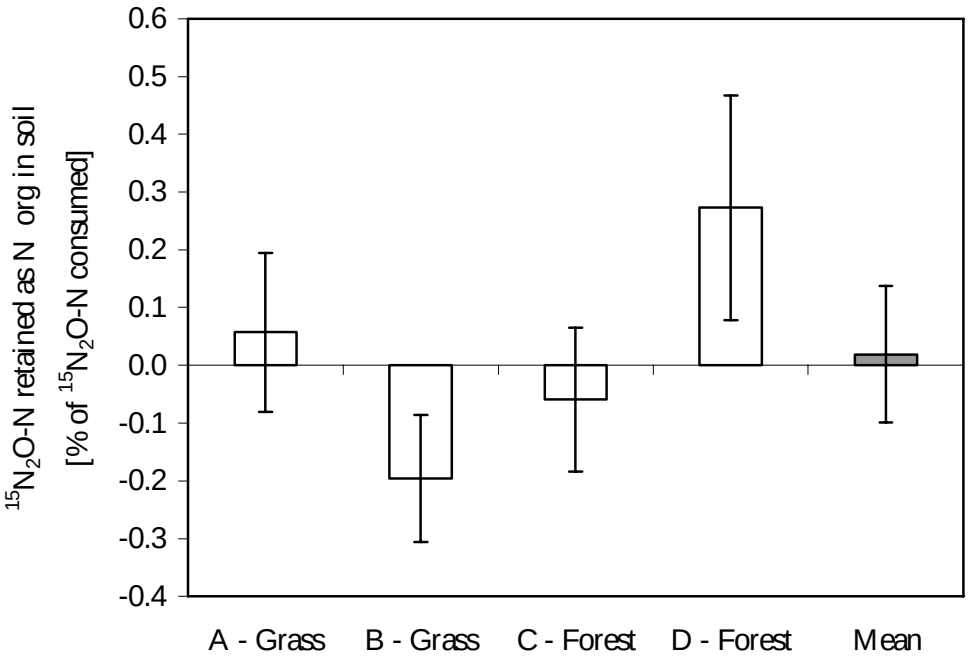

Fig. 3.2: Proportion of consumed $^{15}N_2O$-N that might have been retained in four soil samples. Error bars indicate ± 1 standard error of the measurement as determined by the measurements of $\delta^{15}N$ in soil organic matter on replicate sub-samples before and after the incubation period. The proportion retained has been calculated as: Shift in $\delta^{15}N$ during incubation [‰] / 1000 [‰] * initial mass of ^{15}N in sample [mg] / $^{15}N_2O$ consumed [mg] *100 [%].

The negative result regarding N_2O assimilation is unexpected, considering the observed large rates of N_2O consumption, the thermodynamical advantage of N_2O over N_2 as a substrate for the production of NH_3 (Shestakov and Shilov, 2001), and the evidence of direct N_2O to NH_3 reduction as a biological process (Yamazaki et al., 1987) albeit only for a low affinity enzyme (Jensen and Burris, 1986) so far. One would expect natural selection to have favoured organisms assimilating N_2O rather than N_2. We can not completely rule out that N_2O assimilation may have some ecological importance, at least in N limited soils. Still, our results strongly indicate that in environments similar to those studied here, N_2O assimilation is an ecologically irrelevant pathway in N_2O consumption by soil. Thus, the general assumption that N_2O is exclusively consumed by dissimilatory reduction to N_2 (i.e. complete denitrification) almost certainly applies in these environments.

3.5 Acknowledgements

We thank Thomas Blunier from the Institute of Climate and Environmental Physics of the University of Bern for analysing our labelled $^{15}N_2O$. This study was supported by the Swiss State Secretariat for Education and Research through grant Nr. C04.0254 to COST Action 856 and the Swiss National Science Foundation.

Chapter 4

Kinetics of nitrous oxide reduction in two hydromorphic soils

This chapter is in preparation for publication

4.1 Abstract

Soil microorganisms are known to produce nitrous oxide (N_2O) during nitrification and denitrification. Some of them have the ability to reduce N_2O to N_2 before it emits to the atmosphere. Although N_2O consumption is assumed to occur under anaerobic condition there were observations of N_2O consumption at low O_2 even at atmospheric conditions. Our objective was to define the ecological importance of N_2O consumption in relation to the total respiration in soils at suboxic. An additional objective was to compare two different sizes of soil aggregates in their effect on N_2O consumption. We performed experiments with two hydromorphic soils in continuous flow-through incubation at suboxic condition (here: 0.15% O_2). After the samples had reached steady state, we determined the kinetics for N_2O consumption at different temperatures and oxygen concentrations, the total respiration rate and the N_2O reduction rate for two different aggregate sizes of one soil. We received N_2O reduction rates (V_{max}) of 0.11 - 3.22 pmol s^{-1} g^{-1} dry soil and k_m values of 1.8-10.4 ppm in soil gas phase (0.045 -0.26 μM in soil solution) for all applied temperatures and oxygen concentrations. The k_m values we received fit in a range of k_m values determined for other soils. In contrary, the k_m values measured in pure cultures were between 96 – 300 ppm and therefore 9 times higher than the k_m values we measured. The mean Q_{10} for V_{max}, apparent k_m and total respiration were for the pseudogley 2.4 (± 0.6), 1.5 (± 0.3) and 2.1 (±0.3) and for the peat 2.2 (± 0.2), 1.3 (± 0.2) and 2.5 (±0.2), respectively. We found out that bigger soil aggregates (≤ 6.2 mm) could consume up to 40% more N_2O than smaller soil aggregates (≤ 2 mm) depending on the N_2O substrate concentration. The ratio of N_2O to total respiration was maximum 2.5% for pseudogley and 1.6% for the peat. We could conclude that N_2O and aerobic respiration can coexist and that N_2O can be an alternative electron acceptor in hydromorphic soils under suboxic – in some cases even under aerobic – conditions. Thereby, up to 1.25% of the electrons can be transmitted to N_2O. The size of the soil aggregates has an effect on the potential of N_2O consumption and it seems that the affinity of the enzyme N_2O reductase is higher in soils than in pure cultures.

4.2 Introduction

Nitrous oxide is a climate relevant gas that microorganisms produced during nitrification and denitrification in soils. While N_2O can be emitted as a side product during nitrification, N_2O is an intermediate product during denitrification where nitrate is reduced over several steps to N_2 (Firestone and Davidson, 1989). During the last step of denitrification, N_2O accepts two electrons and is reduced to N_2 (Zumft and Körner, 2007). Zumft (1997) described the N_2O consumption as an autonomous form of respiration – a kind of anaerobic respiration, because several denitrifiers are able to use N_2O as the sole electron acceptor for the oxidation of organic compounds. A complete denitrification is the prevalent sink process for N_2O in soils. Other processes like incorporation of N_2O-N into the soil biomass could not be proven to be relevant (Vieten et al., 2008).

Since several years, the N_2O consumption is in the focus of surveys about its occurrence in the environment. Field studies measured N_2O uptake from the atmosphere into soil and thus could show that soil can be a sink for N_2O (Ryden, 1981; Neftel et al., 2000; reviewed in Chapuis-Lardy et al., 2007). Clough et al. (1999; 2005) or Van Groenigen et al. (2005b) performed experiences with soil cores in labs and found that a large part of the produced N_2O in the soil was reduced on its upward diffusion through the soil. Extraction of the enzyme N_2O reductase from pure cultures, which perform the transformation of N_2O to N_2, were studied and the activity of the enzymes under anaerobic conditions were determined (Kristjansson and Hollocher, 1980; Snyder and Hollocher, 1987; Teraguchi and Hollocher, 1989; SooHoo and Hollocher, 1991). The activity of N_2O reductase of soil slurries was measured under anaerobic conditions in airtight closed bottles (Blackmer and Bremner, 1976; Freney et al., 1978; Holtan-Hartwig et al., 2000; Holtan-Hartwig et al., 2002). Most lab studies were carried out under anaerobic conditions, which promote denitrifiers, from whom only 10 % have the ability to reduce N_2O (Gamble et al., 1977). The potential of N_2O respiration depends on several environmental conditions like the availability of oxygen (Cavigelli and Robertson, 2001), nitrate (Blackmer and Bremner, 1978), temperature (Holtan-Hartwig et al., 2002), soil pH (Stevens et al., 1998), water content of soils (Freney et al., 1978; Ryden, 1981; Bandibas et al., 1994), and microbial taxonomic diversity (Cavigelli and Robertson, 2001; Mei et al., 2004).

During the O_2 respiration, four electrons are transferred to one molecule of oxygen (O_2) with the help of the electron-transport chain. The released energy is stored over several steps and reactions in the molecule ATP, the energy unit of nature (Schlegel, 1992; Alberts et al., 1994). Aerobic respiration is highly competitive for electrons and will out compete denitrification or N_2O respiration to a high proportion. Under anaerobic conditions, the next microbial reduction processes would be theoretically denitrification or after nitrate depletion N_2O reduction following the sequential reduction chain depending on availability of electron

acceptors, pH and temperature (Paul and Clark, 1996). Other anaerobic respiration processes that would come next in sequential reduction chain are manganese reduction, iron reduction, sulphate and sulphur reduction and methanogesis. Recent studies showed that some of these redox processes can occur simultaneously in soil (Alewell et al., 2006; Paul et al., 2006). Therefore, the total respiration of soil is the sum of all redox processes that occur at the same time.

To the best of our knowledge, there are no studies, which dealt simultaneously in their experiments with the N_2O and total respiration. The objectives of this study were to determine if there is any ecological relevance of N_2O respiration in suboxic soils in relation to the total respiration through determining the kinetics for N_2O, the total respiration rate, and the ratio of N_2O to total respiration at suboxic conditions when both respirations processes are active and simultaneous. In addition, we tested, if the size of soil aggregates has any effect on N_2O consumption. The expression suboxic comes from the oceanography that describes oxygen minimum zones in the sea there the dissolved oxygen content is below 4.5 μmol kg^{-1} (0.1 ml l^{-1}) (Karstensen et al., 2008). In our experiments, we defined suboxic conditions at oxygen concentration below 0.3%. We used experimental set-up with a continuous flow of substrate gases through incubators. This allowed us to change individually the O_2 and N_2O gas concentrations of the soil samples. With the continuous flow, it was possible to keep the gas concentrations constant during the experiment, so that the soil samples could reach steady state.

4.3 Material and Methods

4.3.1 Soil

Samples were obtained from the upper 10 cm of soil at two locations, one in Switzerland and the other one in South-West Germany. The first site is located close to Basel (47°28'N, 7°42'E at 476 m a.m.s.l.) in a mixed deciduous forest on pseudogley over limestone; and the second site is an open pine (*Pinus spp.*) forest on a peat bog in the Black Forest located 70 km north of Basel (47°52'N, 8°06'E at 975 m a.m.s.l.). The soils were analysed on texture, pH – measured in 0.01 M $CaCl_2$, C, N and water content, soil dry weight and biomass (Table 4.1). Thereby the microbial biomass was determined with the substrate application technique of Blagodatsky (2000).

Table 4.1: Summary of soil characteristics and chemical data of the samples. The values are the mean over all samples, which were taken for the experiment. The dry weight was calculated from 200g fresh weight. pH was measured in 0.01 M $CaCl_2$.

Ecosystem	*Forest*	*Peat bog*
Soil type	Pseudogley	Histosol
Texture	Loam	Organic
C total [%]	6.7	46.6
N total [%]	0.43	1.68
C:N ratio	14.6	28.8
pH	7.0	2.9
Water content [%]	33.3	89.3
dry weight [g]	133.4	21.4
Biomass [mg/g dw]	0.31	1.44

4.3.2 Incubation system

The fresh samples from the pseudogley soil were broken and sieved to aggregates of ≤ 6.2 mm within 5 hours after collection from the field site. The sample was mixed and 2 sub samples each with 200 g were placed into incubators (glass, 470 cm^3) at 20°C for a 24 hours pre-incubation period. It was not possible to sieve the samples from the peat, because they are fibrous. These samples were picked in approximately 2 x 1 cm pieces and 2 sub samples each with 200g (volume of 165.7 cm^3) of fresh material and were placed into incubation vessels and set under the same conditions as the samples from the pseudogley.

For our flow through incubation experiment, we used three gases (nitrous oxide, oxygen and nitrogen). They were automatically mixed by 3 mass flow controllers and adjusted to a constant oxygen concentration of 0.15 %. The N_2O concentration changed from 0.3 to 12.2 ppm in 6-hour steps, with N_2 being the carrier gas. The continuous gas stream passed a humidifier and was divided into five equal streams with flow rates around 40 ml min^{-1}. Four of them (sample 1 to 4) ran as the incubation vessels containing the soil samples – from the bottom to the top – into a 4-port selecting valve (Flow-through flowpath valve, Valco Instruments Co. Inc.; Houston, Texas, U.S.A.). Every 10 minutes one after another of the 4 samples was sent to a 6-port selection valve. The fifth gas stream (inlet) arrived directly at the selection valve. This valve selected alternatingly between the common gas stream entering and one of the four gas streams leaving the incubation vessels. It sent it through a Nafion® dryer (MD™ Series Gas dryer, Perma Pure LLC.; Toms River, N.J.,U.S.A.) to a 2 ml sample

loop and a 500 µl sample loop on an injection valve (10-port selection valve, Valco Instruments Co. Inc.; Houston, Texas, U.S.A.) for 5 minutes, while the other gas stream was vented to the atmosphere. Concentrations of N_2O from the 2 ml sample loop were measured by GC-ECD and the concentrations of CO_2 from the 500 µl sample loop by GC-FID with methanizer (SRI8610C Gas Chromotograph; SRI Instruments Inc.; Las Vegas, N.V., U.S.A.). The inlet gas sample was measured every 10 minutes and the single sample gas every 40 minutes. The O_2 concentration was maintained at 0.15% (± 0.03%), which was controlled from time to time with an O_2 sensor (Checkmate 9900; PBI Dansensor, Ringsted, Denmark).

4.3.3 Experiments

The N_2O inlet concentration was changed every 6 hours and the temperature every 2-3 days. We allowed the samples to adjust to the new temperature for 24h. In one experiment with the peat soil, we changed the oxygen concentrations. After giving the system 72 hours time to adjust to the new conditions at 2% O_2, the oxygen concentrations was changed in 24 h steps from 4% to 6%, to 4%, to 2% and then to 20%. We used the peat soil, because we could not observe any N_2O consumption at oxygen concentrations higher than 1.5% for the pseudogley.

In another experiment, we sieved fresh samples of pseudogley to aggregate size of ≤ 6.3 mm and ≤ 2 mm. Two samples with 200g of each aggregate size were placed in an incubation vessel with a constant flow of 0.15% O_2 concentration and N_2O concentrations that changed every 6 hours from 2, to 5, to 8 and to 12 ppm with N_2 as the carrier gas.

4.3.4 Calculations

In several blank tests with empty vessels, we received very little difference (about 2 pmol $N_2O\ s^{-1}$) of the N_2O concentrations between the inlet and the outlet of the incubation vessels.

The first 24 h of the experiments and the first 2 hours of the measurement after a change of the N_2O concentration were discarded, giving the system time to adjust to the new conditions and N_2O concentration and to get in steady state (Fig. 4.1). From the remaining four hours of a new N_2O concentration, we calculated the mean of the N_2O inlet and outlet concentrations for further calculations.

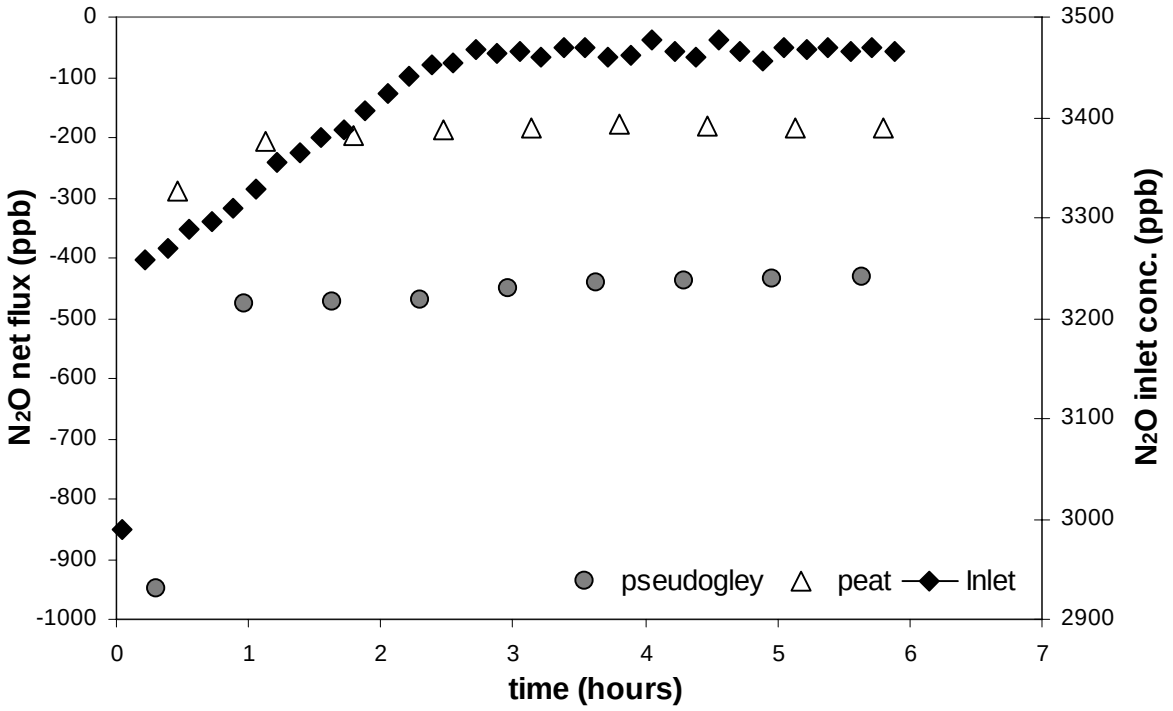

Figure 4.1: Example for a measurement of the N_2O net fluxes for pseudogley and peat after the change of the inlet concentration. It shows the time that the sample needs to adjust to the new condition.

We were able to determine rates of gross production and gross consumption of N_2O from a linear regression fitted through the measured net N_2O fluxes against inlet N_2O concentrations by varying the concentration of N_2O in the gas mixture (Vieten et al., 2007). The inlet N_2O concentrations were in the range of k_m for this fitting and therefore the N_2O reduction rate was not in saturation.

The average of the N_2O concentrations entering (Inlet) and leaving (Outlet) the incubation vessel was considerate as the substrate concentration that was available for N_2O consumption.

With the calculated N_2O reduction rate and the mean substrate concentration, we were able to estimate V_{max} and k_m. V_{max} is the maximum velocity at which enzymes catalyse a reaction at saturated substrate levels. k_m is the Michaelis-Menten constant that express the affinity of the enzyme for the substrate at which the reaction rate is at its half-maximum velocity (1/2 V_{max}). Plotting the observed N_2O reduction rate (velocity) against the mean substrate concentration, resulted in a Michaelis-Menten Curve (Fig. 4.2). The adequate k_m and V_{max} values were calculated with the Excel tool "Solver" that minimised the sum of residuals squared between observation and the function $V_{calculated} = (V_{max}*S)\ (k_m + S)^{-1}$ by changing V_{max} and k_m. We selected a time window for the calculation of V_{max} and k_m, in which microbial activity was relatively stable and included all induced temperature changes. This was due to the decrease of microbial activity over the experimental time.

The differences of the O_2 concentration between the inlet and the outlet of the incubation vessel had the same magnitude of the CO_2 concentration measured with the GC. We assumed that most of the CO_2 emissions are due to aerobic respiration. Through the CO_2 measurement and the assumption of the respiration quotient of 1, we received the total respiration rate.

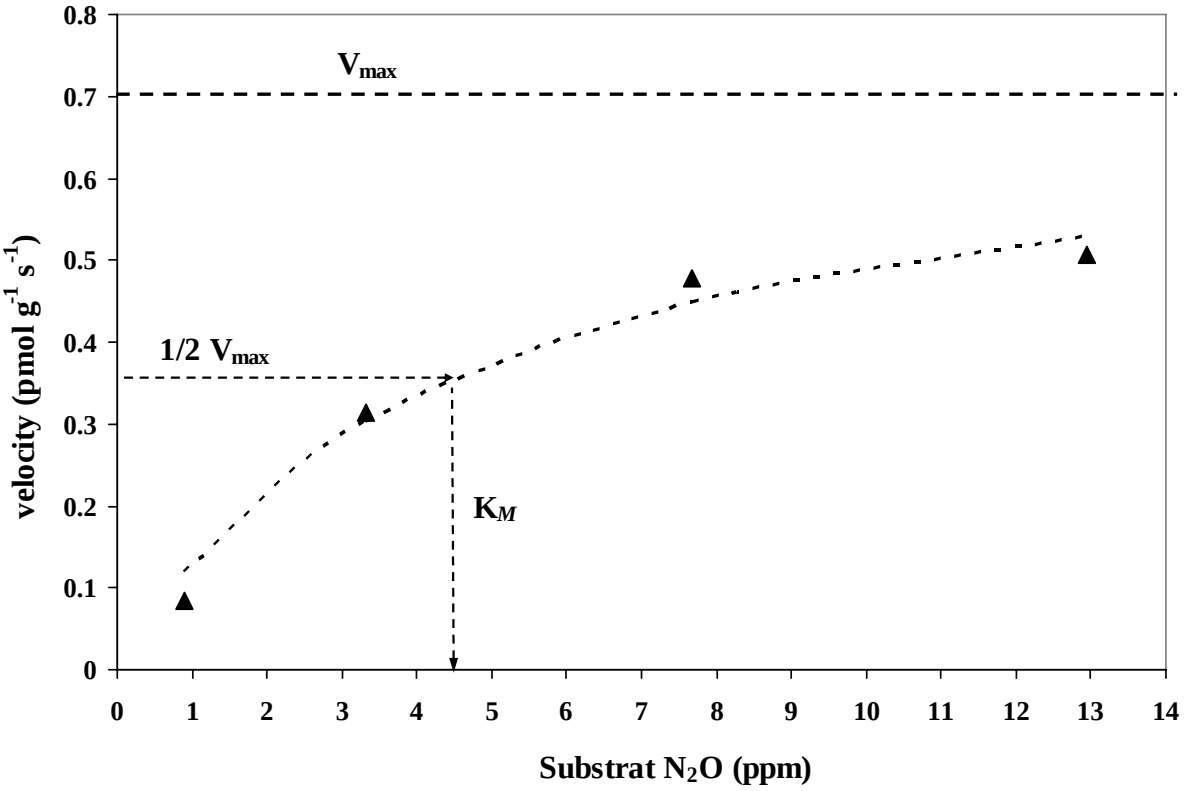

Figure 4.2: Example of the Michaelis Menten Curve for the peat bog soil from the 26.06.07 with V_{max}, ½ V_{max} and k_m. The substrate N_2O is in ppm and the reaction velocity in pmol s^{-1} g^{-1} dry soil.

4.4 Results

The dynamic of all experiments was very similar in the progress (for a typical example see Fig. 4.3). The sample released N_2O, which was about 3 times larger than the N_2O substrate concentration in the beginning of the experiment. This emission was followed by a net N_2O uptake after 24h (Fig. 4.3). At the same time, the CO_2 emission decreased by about 67% in 24 hours following the change from atmospheric to 0.15% O_2 concentration (Fig. 4.3; upper graph). The sample reacted from that time to all changes in substrate N_2O concentrations and temperature or oxygen concentrations. The activity of the soil microbes decreased by 25% (N_2O as well O_2 reduction) within the time of the experiment. The highest N_2O consumption we measured for this sample was about 42% of the substrate N_2O. The average of the N_2O consumption of the substrate N_2O was about 22%.

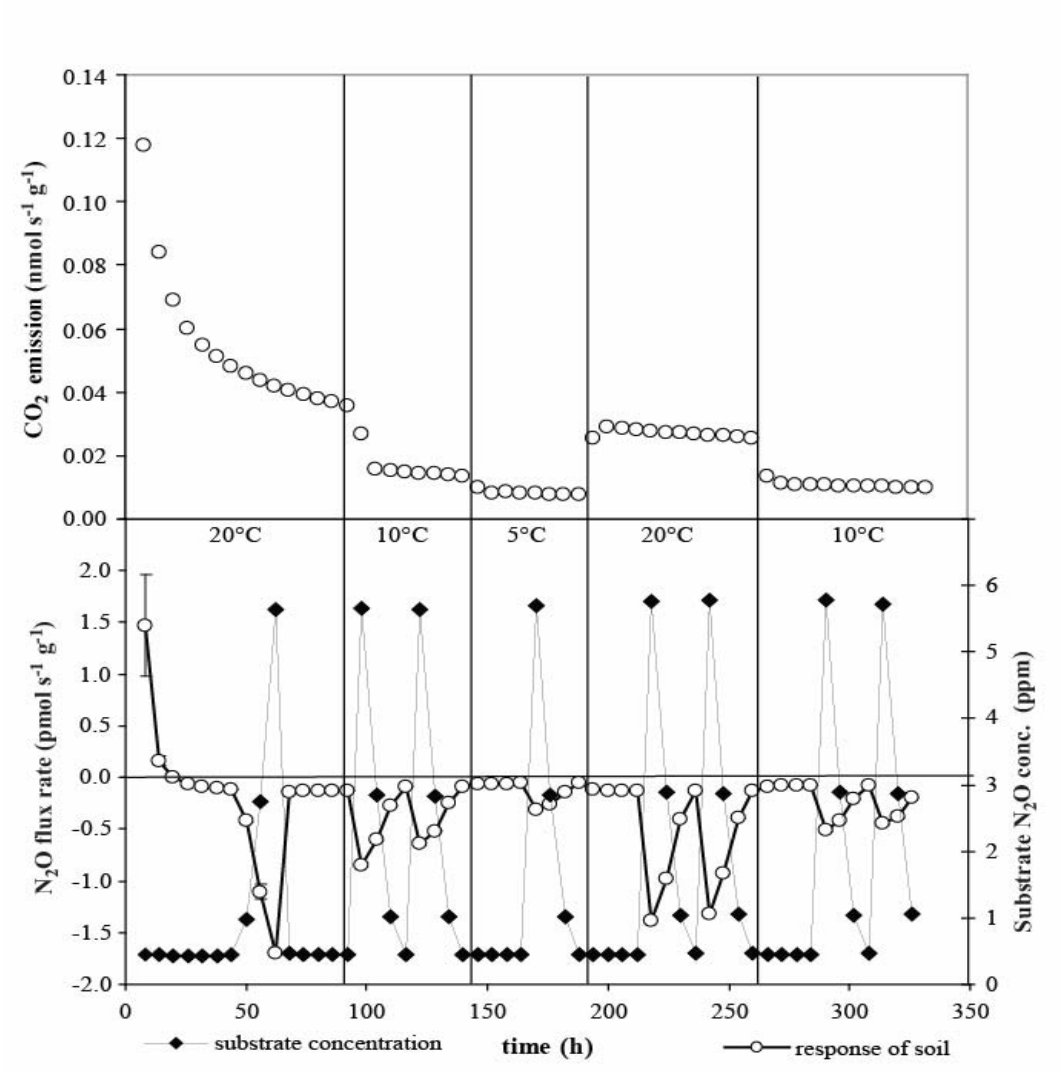

Figure 4.3: N_2O flux rates and mean substrate N_2O concentrations in pmol s^{-1} g^{-1} dry soil and the CO_2 emission in ppm over 14 days. The vertical lines indicate the timing of temperature changes. The data are from the pseudogley.

The k_m and V_{max} values for the pseudogley and peat were not significantly different (t-test, α = 5%; Table 4.2), but they vary fairly between the sub samples and between their collecting dates. The range for the k_m values was between 1.8 and 10.4 ppm N_2O in soil gas phase (0.04 – 0.26 µM N_2O in soil solution) and from 0.11 to 3.22 pmol s^{-1} g^{-1} dry soil for the V_{max} values. The V_{max} and k_m values changed with increasing temperature and lay in between 0.11 – 0.63; 0.18 – 1.37 and 0.55 – 3.22 pmol s^{-1} g^{-1} dry soil for V_{max} at 5, 10 and 20°C, respectively (Table 4.2). The k_m values were between 1.8 – 5.8 ppm, 2.1 – 8.2 ppm and 4.1 – 10.4 ppm at 5, 10 and 20°C, respectively (Table 4.2). The calculated mean temperature coefficients (Q_{10}) for V_{max}, apparent k_m and O_2 reduction for the pseudogley were 2.4 (± 0.6), 1.5 (± 0.3) and 2.2 (±0.1) and for the peat they were 2.2 (± 0.2), 1.3 (± 0.2) and 2.6 (±0.1), respectively (Table 4.2). The total respiration rates seemed to be similar between the seasons and the sampling dates of the soils. However, the total respiration rate in the pseudogley is about half of the rate in the peat. With the exception of the rates from the 6.12.07, there they

were a third of the peat total respiration rates (Table 4.2). The ratio of N_2O to total respiration rate were for pseudogley between 0.6 and 2.5%, average at 1.6% and for peat between 0.5 and 1.6% with an average of 0.8% at half-maximum N_2O and total respiration rate ($½V_{max}$) (Tabel 4.2).

The N_2O reduction rate declined by about 46% after the change of the oxygen concentration from 2 to 4% and it declined by another 27% after the change from 4 to 6% O_2 (Fig. 4.4). It rose after the change of 6 to 4% and 4 to 2% O_2 by about 30% and 45%, respectively, at all N_2O concentration (Fig. 4.4). The N_2O reduction rate was about 90% smaller at atmospheric O_2 concentration than at 2% O_2 concentration (Fig. 4.4). The N_2O respiration rates were at 3000 ppb 88% larger than at 300 ppb; 62% larger than at 750 ppb and 35 % larger than at 1500 ppb. The N_2O respiration rates were at 1500 ppb 79% larger than at 300 ppb and 41% larger than at 750 ppb and at 750 ppb it was 65% larger. The apparent k_m and V_{max} values lay between 4.0 – 9.5 ppm and 0.001 – 0.10 pmol s^{-1} g^{-1} dry soil for N_2O respiration, respectively. The total respiration rates were not significant different (t-test, α = 5%) at 2, 4 and 6% O_2 concentration (about 0.40 nmol s^{-1} g^{-1} dry soil). It decreased slightly to 0.38 nmol s^{-1} g^{-1} dry soil and than to 0.35 nmols^{-1} g^{-1} dry soil with the decrease of the O_2 concentration from 6% to 4 % and then to 2% the total respiration rate, respectively. The total respiration rate increased by about 23% to 0.46 nmol s^{-1} g^{-1} dry soil with the change from 0.2% to 20% O_2 concentration.

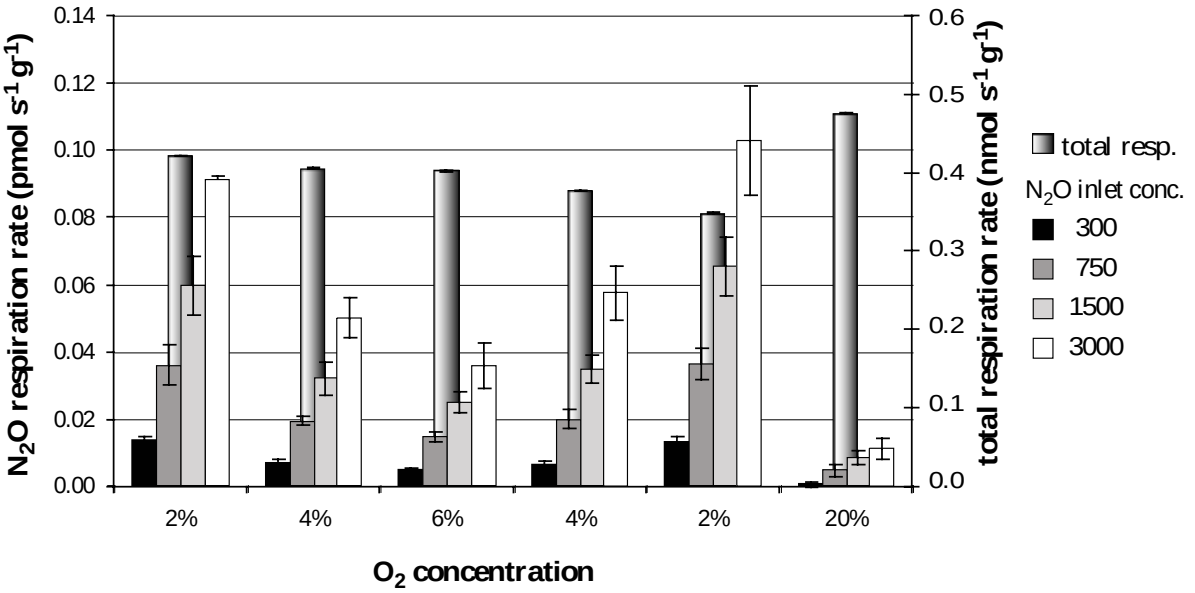

Figure 4.4: Chronological order of the N_2O respiration rate in pmol s^{-1} g^{-1} dry soil and total respiration rate in nmol s^{-1} g^{-1} dry soil (mean with standard deviation between quadruplicates). N2O inlet concentrations are in ppb.

Table 4.2: N_2O respiration values of V_{max} in pmol s^{-1} g^{-1} of dry soil and k_m in ppm in soil gas phase and V of the total respiration in nmol s^{-1} g^{-1} dry soil. Data are from the pseudogley and the peat at different temperatures in °C at their date of sampling. Average standard deviation between duplicates is ± 9.3 % of the mean V_{max} (N_2O), ± 17.9 % of the K_M and ± 0.1 % of the mean V (total respiration). The conversion factor from the k_m of soil gas phase into the k_m of soil solution (µmol) is at 20°C 0.025 (Henry constant).

Pseudogley	V_{max} (pmol s^{-1} g^{-1} dry soil) of N_2O				k_m (ppm) of N_2O				V (nmol s^{-1} g^{-1} dry soil) of total resp.			
Temp. (°C) \Date	26.06.07	12.07.07	06.12.07	08.01.08	26.06.07	12.07.07	06.12.07	08.01.08	26.06.07	12.07.07	06.12.07	08.01.08
5	0.16	0.30	0.11	0.48	2.65	5.16	1.79	4.95	0.008	0.008	0.009	0.015
10	0.34	0.69	0.18	1.17	3.54	8.23	2.11	7.35	0.013	0.014	0.013	0.024
20	0.91	1.24	0.55	2.34	5.07	9.45	4.07	10.07	0.029	0.025	0.034	0.048
Q_{10}	**2.66**	**1.80**	**3.12**	**2.00**	**1.43**	**1.15**	**1.93**	**1.37**	**2.15**	**1.77**	**2.58**	**1.99**
Peat	V_{max} (pmol s^{-1} g^{-1} dry soil) of N_2O				k_m (ppm) of N_2O				V (nmol s^{-1} g^{-1} dry soil) of total resp.			
Temp.(°C)\ Date	26.06.07	12.07.07	06.12.07	08.01.08	26.06.07	12.07.07	06.12.07	08.01.08	26.06.07	12.07.07	06.12.07	08.01.08
5	0.14	0.37	0.40	0.62	2.13	4.26	3.73	5.82	0.016	0.022	0.029	0.024
10	0.29	0.64	0.63	1.37	3.20	5.10	4.25	7.23	0.028	0.038	0.050	0.042
20	0.71	1.25	1.39	3.22	4.56	5.72	5.89	10.37	0.072	0.087	0.130	0.105
Q_{10}	**2.47**	**1.94**	**2.22**	**2.34**	**1.42**	**1.12**	**1.39**	**1.43**	**2.60**	**2.28**	**2.62**	**2.47**

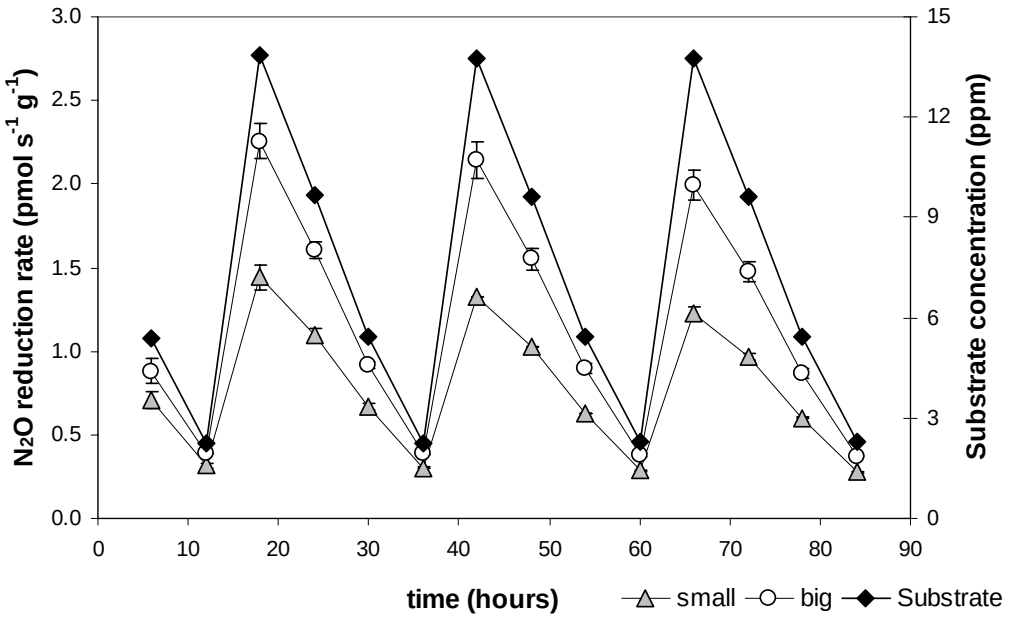

Figure 4.5: The effect of different aggregate sizes on the N_2O reduction rate in pmol s^{-1} g^{-1} dry soil with standard deviation (n = 2) in dependence on the applied substrate concentration in ppm. Small aggregates are ≤ 2mm and big aggregates are ≤ 6.3 mm.

She sample with the bigger aggregates had a N_2O reduction rate that was 40% higher than that of the smaller aggregates at 13.8 ppm N_2O substrate concentration. The percentage of the difference decreased with the change of the substrate to lower N_2O concentrations. At 9.6 ppm the difference was 25.7%, at 5.4 ppm 11.9% and at 2.3 ppm 4.4% (Fig. 4.5). The total respiration rate were

4.5 Discussion

We observed in almost all samples a large increase in the N_2O concentration with the onset of suboxic concentration at the beginning of the experiments. These observations are in agreement with whose of Ferney et al. (1978) and Bandibas et al. (1994). The reduction of the available nitrate to N_2O or N_2 most likely started with a delay when the soil reached a low oxygen level, because of the oxygen sensitive denitrification process. Bollmann an Conrad (1998) observed an increase of N_2O release through denitrification by a factor of 200 with the change of oxygen from 20 to 0.2%. We applied an oxygen concentration of about 0.15% and received a comparable steep increase of N_2O release. This N_2O emission is probably only due to denitrification, because nitrification is very low at 0.15 % O_2 concentrations (Bollmann and Conrad, 1998), if there was any. The change from N_2O production to net consumption took

place after about 24 hours. This change might be due to the exhausted availability of nitrate for the microorganisms in the micro pores (Robertson and Groffman, 2007). Another reason for the late N_2O net consumption could be that the enzyme N_2O reductase is highly sensitive towards oxygen. Thus the formation and the activity of the enzyme that occur at suboxic or anaerobic conditions started with a delay and reached the maximum of its activity after 20-30 hours (McKenney et al., 1994; Dendooven et al., 1996). It seemed that our soil sample with steady substrate concentration approached its activity maximum after 24 hours. The samples reacted sensitively to all the applied changes of N_2O concentrations and of temperatures or of oxygen concentrations (Fig. 4.3 and 4.4). Although the samples were not in steady state for the whole experiment, it was in a relatively constant condition over the time, in which we applied a constant N_2O concentration (Fig. 4.1). A reason for the decrease in the activity of the microorganisms could be that the availability of energy sources like easily degradable carbon compounds is limited and that over the time of the experiment they were less obtainable (Amundson and Davidson, 1990; Demoling et al., 2008).

Through the possibility to apply different N_2O inlet concentrations that was constant over a time interval, we could obtain k_m and V_{max} values of N_2O respiration under stable suboxic condition. The N_2O reduction rates (V_{max}) varied between sub samples with the same treatment and temperature (Table 4.2). Holtan-Harwig et al. (Holtan-Hartwig et al., 2000) as well observed that V_{max} values have large differences to each other and concluded that V_{max} values are limited suitable to compare different soils. McKenney (1994) argued that V_{max} values depend on the amount of active microorganisms and that they are therefore not very. The k_m values, on the other hand, are independent of the enzyme concentrations in the soil and so better suitable for comparing different soils (McKenney et al., 1994). We received k_m values, which were not significant different between the soils despite their contrasting soil characteristics, especially in their pH values (Table 4.1: pseudogley 7.0 and peat 2.9). This result is surprising because of the observations from Stevens et al. (1998) that N_2 emission increased with the increase of pH. Their observation leads to the assumption that high pH promote N_2O reduction to N_2. However Stevens et al. (1998) tested soils with pH from 5.6 to 8.0, and there are no data of the effect of low pH (< 5) on N_2O consumption.

All received k_m values at 20°C (4.1 - 10.4 ppm) were in the same range as the soils measured by Holtan-Hartwig et al. (2000) (0.1 – 0.4 µM, converted: 4 - 16 ppm). The k_m values measured in pure cultures like *Pseudomonas aeruginosa, Paracoccus denitrificans, Wolinella succiongenes* were from 2.4 to 7.5 µM (converted: 96 - 300 ppm) (Kristjansson and Hollocher, 1980; Snyder and Hollocher, 1987; Teraguchi and Hollocher, 1989; SooHoo and Hollocher, 1991). These k_m values were at least 9 times larger than the k_m values Holtan-Hartwig (2000) or we received in our studies. The affinity of the enzyme N_2O reductase seemed to be larger in soil microbe communities than in pure cultures. A reason for a better affinity in soils could be that wide variations of different denitrifiers live in soils, which are in

a constant competition for substrate to each other (Alewell et al., 2006). Cavigelli and Robertson (2001) and Mei et al. (2004) assumed that the organisation of the denitrifying taxa in soils might have functional significance to the N_2O consumption. Another reason could be that the studies that dealt with the pure cultures extracted and analyzed the enzyme without the cell (Kristjansson and Hollocher, 1980; Snyder and Hollocher, 1987; Teraguchi and Hollocher, 1989; SooHoo and Hollocher, 1991). The enzymes in solution might be more sensitive to environmental factors.

We received k_m values (4.0- 9.5 ppm) during the oxygen changing experiment for the peat at 2, 4, 6 and 20% O_2 concentrations that were in the same range as the k_m values of the temperature experiment. The k_m values seemed to be independent on oxygen concentration. N_2O consumption at aerobic conditions were already observed in wetland soils by Hemond (1983) and in pure cultures by Bell and Ferguson (1991) and McKenney et al. (1994). Bell and Ferguson (1991) presumed that some microorganisms use special mechanisms that protect the enzyme N_2O reductase from the disabling effect of O_2, as it was observed in the *Azotobacter* species. McKenney et al. (1994) argued that O_2 was limited in aqueous phase due to the kinetic effects and the low solubility of O_2 into water. This can cause formations of various microhabitats in soils that allow microorganisms to perform different redox processes at the same time (Paul et al., 2006; Alewell et al., 2007). This would explain our result that bigger soil aggregates consumed more N_2O (up to 40%) than the smaller soil aggregates in the pseudogley. It is more likely that big soil aggregates can develop anaerobic areas inside the aggregates than the small ones. The peat again has bigger aggregates and in addition higher water content than the pseudogley. Both parameters seemed to be important for the development of anaerobic microhabitats in which N_2O can be consumed independent of the applied oxygen concentrations. This would explain that we could measure N_2O consumption in the peat at atmospheric oxygen concentrations, while N_2O consumption in the pseudogley was not detectable at O_2 concentration above 1.5%. We measured the same CO_2 emission rate (total respiration rate) at 2, 4, and 6% O_2 concentration in the peat in the beginning of the experiment. With the decrease of the O_2 concentration, we received a slight decrease in the total respiration rate (Fig. 4.3). With a decrease of about 90% of the O_2 concentration (20% to 2% O_2 concentration), we would expect a similar high decrease in the total respiration rate. Reasons for the relative large total respiration might be that the microorganisms are adapted to natural occurring low O_2 concentration in the peat and that some denitrifiers in this community do not have a high sensitivity towards O_2 (Cavigelli and Robertson, 2001).

The measured CO_2 emission had the same magnitude as the measured difference of the O_2 concentration between the inlet and outlet of the incubation vessel. We assumed that aerobic respiration was still active and that it was mainly and in a smaller extent N_2O respiration responsible for the CO_2 emission. This assumption is supported through the depletion of easy accessible nitrate after the first 24 hours and that the soil had no putrefactive smell after the

experiment, which other anaerobic redox reaction would induce. We concluded that the total respiration rate was at its half-maximum rate at 0.15% O_2 concentration, as the decrease of the CO_2 production was about 50% in both soils after the first 24 hours. We calculated the ratio of N_2O to O_2 respiration rates at their half-maximum velocity ($½V_{max}$) (Table 4.2). At this rate, all participating enzymes of N_2O and O_2 respiration were in a similar active phase and therefore the respiration rates were comparable. The N_2O/O_2 respiration rate ratios for our soils were from 0.5 and 2.5% depending on the soil. Since O_2 accepts 4 electrons (Alberts et al., 1994) and N_2O 2 electrons during their reduction (Zumft and Körner, 2007), we could conclude that in the pseudogley up to 1.25% and in the peat up to 0.8% of the electrons were transmitted to N_2O. N_2O can be an alternative electron acceptor in soils at suboxic conditions that probably occur in anaerobic microhabitats of the soil.

4.6 Conclusion

N_2O respiration was clearly measurable in the soils under suboxic condition and even at atmospheric O_2 concentration in peat. The potential of the N_2O respiration rate depends on the substrate and oxygen concentration, the size of the soil aggregates, and temperature. The size of the soil aggregates seemed to be important for the formation of anaerobic microhabitats within the soil and inside the soil aggregates. N_2O can be an alternative electron acceptor under suboxic conditions in hydromorphic soils even if aerobic respiration is still active. These redox processes most likely occur separated in different microhabitats.

The similar k_m values in our two soils and the three soils of Holtan-Hartwig et al (2000) lead to the assumption that there might be a common range of k_m values for N_2O consumption in soils. We determined k_m values at different oxygen concentrations in peat that fit this range. We concluded that the k_m values of N_2O consumption are independent of the oxygen concentrations in soils, which have high water content and big aggregates. Further researches should prove if there is a common range of k_m values for N_2O consumption with various soils from different climate zones.

4.7 Acknowledgements

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Chapter 5

Molecular genetic analysis of soils

This work was due to an one week training at the institute for soil science and land evaluation at the university Hohenheim, Stuttgart.

5.1 Introduction

The molecular analysis of soils is another approach to get more knowledge about the N_2O formation and reduction processes in soils as well as the N_2O reducing bacteria and the microbiological community in which they live.

It is already known that the enzyme N_2O reductase (N_2OR) is encoded in the gene nosZ. Not all bacteria possess this gene, not even all denitrifiers. Gamble et al. (1977) found that 146 bacteria out of 1500 have the possibility to conduct a complete denitrification. An analysis of soils that showed large N_2O reduction rates, might give information between observations of N_2O concentrations in soils and the soil microbiology.

The objective of this training was to find out if the microbiological community change by N_2O treatment and if N_2O treatment has any effects on the amount of N_2O reducing bacteria. Therefore, 4 different soils with N_2O treatment and a control were analysed with the help of two DNA fingerprinting methods: RISA (ribosomal RNA intergenic spacer region analysis) and DGGE (denaturation gradient gel electrophorese).

5.2 Material and Methods

5.2.1 Soils

Samples were obtained from the upper 10 cm of soil at three locations in Switzerland and one location in South-West Germany. The first site, grassland 1, is located in central Switzerland (47°17'N, 7°44'E at 450 m a.m.s.l.), the second site, grassland 2, is located at Zurich-Reckenholz (47°26'N, 8°32'E at 491 m a.m.s.l.). Both sites have soil types classified as cambisol and are experimental grassland sites of the Research Station ART (Agroscope Reckenholz-Tänikon), Switzerland. The third site, forest, is located close to Basel (47°28'N, 7°42'E at 476 m a.m.s.l.) in a mixed deciduous forest on pseudogley over limestone; and the fourth site, peat, is an open pine (*Pinus spp.*) forest on a peat bog in the Black Forest located 70 km north of Basel (47°52’N, 8°06’E at 975 m a.m.s.l.). The soils of these sites range in texture from sandy loam to clay loam and organic, in pH from 2.9 to 7.0, in the C:N ratio from 9.4 to 28.8 and in the soil moisture from 23.0 to 93.6% (Table 3.1 in Chapter 3).

5.2.2 Preliminary tests

The fresh soil samples from grassland 1, grassland 2 and forest were broken and sieved to aggregates of ≤ 6.2 mm within 5 hours after collection from the field site. The sample from the peat soil is fibrous and could therefore not be sieved. It was picked in approximately 2 x 1 cm pieces. 200 g of each soil sample were placed into an incubation vessel (glass, 415 cm^3). The samples were exposed for several days to a stream of changing concentrations of N_2O. O_2 concentration stayed constant at 0.2% over this time and the carrier gas was N_2. The experiment set-up was the same as described in Chapter 4.

Each soil showed a clear N_2O uptake during the experiment and they were therefore suitable for the molecular analysis. Altogether, we had 4 different soils with a control and a sample that was in the N_2O experiment for the molecular analysis, except grassland 2. From this soil we had instead a control two samples with the N_2O treatment collected at different dates (June and November). After the experiments, the samples were frozen at -20°C. The controls were not incubated in any way and were already placed in the freezer before the experiment started at t = 0.

5.2.3 Methods

From each sample, we took one replicates (2 sub samples) from the control and the N_2O treated soil – altogether 16 sub samples – for the molecular analysis. The total DNA was extracted with the FAST DNA® Spin Kit for Soil (Bio 101) and the Fast Prep® equipment in the beginning. Afterwards, the DNA was purified over PVPP Column (Ployvinylpolypyrrolidon, Sigma P6755) following a dilution of the DNA to the needed concentration of 5 ng/µl.

5.2.3.1 Polymerase Chain Reaction (PCR)

The PCR is an enzymatic amplification of a certain DNA section between two primer-binding sites of a known nucleotide sequence. It is a chain reaction in three steps: denaturation, annealing and extension (Fig. 5.1). During the denaturation, the DNA will be denatured at 94°C, so the double Helix of the DNA brakes in single strands. This allows specific oligonucleotid-primer to attach at the matching single strand sector in the following annealing step. Thereby, the annealing temperature conforms to the primer sequence (for our primers it was 55°C). In the third step, the thermo stable Taq-polymerase extends the attached primer DNA by adding nucleotides at 72°C, thereby the single strand DNA is used as a template. In the end of one cycle, we duplicated the wanted gene. These steps will be repeated about 35 times in a programmed thermocycler.

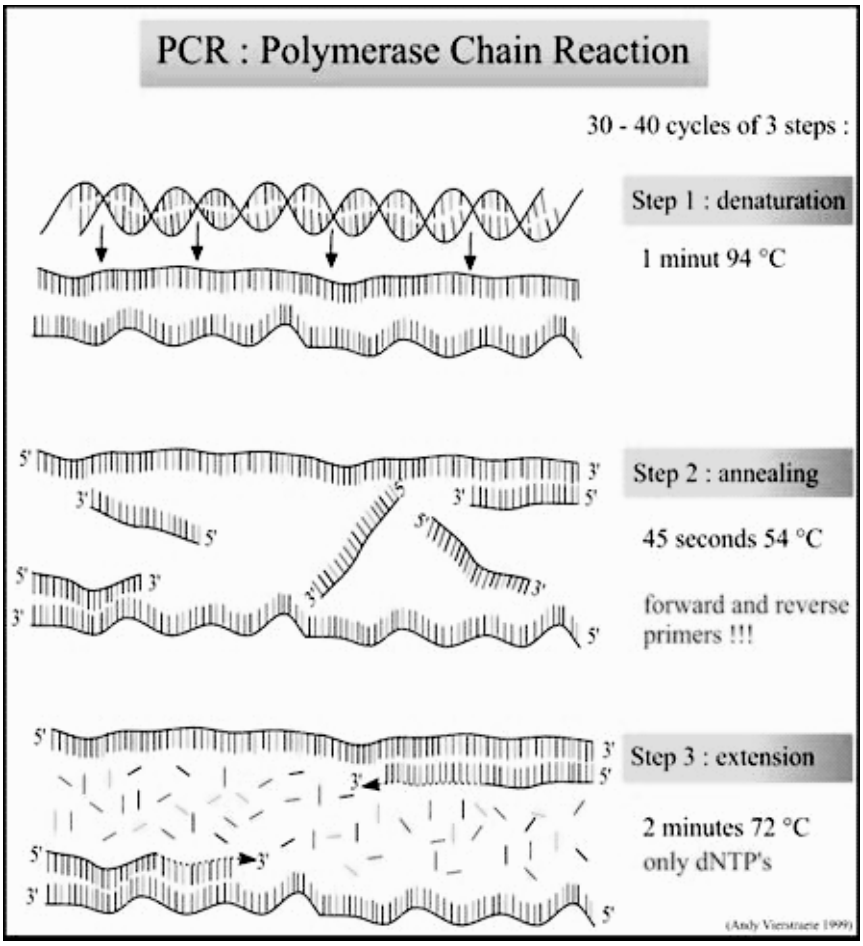

Fig. 5.1: Principle of the PCR (from http://users.ugent.be/~avierstr/principles/pcr.html)

The primer RISA (ribosomal RNA intergenic spacer region analysis) that was used in our experiment is specific for sections on the 16S rDNA of Bacteria. Thereby, it is for the analysis of the bacterial total community that binds in the area of 16S and 23S rDNA, the intergenic spacer region (ISR, Fig. 5.2). The length of the ISR differs a lot between organisms. The primer for nosZ on the other hand is for the functional gene on the bacteria DNA. Following primer sequences were used:

1. For RISA: primer F (RISA-38r: 5'-CCG GGT TTC CCC ATT CGG-3') and primer R (RISA-72f: 5'-TGC GGC TGG ATC TCC TT-3')
2. For nosZ: primer F (nosZ-F: 5'-CG(C/T) TGT TC(A/C) TCG ACA GCC AG-3') and primer R (noZ-R: 5'-CAT GTG CAG (A/C/G/T)GC (A/G)TG GCA GAA-3')

After the PCR, the fragments were separated with an acryl amid-gel electrophoresis (Fig. 5.3). The nosZ primer amplifies the nosZ gene and is special for the analysis of denitrifiers. After PCR, 5 µl of the PCR product was checked on a 2% agarose gel and purified with QIAquick Purification Kit (Qiagen).

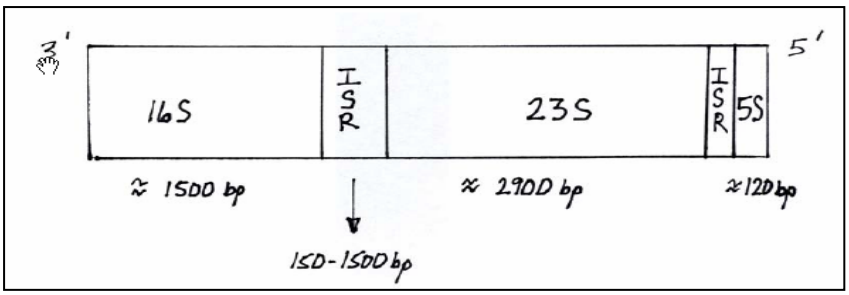

Fig. 5.2: ISR area of the gene

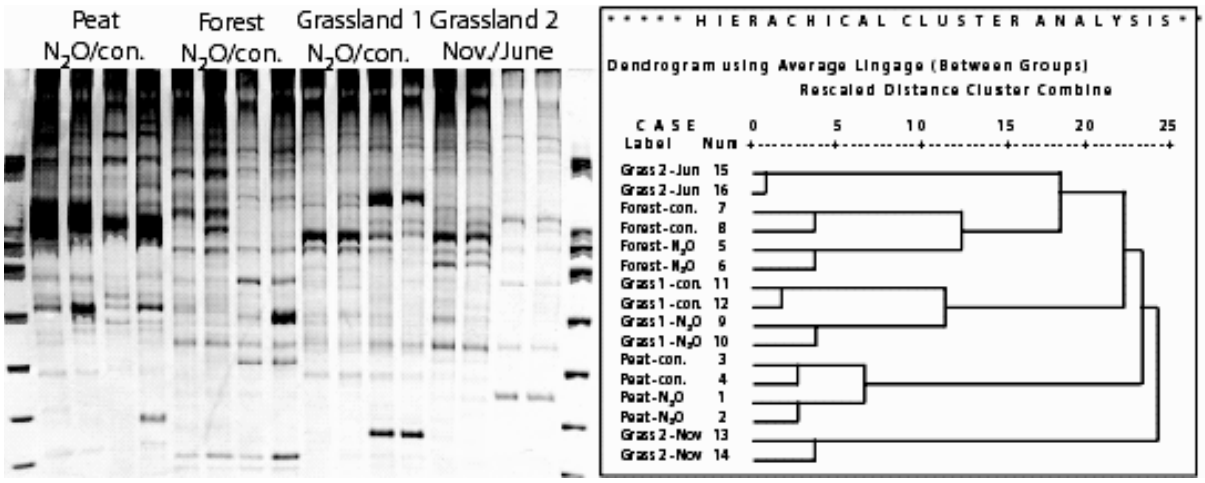

Fig. 5.3: Non-denaturing gel electrophoresis for RISA and dendrogram for the intensity of the bands. con. stands for the control of the samples without any treatment.

5.2.3.2 Denaturing Gradient Gel Electrophoresis (DGGE)

The received and purified PCR-product was analysed with a DGGE (Fig. 5.4). Thereby the DNA fragments with high GC ratio denature later than the fragments with lower GC ratio. In the electrophoresis, the various denaturing condition split the fractions to their GC ratio. With a silver-dye, the fragments in the gel get visible as bands.

The pictures of the electrophoresis gel were scanned with the software “Quantity One” and transferred in a numerical form (1/0 Matrix). The statistical analysis were done with a cluster-analysis (Fig. 5.3 and 5.4 dendogram on the right side respectively).

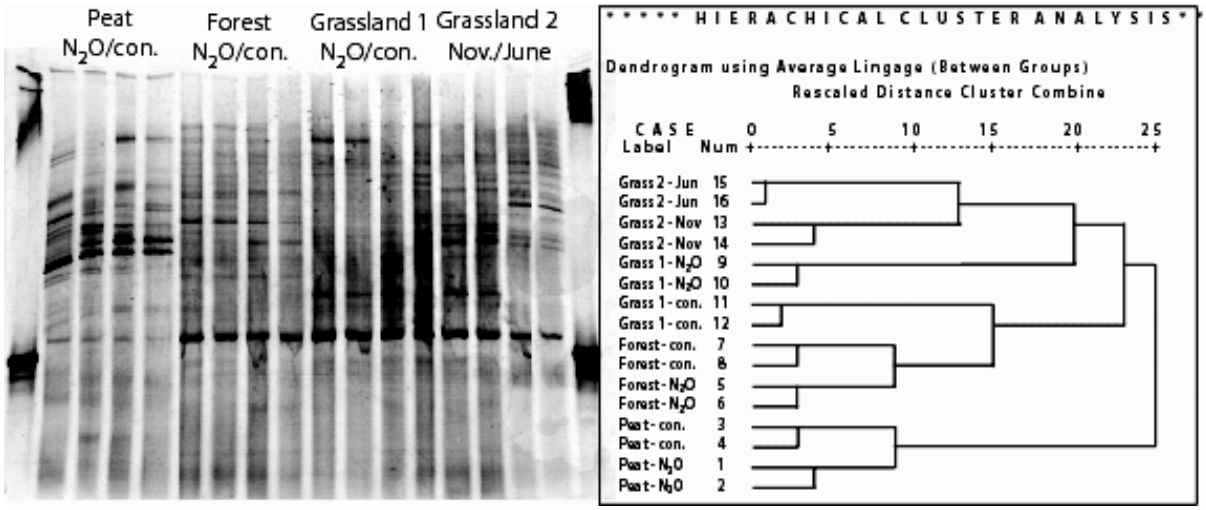

Fig. 5.4: DGGE for nosZ and dendrogram for the intensity of the bands. con. stands for the control of the samples without any treatment.

5.3 Results and Discussion

In both methods, the replicates do not differ from each other, which mean that the samples had no contamination. There are clear differences between the sites and both peat and forest form individual groups independent from the treatment in both gels (dendogram in Fig. 5.3 and 5.4). The peat has the most analogies between the treatments and has the most obvious differences to all the other sites. In comparing to all the other sites, the peat seems to have a very different bacteria community (RISA, Fig. 5.3) and different bacteria, which have the nosZ gene (DGGE, Fig. 5.4).

Grassland 2 had the greatest heterogeneity, although both samples were treated with N_2O. Here the season might play a dominant role for the bacteria community (RISA Fig. 5.3). The discrepancy was on the DGGE gel and therefore for the distribution of the nosZ gene owning bacteria not so peculiar. In this gel, grassland 2 has some similar bacteria strands with the N_2O treated sample of grassland 1. The N_2O treatment might promote bacteria that are in both grasslands common, although the bacteria community is very different between both sites (Fig. 5.3). It seems that in grasslands they have common bacteria with the nosZ gene. In contrary does the control of grassland 1 show in the DGGE gel more similarities with the forest, independent of the treatments.

The DNA-fingerprinting methods demonstrated clearly, that the soils differ strongly in their microbial community. It seems that the special treatment in the experiment of the soil samples caused a shift of the soil community. Reason for this shift can be the relative high temperature (20°C), the low oxygen concentrations, and/or the relatively high N_2O concentration the soils were exposed for several days during the experiment. The controls on the other hand were frozen immediately before the experiment started. Therefore, the controls were exposed to

atmospheric oxygen concentration and to a temperature above 20°C for some hours (up to 5 hours). It is most likely that the shift in the microbial community in the soil samples, which were in the experiment, was caused through the low oxygen concentrations and/or the temperature of 20°C during the experiment. It is most unlikely that the N_2O treatment has such a high impact on the microbial community, as only 1% of all soil microbes have the N_2O reducing gene nosZ. The effect of low oxygen concentration and/or temperature would also explain the slight differences in the DGGE band pattern between the control and the samples in the experiment.

5.4 Outlook

The DNA fingerprinting method is a good tool for comparing different soils and their bacteria communities. It is also suitable to recognise changes in bacteria communities caused through different treatments.

For future studies, it is important to define the influence of the temperature and the low oxygen concentration on the soil microbe community. The experiment set up can be that a soil sample would be frozen before the experiment starts, one sample should be fumigated with the $N_2O/O_2/N_2$ mixture, another sample should be fumigated without N_2O, and a last sample should be kept at the same temperature as the others, but without any gas fumigation. With this experiment, we can determine which of these parameters have the most effect on the bacterial community.

Additional analysis of the DNA material like cloning and the determination of the bacteria stems could give some new facts about soils that have active N_2O reducing bacteria.

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Chapter 6

Overall conclusion and outlook

In this thesis, we observed in all tested soils N_2O consumption at different oxygen concentrations. All soils had the ability to consume large portions of N_2O. The magnitude of the N_2O consumption process depends among others on the N_2O and O_2 concentrations, size of the soil aggregates, and temperature (Chapter 4). Therefore, we can confirm our overall hypothesis that great portions of produced N_2O can be consumed within the soil. In addition, we could verify the hypothesis that the N_2O consumption process follows the first-order kinetic, as long as the N_2O concentration is below the k_m values of 4 ppm at 20°C. We also could conclude that N_2O consumption and aerobic respiration can occur at the same time probably spaciously separated in different microhabitats.

We concluded that the prevalent N_2O concentration influences the N_2O reduction rate and that the N_2O reduction rate constant controls the fractionation of isotopic signature of N and O in the N_2O. However, the ratio of O to N isotope fractionation is independent of the reaction rate constant, so that this ratio can help to detect N_2O reduction where reduction is the dominating process in the turnover of N_2O (Chapter 2).

Further, we learnt that assimilatory denitrification plays only a negligible role in the consumption of soils in environments similar to those studied here. Dissimilatory reduction of N_2O to N_2 (i.e. complete denitrification) is probably the exclusive type of N_2O consumption in these environments (Chapter 3).

We could determine that up to 1.25% of the electrons were transmitted to the N_2O molecule instead to the O_2 molecule at suboxic conditions. In addition, we received k_m values for N_2O reduction enzyme that might point to a common range of k_m values for the N_2O reductase in soils independent of the O_2 concentration (Chapter 4).

Different treatments of the soils like low oxygen concentrations, high temperature and maybe also high N_2O concentrations in soils have an effect on the soil microbial community and cause a shift in the bacteria diversity (Chapter 5).

Overall, N_2O consumption has a higher importance within soils as assumed and therefore it is a process of ecological relevance. For the future, it would be interesting to determine the amount and dispersal of anaerobic microhabitats in soils. Together with the determination of the N_2O reducing bacteria in the soils, this information can improve estimation on the potential of the N_2O consumption process in a soil.

The cause of the shift in the microbial community in soils we could observe in our experiment should be determined with a special designed experiment with that we can define the parameter, which has the most effect on the microbial community.

Further investigations of various soils in different climate zones can show if the k_m values have a common range. If such a range exists, the k_m values for N_2O consumption in soils can be a helpful tool for models calculating N_2O emission from soils.

With the fractionation factors of stable isotopes during the N_2O reduction, it will be possible to determine, if N_2O reduction and N_2O emission exist at the same time in the field and therefore no N_2O concentration flux is measurable.

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