Nitrous oxide from fungal denitrification - Pure culture and soil studies using stable isotope and microbial inhibitor approaches

Dissertation

zur Erlangung des Doktorgrades

der Fakultät für Agrarwissenschaften

der Georg-August-Universität Göttingen

vorgelegt von

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geboren in Haselünne

Göttingen, 20. März 2014

D 7

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Tag der mündlichen Prüfung: 22.05.2014

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General introduction

Introduction

Environmental impact of nitrous oxide

The impact of nitrous oxide (N_2O) in the atmosphere became clear in the 1970s, when Crutzen observed its contribution to the depletion of the stratospheric ozone layer (Crutzen, 1970, 1972; Crutzen and Ehhalt, 1977). It was the same decade when N_2O was found to be one of the heat-trapping greenhouse gases (Wang et al., 1976). Nowadays, besides carbon dioxide (CO_2), methane (CH_4) and halocarbons, the importance of N_2O contribution to the global climate change induced by human activity is well known (IPCC, 2013). While its concentration in the atmosphere is about one thousand times smaller than that of CO_2, the warming potential of N_2O is about 298 times greater than that of CO_2. Thus, N_2O has the third largest impact on anthropogenic greenhouse gas emissions (IPCC, 2013). Prior to the beginning of the industrial era, N_2O levels in the atmosphere were relatively constant. Natural sources for N_2O are oceans (35%), soils under natural vegetation (60%) and atmospheric chemical reactions (5%) (IPCC, 2013) and its atmospheric concentration amounted in pre-industrial times about 270 ppb. However, until 2011 the N_2O concentration in the atmosphere increased up to 324 ppb (IPCC, 2013). Anthropogenic sources account for up to 39% of all global N_2O emissions in 2006, while agricultural soils comprised most of it (IPCC, 2013) (Figure 1). Direct anthropogenic N_2O emissions from agriculture come from the N fertilization of soils and livestock manure (59%, Figure 1), while rivers, estuaries and coastal zones contribute to indirect anthropogenic emissions of agriculture due to fertilizer leaching and runoff (9%) (Del Grosso et al., 2008; IPCC, 2013; Well and Butterbach-Bahl, 2010). Consequently agricultural activity constitutes the most anthropogenic emissions of N_2O (68%). Other anthropogenic sources for N_2O are industry and fossil fuels, burning of biomass, human excreta and atmospheric deposition, which contribute about 32% (IPCC, 2013).

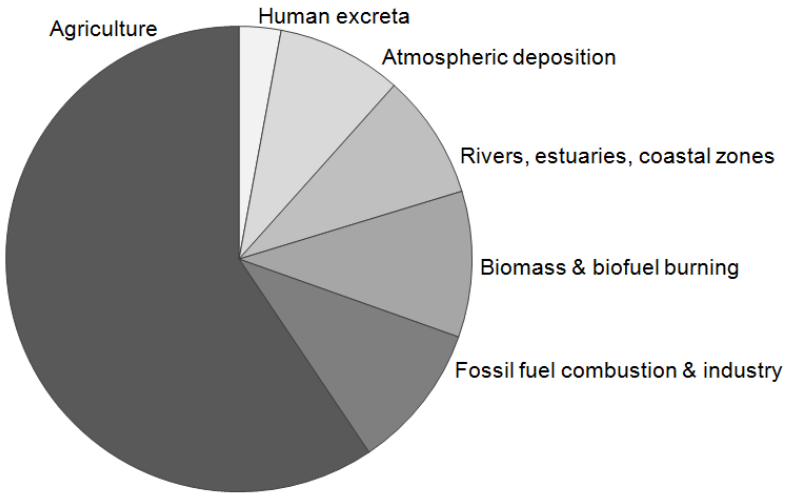

Figure 1: Percentage of global anthropogenic emissions of nitrous oxide (N_2O) based on 6.9 Tg N yr^{-1} total anthropogenic N_2O emissions in 2006 (IPCC, 2013).

Improving knowledge about process dynamics controlling N_2O emissions is one of the goals to find mitigation strategies of N_2O emissions from anthropogenic sources. There is only minor consideration of chemical N_2O production in soil, called chemodenitrification. It occurs under acidic conditions (pH < 5) and describes the destruction of nitrite (NO_2^-) and produces predominantly nitric oxide (NO) (Chalk and Smith, 1983; Ussiri and Lal, 2013). This is the reason why studies focused on microbial sources in soils. Microbial processes in soil are the main source of N_2O from soils, which are enhanced by high nitrogen fertilization and depend on soil conditions (pH, water content, oxygen (O) availability, mineral nitrogen, C content and temperature) (Stehfest and Bouwman, 2006). Microbial sources of N_2O differ in production pathways depending on ambient conditions and the organisms involved.

Microbial N_2O production pathways in soil

Soil microorganisms produce N_2O as intermediates or end products during different biochemical processes depending on the prevailing soil conditions. Autotrophic nitrification, nitrifier denitrification and denitrification represent the major pathways of N_2O production, while codenitrification and heterotrophic nitrification are supposed to play a minor role in N_2O production in soil (Figure 2) (Ussiri and Lal, 2013).

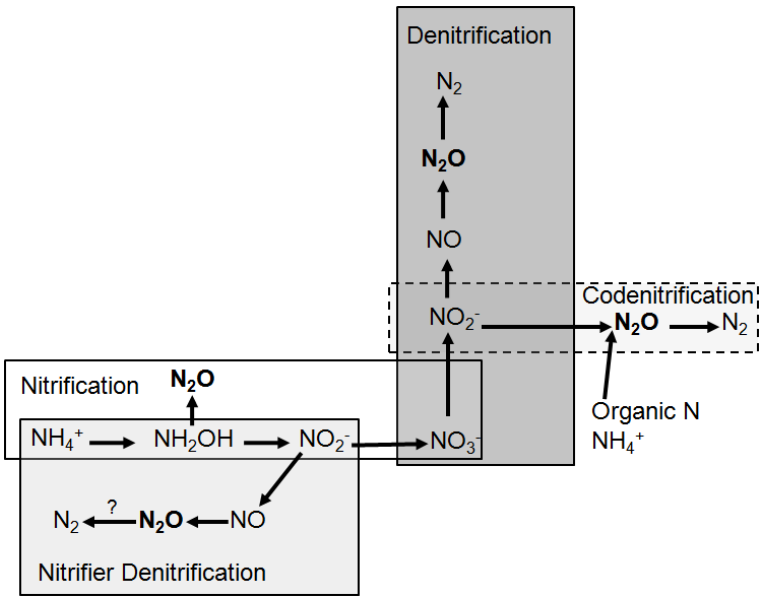

Figure 2: Microbial production pathways of nitrous oxide in soil by microorganisms (after Wrage et al. 2001, modified): aerobic nitrification, nitrifier denitrification and anaerobic denitrification are the main production pathways and codenitrification (dotted box) is a less important pathway producing N_2O in soil.

Under aerobic soil conditions, nitrification is the predominant process of N_2O production (Figure 2). Generally autotrophic bacteria are capable of ammonium (NH_4^+) oxidation with the end product nitrate (NO_3^-) and the intermediates hydroxylamine (NH_2OH) and NO_2^-, while ammonium oxidation can evolve N_2O as a by-product (autotrophic nitrification) (Baggs and Philippot, 2010). Heterotrophic nitrification might produce significant amounts of N_2O only under certain environmental conditions (low pH, high O_2 amounts and organic material) (Anderson et al., 1993; Müller et al., 2014; Papen et al., 1989) and is more common among fungi than among bacteria, although some heterotrophic bacteria are capable of nitrification (Odu and Adeoye, 1970; Papen et al., 1989). Nitrifier denitrification has received more attention since its contribution to N_2O production from soil was found some years ago (Kool et al., 2010). In this process, nitrifiers (autotrophic oxidizing bacteria) reduce NO_2^- to N_2O or N_2 (Wrage et al., 2001).

Beside nitrification, denitrification is known to significantly produce N_2O emissions from soil, but under anaerobic or low oxygen conditions in soil, which can occur in aerated soils in anaerobic micro-sites, e.g., within soil aggregates (Sexstone et al., 1985). NO_3^- is stepwise reduced to N_2, via NO_2^- and gaseous NO and N_2O by heterotrophic organisms (Firestone and Davidson, 1989; Knowles, 1982; Zumft, 1997) (Figure 2). N_2O can be emitted as an intermediate or is further reduced to N_2. NO_3^- is abundant in soil, originating from co-occuring nitrification (nitrification-coupled-denitrification) as well as from fertilizer-NO_3^- (fertilizer denitrification) (Ussiri and Lal, 2013). For a long time, heterotrophic bacteria were

thought to be the only organisms capable of denitrification. However, fungi are also capable of denitrification and even some archaea were found to produce N_2O under anaerobic conditions (Bollag and Tung, 1972; Hayatsu et al., 2008; Shoun et al., 1992). But archaeal denitrification and its product ratio is relatively unknown. First evidence for fungal denitrificartion was given by Bollag and Tung (1972) and ever since interest in this process increased and several years later Shoun et al. (1992) found that most fungi lack the enzyme N_2O reductase (Nos), which was followed by several pure culture studies to get more information about the fungal pathway of denitrification (e.g. Kim et al., 2009; Shoun et al., 2012; Zhang and Shoun, 2008). Precise information about the contribution to N_2O emissions from fungal denitrification as well as reliable methods to quantify fungal origin of N_2O from a soil community are lacking so far.

Fungi not only produce N_2O via denitrification, but are also able to produce a hybrid N_2O from NO_2^--N and N from another source (NH_4^+or organic N) by codenitrification under anaerobic conditions (Spott and Stange, 2011). First evidence of codenitrification was observed by Tanimoto et al. (1992). The fungus *Fusarium oxysporum* produced N_2O as the product of codenitrification (Tanimoto et al., 1992). Beyond that, Shoun et al. (1992) showed that *Fusarium solani* and C*ylindrocarpon tonkinense* formed N_2 as end-product of codenitrification. Several years later, Spott et al. (2011) summarized that apart from the mentioned three fungal species at least 12 bacterial species and one archaeal species were found to be capable of codenitrification. However, information about the role of codenitrification in soil is very rare.

Studying N_2O production under anaerobic conditions is of great importance to achieve more information about the contributing microorganisms. Several incubation studies indicated that fungi dominate N_2O production from denitrification in soils (Blagodatskaya et al., 2010; Crenshaw et al., 2008; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). If dominance of fungal denitrification was common in soil, this would have to be considered in N_2O flux modeling methods and in evaluating mitigation strategies.

Enzymes involved in fungal denitrification

Every reduction step of denitrification in microorganisms is catalyzed by specific enzymes (Ferguson, 1994; Knowles, 1982; Zumft, 1997) (Figure 3). Most information about these enzymatic steps was attained by studies with pure bacterial cultures (e.g. Allen and Niel, 1952; Sutka et al., 2004; Toyoda et al., 2005; Ye et al., 1994). As denitrification is a characteristic of anaerobic respiration, enzymes of denitrification compounds receive

electrons (e^-) during the transport through the enzymes (Berks et al., 1995). Most enzymes of the anaerobic respiratory system of bacteria are located in the periplasm (Bothe et al., 2006; Ferguson, 1994; Zumft, 1997) and the resembling enzymes were found in the mitochondria of fungi (Kobayashi et al., 1996). In bacteria the NO_3^- reductase (Nar) was found soluble in the periplasm (Nap), but exists as well membrane-bound with its active site in the cytoplasm to catalyze the reduction of NO_3^- to NO_2^-. In the latter case, NO_3^- requires a transport through the membrane into the cytoplasm (Ferguson, 1994). Due to this transport, a concentration gradient between cytoplasm and periplasm, the returning transport of NO_2^- to the periplasm of the cell, and the on-going reduction is possible. All other enzymes (NO_2^- reductase (Nir), NO reductase (Nor) and N_2O reductase (Nos)) are located inside the periplasm of the bacterial cells. Although the locations of the enzymes were found to be similar in bacteria, pure culture studies revealed different enzyme types for Nir, heme-cd1 and copper Nir, as well as for Nor, cNor, qNor or qCuNor, depending on the species. Enzymes involved in fungal denitrification were found to be similar to bacterial enzymes, except for Nor, which was the only enzyme differing between bacteria and fungi (P450nor) (de Vries et al., 2007; Kim et al., 2009; van Spanning et al., 2007). Fungal P450nor are similar to bacterial cytochrome P450 and probably both types are evolutionarily related (van Spanning et al., 2007; Zumft, 1997). As mentioned previously Nar and Nir of *Fusarium oxysporum* were found to be located in the mitochondria and this location enables fungi to receive e^- from sources of energy (e.g., succinate, formate, malate or pyruvate). Regarding formate as supplier of e^-, fungal Nar might be more equal to NO_3^- respiration by *E. coli* than to that of bacterial Nar (Bothe et al., 2006). Until now, there has been no study that could prove the existence of N_2O reductase (Nos) existing in fungi (Zumft and Körner, 2007), although some fungal pure cultures produced minimal amounts of N_2 during denitrification or codenitrification (Shoun et al., 1992). The lack of Nos enables fungi to potentially release much more N_2O compared to bacteria assuming both groups have the same production rates and thus this observation gave hints of a potentially great contribution of fungi to N_2O emissions from soil (Sutka et al., 2008).

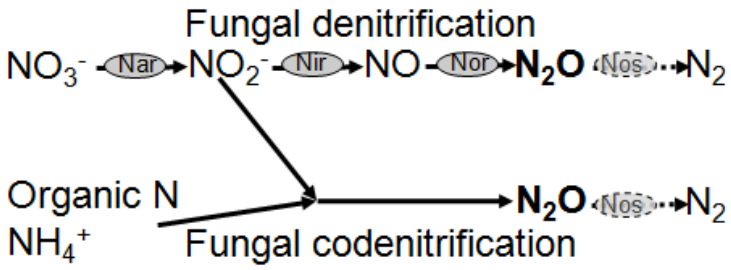

Figure 3: Fungal pathways of denitrification and codenitrification producing nitrous oxide (N_2O) in soils under anaerobic conditions and the associated enzymes. Pure culture studies revealed that most fungi lack Nos (marked in light gray) (Shoun et al., 1992). For further information see text.

Isotopic analysis of N_2O to distinguish between sources of N_2O from denitrification

Analysis of stable isotopes of N_2O turned out to be useful in isotope tracing as well as natural abundance studies to understand the contribution of different N_2O processes and sources. N_2O can be used for isotope analysis, because it contains two elements (N and O) with several stable isotopes. Under natural condition, isotopes of an element vary in the number of neutrons and thus in the mass (Fry, 2006). For N, the natural abundance of the low mass isotope ^{14}N is 99.64%, whereas the high mass isotope ^{15}N is less abundant (0.36%) (Fry, 2006). For O, three stable isotopes exist, differing in the natural abundance: ^{16}O (99.76%), ^{17}O (0.04%) and ^{18}O (0.20%) (Fry, 2006).

Under natural conditions, isotope studies take advantage of the natural isotopic fractionation during reaction steps for lower mass isotopes (^{14}N or ^{16}O) (Fry, 2006). For example, the more abundant $^{14}N^{14}N^{16}O$ molecule with lower mass shows higher reaction rates and less bonding force compared to the less abundant $^{14}N^{15}N^{16}O$, $^{15}N^{14}N^{16}O$ or $^{14}N^{14}N^{18}O$ molecules with higher mass. Thus, N_2O from nitrification is generally composed of isotopes with lower mass relative to the electron acceptor compared to N_2O from denitrification, due to (i) different fractionation effects of N_2O production, probably because of different enzymatic steps involved in both processes (Toyoda et al., 2002), (ii) the reduction step of N_2O to N_2 during denitrification results in N_2O relatively enriched in ^{18}O and ^{15}N compared to denitrification without N_2O reduction step (Popp et al., 2002) and (iii) the intramolecular isotopic fractionation, which prefers cleavage of ^{14}NO compared to ^{15}NO (Baggs, 2008; Toyoda et al., 2005; Yoshida and Toyoda, 2000). The ratios of $^{15}N/^{14}N$ or $^{18}O/^{16}O$ in molecules of a sample (R_{sample}) can be compared to the corresponding ratios of an internationally accepted standard ($R_{standard}$) and expressed as delta-values (δ) in permil (‰) (Fry, 2006).

$$\delta = \frac{R_{Sample} - R_{Standard}}{R_{Standard}} \cdot 1000 \qquad \text{(Eq. 1)}$$

Several studies could show specific isotopic values of N_2O for different production pathways (Table 1). Molecules that differ in the isotopic composition are called “isotopologues”, while molecules that contain the same isotopes (and thus have the same mass), but differ in the position of the tracing isotopes within the molecule are termed “isotopomers” (Ostrom and Ostrom, 2011). Regarding the intramolecular distribution of ^{15}N in N_2O, there are two possibilities for its position in the asymmetric and linear molecule: the central alpha (α) position and the peripheral beta (β) position (Brenninkmeijer and Röckmann, 1999; Toyoda and Yoshida, 1999). This distribution was first analyzed and independently published by two groups in the same year (Brenninkmeijer and Röckmann, 1999; Toyoda and Yoshida, 1999). The ^{15}N distribution in N_2O serves to calculate the ^{15}N site preference of N_2O (SP) (Toyoda and Yoshida, 1999):

$$^{15}N\text{-}SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \qquad \text{(Eq. 2)}$$

Analyzing the isotopic composition of N_2O, including SP, revealed that different production pathways of N_2O differ in isotopologue values (Table 1). Many pure culture studies focusing on N_2O production pathways showed that N_2O from nitrification is characterized by a SP of N_2O of 33 ‰ (Sutka et al., 2006), whereas N_2O from bacterial denitrification showed much lower SP values between -11 and 0 ‰ (Frame and Casciotti, 2010; Sutka et al., 2006). Cultivating fungi showed that two denitrifying species produced N_2O with substantially higher SP values of 37 ‰ compared to several bacteria (Frame and Casciotti, 2010; Sutka et al., 2008; Sutka et al., 2006). Thus, under standard and designed laboratory conditions, fungal and bacterial contribution to N_2O produced by denitrification might be distinguishable. Under natural soil conditions, however, SP of N_2O produced by fungi might overlap with SP of N_2O from nitrification (Table 1) (Ostrom and Ostrom, 2011; Sutka et al., 2006). Additionally, isotopologues of N_2O produced during denitrification in soil are altered by N_2O reduction to N_2. The residual N_2O contains relatively more isotopes with high mass (^{15}N and ^{18}O) compared to N_2O that is not further reduced by microorganisms. However, despite these limitations, the specific isotopic characteristic of N_2O from different sources (e.g., nitrification and denitrification) and different microorganisms (e.g., fungi and bacteria) might be a promising tool to obtain necessary information for developing new methods and strategies for agricultural practices to reduce the emissions of N_2O.

Table 1: Overview about ranges of $\delta^{15}N_{bulk}$, $\delta^{18}O$ and SP of N_2O [‰] produced during different processes by pure cultures or soil incubations with constrained conditions for nitrification or denitrification from literature.

Production process	**$\delta^{18}O$ [‰]**	**$\delta^{15}N$ [‰]**	**SP [‰]**	**Source**
Nitrification	+22 to +61	-3 to 3	+25 to +40	a
	+22 to +25	-47 to -46	+28 to +37	b
	+33 to +35	+6 to +10	+29 to +32	c
Nitrifier denitrification	+7 to +12	-54 to -23	-14 to +9	d
Codenitrification	not investigated yet			
Fungal denitrification	+30 to +39	-3 to -20	+23 to +40	e
Bacterial denitrification	+7 to +47	-38 to -8	-9 to -1	f
$Nitrification_{soil}$	+8 to +31	-58 to +9	-7 to +36	g
$Denitrification_{soil\ (NO3-N2)}$	+28 to +52	-38 to -6	+3 to +26	h
$Denitrification_{soil\ (NO3-N2O)}$	+4 to +23	-51 to -27	-4 to +18	i

[a] Sutka et al. (2004) and Sutka et al. (2006): *Methylococcus capsulatus* and *Nitrosomonas europaea* (NH_2OH)
[b] Sutka et al. (2006): *Nitrosomonas europaea* (NH_4^+)
[c] Santoro et al. (2011): ammonia oxidizing archaea (AOA)
[d] Frame and Casciotti (2010): *Nitrosomonas marina*; Sutka et al. (2004): *Nitrosomonas europaea*, Sutka et al. (2006): *Nitrosospira multiformis*
[e] Sutka et al. (2008): *Fusarium oxysporum*, *Cylindrocarpon tonkinense* (NO_2^-)
[f] Toyoda et al. (2005): *Pseudomonas fluorescens* and *Paracoccus denitrificans* (NO_2^-), note: without values of *Pseudomonas fluorescence*; Sutka et al. (2006): *Pseudomonas chlororaphis*, *Pseudomonas aureofaciens*
[g] Well et al. (2006) 55% WFPS, (Well et al., 2008)
[h] Well et al. (2006) 75 and 85% WFPS; Snider et al. (2009) $\delta^{15}N$ values only; Perez et al. (2006) and Park et al. (2011)
[i] Well and Flessa (2009); Lewicka-Szczebak et al. (2014) with C_2H_2 to inhibit the N_2O reduction; Park et al. (2011); Perez et al. (2006) SP only

Isotope tracer experiments are useful to quantify the contribution from different sources of substrates for N_2O production. In such experiments, substrates that are enriched in ^{15}N (commonly ^{15}N labeled NH_4^+ or NO_3^-) or ^{18}O (commonly ^{18}O labeled NO_3^- or water) only or in both isotopes, can be applied to trace their fate by analyzing isotopic enrichment of produced compounds (Kool et al., 2011; Ostrom and Ostrom, 2011): e.g. ^{15}N-NH_4^+ or ^{15}N-NO_3^- application can be used to distinguish between N_2O fluxes from these sources by ^{15}N-N_2O analysis (Baggs, 2008). Tracing ^{15}N-NO_3^- is also suitable for quantifying N_2O formation during codenitrification under controlled anaerobic conditions as N_2O produced is a hybrid molecule from NO_3^- and another N source (Laughlin and Stevens, 2002; Spott et al., 2011). When ^{15}N-NO_3 is applied N_2O produced by denitrification is highly enriched in ^{15}N, because both N atoms are obtained from NO_3^-, whereas N_2O produced by codenitrification is relatively less enriched in ^{15}N, because the hybrid N_2O is formed from an N of NO_3^- and of another unlabeled N compound (Shoun et al., 1992; Spott et al., 2011). A combined labeling

approach of substrates (^{15}N-NO_3 and/or ^{15}N–NH_4 and ^{18}O-water) was proposed by Wrage et al. (2005) for the first time to (i) differentiate between denitrification and nitrification by ^{15}N application and to (ii) analyze the origin of O in N_2O by ^{18}O application: the O of N_2O from nitrification originates from atmospheric O_2, whereas O of N_2O during nitrifier denitrification originates from water, which can be observed in N_2O produced from ^{18}O-labeled water and can thus be distinguished from nitrification.

Besides the differentiation between N sources of N_2O, tracer experiments with ^{18}O-labeling of water have been used to quantify O exchange during denitrification in bacteria (e.g. Aerssens et al., 1986; Kool et al., 2011). In case this O exchange is almost complete, the ^{18}O signature of N_2O would be almost unaffected by isotopic fractionation during denitrification, but would be dependent on the $\delta^{18}O$ of soil water and the isotope effect during O exchange. Until now, O exchange during denitrification was found in several bacteria and it varied between 4 and 100%, while its occurrence is completely uninvestigated for fungi (Aerssens et al., 1986; Garber and Hollocher, 1982; Kool et al., 2007; Ye et al., 1991).

Modified substrate induced respiration with selective inhibition (SIRIN) to distinguish between sources of N_2O from denitrification

Until now no reliable method to differentiate N_2O produced during denitrification by bacteria or fungi is available for soil incubation experiments. Several studies used a modification of the substrate induced respiration with selective inhibition (SIRIN) (Anderson and Domsch, 1973) to distinguish between N_2O produced during denitrification by bacteria or fungi (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). This method is based on the application of selective antibiotics, that inhibit the specific protein biosynthesis of either fungi or bacteria (Anderson and Domsch, 1973). Analyzing the evolved CO_2 gave information about contribution of bacteria or fungi on CO_2 respiration (Anderson and Domsch, 1973). Laughlin and Stevens (2002) modified this approach and first published results of soil incubation experiments where N_2O from denitrification produced by bacteria or fungi was determined. They found that inhibition of bacterial growth resulted in 23% reduced N_2O production compared to N_2O produced without growth inhibition, whereas with fungal growth inhibition N_2O production decreased about 89% (Laughlin and Stevens, 2002). Similar growth inhibitor effects on N_2O production from denitrification were observed in the following years by other groups (Blagodatskaya et al., 2010; Crenshaw et al., 2008; Long et al., 2013; McLain and Martens, 2006), confirming the assumption that fungi might play a dominant role in producing N_2O during denitrification

under natural conditions. Until now, the validity of these results was not tested by independent approaches. Moreover, in view of the need to establish anaerobic growth conditions in the laboratory, it is not clear to which extent responses to inhibitors represent fungal and bacterial N_2O production under normal soil conditions. A comparison between the SIRIN approach and isotopologue values of N_2O emissions as indicators of fungal denitrification has not been conducted so far, but would be a useful step in the study of fungal denitrification.

Objectives

The present study aimed at enhancing the knowledge about N_2O produced during fungal denitrification. As explained earlier, previous studies indicated that fungal contribution to N_2O emissions from denitrification has to be taken into account when distinguishing between N_2O sources and developing strategies to mitigate N_2O fluxes from soils. Therefore extended knowledge about fungal denitrification is absolutely essential. Hence, the objectives were:

1. to analyze the O exchange between denitrification intermediates and water during N_2O production by fungal denitrification
2. to verify high SP values of N_2O from fungal denitrification reported in the literature for four additional fungal species,
3. to investigate if fungi existing in a soil community produce N_2O isotopologue values comparable to values known from pure fungal cultures, and
4. to determine fungal N_2O production in agricultural soils comparatively using SIRIN and the isotopologue approach.

Experimental concept

Fungal pure cultures

For pure culture experiments six different fungal strains were incubated under denitrifying conditions to measure the isotopic composition of N_2O produced and to determine the O exchange between denitrification intermediates and water. Stem cultures were ordered from the Japan Collection of Microorganisms (JCM, Saitama, Japan) and Nite Biological Research Center (NBRC, Chiba, Japan). The three species from JCM were *Chaetomium funicola, Fusarium oxysporum* and *Trichoderma hamatum* (Catalogue numbers JCM 22733, JCM

11502 and JCM 1875, respectively) and the three species from NBRC were *Cylindrocarpon lichenicola, Fusarium solani* fsp. *pisi* and *Fusarium decemcellulare* (catalogue numbers NBRC 30561, NBRC 9425 and NBRC 31657, respectively). These fungal species typically occur in soil (Domsch et al., 1980) and are known from previous pure culture studies by Shoun et al. (1992) to be capable of denitrification.

Soil incubations

Agricultural soils from three different locations in Germany (Braunschweig, Wennebostel, and Reinshof) were used for anaerobic incubations. The soil texture and chemical properties of the three soils differed. Hence, the impact of different soil types on microbial community and the isotopologues of N_2O produced could be investigated. Isotopologue analysis of N_2O fluxes was combined with a modification of SIRIN to get a best estimate of the bacterial and fungal contribution to total soil denitrification and to compare both approaches, and thus to evaluate their consistency. An important question of the methodical evaluation was, if the distinct isotopologue values of N_2O produced by pure cultures are found as well in N_2O produced by a microbial soil community with selective growth inhibition of bacteria or fungi.

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Fungal oxygen exchange between denitrification intermediates and water

This chapter is an article published in Rapid Communications in Mass Spectrometry, with a modified reference style:

Rohe, L., Anderson, T.-H., Braker, G., Flessa, H., Giesemann, A., Wrage-Mönnig, N. and Well, R. (2014). Fungal oxygen exchange between denitrification intermediates and water. Rapid Commun. Mass Spectrom., 28: 377–384. doi:10.1002/rcm.6790

Abstract

RATIONALE: Fungi can contribute greatly to N_2O production from denitrification. Therefore, it is important to quantify the isotopic signature of fungal N_2O. Isotopic composition of N_2O can be used to identify and analyze processes of N_2O production and N_2O reduction. In contrast to bacteria, information about the oxygen exchange between denitrification intermediates and water during fungal denitrification is lacking, impeding the explanatory power of stable isotope methods.

METHODS: Six fungal species were anaerobically incubated with the electron acceptors nitrate or nitrite and ^{18}O-labeled water to determine the oxygen exchange between denitrification intermediates and water. After seven days of incubation, gas samples were analyzed for N_2O isotopologues by isotope ratio mass spectrometry.

RESULTS: All fungal species produced N_2O. N_2O production was greater when nitrite was the sole electron acceptor (129 to 6558 nmol N_2O g dw^{-1} h^{-1}) than when nitrate was the electron acceptor (6 to 47 nmol N_2O g dw^{-1} h^{-1}). Oxygen exchange was complete with nitrate as electron acceptor in one of five fungi and with nitrite in two of six fungi. Oxygen exchange of the other fungi varied (41 to 89% with nitrite and 11 to 61% with nitrate).

CONCLUSIONS: This is the first report on oxygen exchange with water during fungal denitrification. The exchange appears to be within the range previously reported for bacterial denitrification. This adds to the difficulty of differentiating N_2O producing processes based on the origin of N_2O-O. However, the large oxygen exchange repeatedly observed for bacteria and now also fungi could lead to less variability in $\delta^{18}O$ of N_2O from soils, which could facilitate the assessment of the extent of N_2O reduction.

KEYWORDS: nitrous oxide, nitrite reduction, fungal denitrification, ^{18}O isotope, isotope tracer

Introduction

Nitrogen (N) fertilizer application in agriculture is the main anthropogenic source for the greenhouse gas emissions of nitrous oxide (N_2O), with detrimental environmental impacts: N_2O contributes to global warming and to the depletion of the stratospheric ozone layer (IPCC, 2007). N_2O from soil predominantly results from microbial processes (e.g., nitrification, nitrifier denitrification and denitrification) (IPCC, 2007). During denitrification, nitrate (NO_3^-) is reduced to nitrite (NO_2^-), gaseous nitric oxide (NO), N_2O and finally dinitrogen (N_2) under anaerobic conditions (Knowles, 1982). Shoun et al. (1992) showed that under pure culture conditions fungi are capable of denitrification. Every reduction step during denitrification is characterized by a specific enzyme: dissimilatory NO_3^- reductase (dNar), dissimilatory NO_2^- reductase (dNir), NO reductase (Nor) and N_2O reductase (Nos) (Kim et al., 2009). Apart from the fungal Nor (P450nor), which differs from the bacterial enzyme, all other enzymes involved in fungal denitrification are similar to bacterial enzymes of this process (Kim et al., 2009; Shoun et al., 2012). However, most fungi lack Nos and N_2O is the major end product of fungal denitrification (Shoun et al., 1992). While research activities had concentrated on bacterial denitrification processes in earlier decades, fungal denitrification received more attention lately. Recent studies indicated that in some soils, fungal N_2O production from denitrification might even be greater than that of bacteria (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). However, the fungal production pathway of N_2O has not yet been sufficiently investigated and to our knowledge there is no study focusing on fungal oxygen (O) exchange between water (H_2O) and denitrification intermediates. In the following we briefly describe (I) the enzymatic steps of bacterial NO_3^- reduction to N_2O and its O exchange, (II) existing hypotheses about the O exchange mechanism and (III) possibilities of using ^{18}O isotopic analysis as a tool to understand production processes in order to investigate parallels to fungal denitrification and its associated O exchange.

Bacterial denitrification and its metabolic steps including enzymes and the O exchange between H_2O and denitrification intermediates have been investigated in several studies (Aerssens et al., 1986; Casciotti et al., 2002; Garber and Hollocher, 1982; Kool et al., 2007; Ye et al., 1991). Bacterial O exchange varies from 4 to 100% depending on species (Aerssens et al., 1986; Garber and Hollocher, 1982; Ye et al., 1991). There is no study so far that specifically targeted O exchange with Nar during NO_3^- reduction to NO_2^-. However, the active site of Nar is located in the cytoplasm, whereas the active

site of Nir and Nor is in the periplasm (Kool et al., 2007). Kool et al. (2007) suggested that the required transport of NO_3^- as well as of H_2O during NO_3^- reduction through the cytoplasmic membrane could cause smaller O exchange rates, which has not yet been confirmed. So far, studies have focused on bacterial O exchange with H_2O during NO_2^- (4 to 94% O exchange) and NO reduction (4 to 84% O exchange) (e.g.Aerssens et al., 1986; Ye et al., 1991)). During NO_2^- reduction the Nir type did not affect the O exchange, whereas during NO reduction, bacteria with copper Nir type showed larger O exchange compared to bacteria with heme-cd1 Nir type (Aerssens et al., 1986). Ye *et al.* did not find an O exchange when incubating bacterial denitrifiers with N_2O as sole electron acceptor, showing that O exchange occurs during previous reduction (NO_3^- to N_2O) steps and does not result from abiotic equilibrium between H_2O and N_2O (Ye et al., 1991). However, Casciotti et al. (2007) examined the storage effect on O exchange between NO_2^- and H_2O in autoclaved seawater and freshwater samples at pH 7.9 during 24 days. They found remarkable O exchanges between 15 and 38% at +4 °C, which may indicate that a potential chemical O exchange in soil could exist as well, assuming that the water samples were completely free of microbial growth (Casciotti et al., 2007). During microbial reduction of NO_3^- to N_2O, O exchange probably occurs due to equilibration between O of cell-internal intermediates and the ambient water and seems to be accelerated by enzymatic activity (Brunner et al., 2005; Knöller et al., 2011). During denitrification of bacterial species with the heme-cd1 Nir type, Aerssens et al. (1986) found that O exchange decreased with increasing NO_2^- concentration when NO_3^- was absent from the medium. Their explanation was that NO_2^- was reduced by Nir to an enzyme-bound nitrosyl intermediate ($E\text{-}NO^+$ or $E\text{-}NO^-$) with a reversible dehydration (Aerssens et al., 1986). They concluded that high NO_2^- concentration enhances the NO_2^- reduction rate to the enzyme-bound nitrosyl intermediate. In this case the reduction rate is high and limited by the reversible dehydration step (Aerssens et al., 1986) in such a way that after dehydration and the formation of the nitrosyl intermediate there is no time left for rehydration. Correspondingly, with small NO_2^- concentrations the production rates of enzyme-bound nitrosyl intermediates decreased, resulting in sufficient time for a more reversible dehydration step and thus in greater O exchange between the enzyme-bound intermediates ($E\text{-}NO^+$ or $E\text{-}NO^-$) and H_2O (Aerssens et al., 1986). Information on the O exchange mechanism in the copper Nir type is lacking, but due to the different O exchanges observed by Ye *et al.*, the enzymatic step of NO_2^- reduction might differ from the heme-cd1 Nir type (Ye et al., 1991). Consequently, the

bacterial O exchange seems to be dependent on reaction rates and the enzyme types involved in microbial NO_3^- reduction to N_2O. Based on ^{18}O tracer studies and calculated O exchange, Snider et al. suggested that O exchange may depend on the microbial community, but not on environmental conditions like temperature and soil moisture or N_2O production (Snider et al., 2009; Snider et al., 2013).

Like the N isotopes, O isotopes of N_2O can principally serve as tracers for the determination of N_2O production pathways. Natural O sources of N_2O from the different production pathways are (I) air oxygen (O_2) during ammonia oxidation of nitrification with N_2O formation from the hydroxylamine intermediate; (II) H_2O-O and O_2 during nitrifier denitrification, and (III) NO_3^--O and H_2O-O (due to O exchange) during denitrification (Kool et al., 2007; Ostrom and Ostrom, 2011; Wrage et al., 2005). The O of NO_3^- can be affected by H_2O-O and O_2 as well, if the NO_3^- comes from nitrification (Kool et al., 2007). Hence, differences in isotopic compositions of all O precursors, O exchange with H_2O, and isotope effects of enzymatic reaction and of O exchange affect $\delta^{18}O$-values of produced N_2O ($\delta^{18}O$-N_2O). Snider et al. summarized studies focusing on $\delta^{18}O$-N_2O from nitrification and they specified a $\delta^{18}O$-N_2O range from +13 to +35 ‰, whereas $\delta^{18}O$-N_2O from denitrification might reach larger $\delta^{18}O$-N_2O (> 35 ‰) due to isotopic fractionation during NO_3^- reduction (Snider et al., 2012, 2013). However, in cases of larger O exchanges with H_2O during denitrification, indistinguishable $\delta^{18}O$-N_2O could occur from different N_2O sources. Moreover, N_2O reduction to N_2 leads to an increase in $\delta^{18}O$-values of residual N_2O (Ostrom and Ostrom, 2011). Estimation of N_2O reduction might be possible by analyzing $\delta^{18}O$-N_2O. To this end, effects on $\delta^{18}O$-N_2O during N_2O production by nitrification and bacterial/fungal denitrification must be known. However, the interpretation of $\delta^{18}O$-N_2O is difficult due to the mentioned possible O exchange that could occur between denitrification intermediates and H_2O (Kool et al., 2007). These factors complicate the use of $\delta^{18}O$-N_2O for differentiation between nitrification and denitrification processes.

So far, information on O exchange between H_2O and intermediates during fungal denitrification is lacking. Particularly the fungal P450nor, which is different from bacterial Nors, could cause differences between fungal and bacterial O exchange. Information about the factors controlling $\delta^{18}O$-N_2O produced by fungal denitrification is necessary to differentiate between the N_2O production pathways and to elucidate N_2O reduction to N_2 (Well et al., 2012).

The aim of this study was to quantify O exchange with H_2O during fungal denitrification and to determine the influence of the two electron acceptors NO_3^- and NO_2^- on N_2O production and O exchange. The mode of fungal O exchange was compared with bacterial O exchange known from literature to see if differences exist (Aerssens et al., 1986; Garber and Hollocher, 1982; Ye et al., 1991). To this end, we incubated six different fungal species under denitrifying conditions with ^{18}O-labeled H_2O and analyzed $\delta^{18}O$-N_2O.

Experimental

Microorganisms

Six ubiquitous soil fungi (Domsch et al., 1980) known to be capable of denitrification (Shoun et al., 1992) were used to investigate the O exchange between ^{18}O-labeled H_2O and denitrification intermediates. *Chaetomium funicola* JCM 22733, *Fusarium oxysporum* JCM 11502 and *Trichoderma hamatum* JCM 187 were obtained from the Japan Collection of Microorganisms (Saitama, Japan), while *Cylindrocarpon lichenicola* NBRC 30561, *Fusarium solani* fsp. *pisi* NBRC 9425 and *Fusarium decemcellulare* NBRC 31657 were received from the NITE Biological Research Center (Chiba, Japan). Except for *C. funicola*, which belongs to the order *Sordariales*, all other species belong to the order *Hypocreales*. Shoun *et al.* did not find N_2 production by *F. oxysporum* and *F. decemcellulare* and only a small N_2 production by the other fungi compared to bacteria capable of N_2O reduction (Shoun et al., 1992).

Experimental procedure

According to the protocol of Shoun et al. (1992) the supplied stem cultures were transferred to 100 mL sterile fungal medium in an Erlenmeyer flask with cotton wool plugs. The culture medium contained 1% glucose, 0.2% peptone, 0.02% $MgSO_4 \cdot 7 H_2O$, 2 ppm $CoCl_2 \cdot 6 H_2O$, 2 ppm $FeSO_4 \cdot 7 H_2O$ and 0.01 mol potassium phosphate (pH 7.4) (Shoun et al., 1992). The inoculated medium was incubated aerobically at 22 °C in the dark in a rotary shaker for three to seven days (100 rpm) as a preparatory culture (Shoun et al., 1992). Incubation time depended on the growth increase. When the fungal mycelium had grown well (mycelium was visible in the medium), triplicates

of 10 mL pre-culture were transferred to fresh medium (38.47 mL) each in a 120 mL crimp neck vial (La-Pha-Pack GmbH, Langerwehe, Germany) with a butyl stopper; adopting the protocol of Shoun *et al.*, either 10 mmol potassium nitrate (KNO_3) or 5 mmol sodium nitrite ($NaNO_2$) were added as electron acceptor for denitrification (Shoun et al., 1992). This medium was supplemented with 1.53 mL filter-sterilized (0.2 µm) ^{18}O-labeled H_2O (10 atom% (at%), Sigma-Aldrich, Taufenstein, Germany) to reach a 0.5 at% ^{18}O-labeling of H_2O in the medium, giving a total volume of 50 mL in every vial. To obtain anaerobic conditions, the headspace of the vials was purged for 10 min with filter-sterilized N_2. During the purging, the vials were stirred manually to expulse the dissolved oxygen. Following Shoun *et al*. after seven days of incubation in the dark (22 °C) in a rotary shaker (120 rpm), duplicates of gas samples (14 mL) were collected and transferred to evacuated sampling vials (12 mL Exetainer®, Labco Ltd., Lampeter, UK) with a syringe (Shoun et al., 1992).

To determine the N_2O production per fungal biomass the complete medium of each vial at the end of the 7-day incubation was centrifuged (5236 x g for 30 min) and washed twice with distilled H_2O. Subsequently, the supernatant was decanted and the cells oven dried at 105 °C for 48 hours to determine the dry weight (dw) of the fungi.

In parallel, triplicates of 50 mL fresh medium containing 5 mmol $NaNO_2^-$ or 10 mmol KNO_3^- without fungi were incubated according to the same protocol as above to analyze the chemical production of N_2O. After seven days of incubation, the controls without fungal species did not show any N_2O production with NO_3^- as electron acceptor. However, in the presence of NO_2^- as electron acceptor there was minute N_2O production, < 0.01 nmol h^{-1}. This small chemical production was not enough N_2O for isotopic analysis and negligible compared to the observed fungal production of N_2O. The medium was clear and did not show bacterial or fungal growth after seven days of incubation.

Analysis of gas samples

Headspace samples were analyzed by gas chromatography (GC, Agilent 7890A, Agilent, Böblingen, Germany) for N_2O concentration. The detection limit of GC analysis of our incubation was 0.01 nmol h^{-1} with a measurement precision of 1%. Dissolved N_2O in the medium was calculated by Henry's law (Davidson and Firestone, 1988). In the following, the N_2O production implies gas produced in the headspace as

well as the calculated dissolved N_2O. Afterwards, samples were analyzed for N_2O isotopologues by isotope ratio mass spectrometry (IRMS, Thermo Fisher Scientific, Bremen, Germany) using a pre-concentrator (PreCon, Thermo–Finnigan, Bremen, Germany) interfaced to a Delta V isotope ratio mass spectrometer (Brand, 1995; Köster et al., 2013; Toyoda and Yoshida, 1999). The typical analytical precision for natural abundance samples of IRMS analysis was 0.1 ‰ and 0.2 ‰ for $\delta^{15}N_{bulk}$ and $\delta^{18}O$-values, respectively. There are no commercially available N_2O standards enriched with ^{18}O. To check the accuracy of ^{18}O-analysis of enriched N_2O using non-enriched standards, we evaluated linearity by diluting the N_2O sample with the largest ^{18}O enrichment of a pre-experiment (measured value of 0.552 at%) with non-labeled N_2O. A dilution series of eight dilution levels was prepared with ^{18}O enrichments between 0.208 and 0.552 at% and analyzed in triplicates. The non-labeled N_2O was our laboratory working standard with 0.208 at% ^{18}O. A close correlation between measured and expected ^{18}O-values ($R^2 = 0.992$) showed that the response was linear and the calibration with our non-labeled working standard was thus valid. The standard deviation within triplicate measurements of ^{18}O enriched samples varied between 0.001 and 0.004 at%, which can be attributed to influences of dilution and to analytical errors.

^{18}O analysis of H_2O

We made a plausibility check of the ^{18}O enrichment of H_2O in the medium to verify our labeling target of 0.5 at% ^{18}O. We used a cavity ring-down laser spectrometer (model L 1115-I, Picarro, Santa Clara, USA) suitable for analyzing H_2O as liquid or vapor. For calibration, we used three working standards between 0.201 and 0.198 at% ^{18}O (standard deviation < 1.4×10^{-5} at%) measured against Vienna Standard Mean Ocean Water (VSMOW). The ^{18}O enriched medium was distilled to obtain pure H_2O for liquid analysis. Due to the high ^{18}O enrichment, isotopic fractionation during distillation was assumed to be negligible. Samples were analyzed in triplicates in a dilution series with three dilution levels produced with distilled H_2O with known ^{18}O-value from the laboratory ($0.198 \pm 2 \times 10^{-5}$ at%): 1) pure distilled water and dilutions of 2) 1:100 and 3) 1:10 (medium: distilled water). We found a linear response between analyzed ^{18}O-values and the dilution levels (0, 0.01 and 0.1) ($R^2 = 0.999$). The ^{18}O analysis of the ^{18}O-labeled medium showed the expected value of 0.49 ± 0.01 at% ^{18}O.

Calculations

Assuming there is no N_2O reduction (Shoun et al., 1992) and no loss of gas (e.g., loss of NO), the reaction progress of denitrification can be calculated as follows:

$$Reaction\ progress\ (\%) = \frac{final\ N_2O{-}N}{initial\ NOx{-}N} * 100 \qquad \text{(Eq. 1)}$$

where *final N_2O-N* is the amount of N_2O-N and the *initial NO_x-N* is the offered precursor amount (5 mmol $NaNO_2$ or 10 mmol KNO_3).

The ^{18}O enrichment of N_2O was used to determine the incorporation of O from ^{18}O enriched H_2O into N_2O produced during denitrification. Isotope fractionation during O exchange and reduction of N-oxides can be assumed to be negligible because of the high ^{18}O enrichment in H_2O. The extent of O incorporation from H_2O into N_2O can thus be calculated as (Kool et al., 2009a):

$$^{18}O\ exchange\ (\%) = 100 * \frac{^{18}O_{(N_2\ O)}}{^{18}O_{(H_2\ O)}} \qquad \text{(Eq. 2)}$$

where $^{18}O_{(N2O)}$ and $^{18}O_{(H2O)}$ denote O enrichments of H_2O and N_2O, respectively in terms of atom% excess (at%exc). The latter is given as

$$at\%exc\ = at\%_{sample} - at\%_{background} \qquad \text{(Eq. 3)}$$

where $at\%_{sample}$ denotes the ^{18}O abundance of the N_2O sample and $at\%_{background}$ denotes the ^{18}O abundance of the N_2O precursors NO_3^- and NO_2^-, which were analyzed with the denitrifier method according to Casciotti *et al.* using three working standards (USGS34, USGS35 and IAEA-NO-3), with a precision of ≤ 1.3 x 10^{-4} at% (Casciotti et al., 2002). All three working standards are NO_3^- salts, but Casciotti *et al.* found that the O exchange between H_2O and NO_3^- or NO_2^- was indistinguishable during denitrification by *P. aureofaciens,* while the O fractionation from NO_2^- was less than that from NO_3^- (Casciotti et al., 2007). Consequently, in the absence of NO_3^-, the denitrifier method can be used for NO_2^- if complete conversion of the substrate is given.(Casciotti et al., 2007) With N isotopes, we ensured the complete conversion of the NO_3^- standards to N_2O by *P. aureofaciens.* The resulting ^{18}O-values for NO_3^- and NO_2^- were 0.206 and 0.196 at%, respectively. Some fungi yielded an O exchange slightly above 100%. This could be caused by cumulative errors, i.e. derived from precision of dilution of ^{18}O-labeled and non-labeled H_2O (about 4%), analytical precision (about 1% of IRMS measurement),

inaccuracy in transferring the viscous medium to the fresh autoclaved medium after fungal growth and ^{18}O-labeled H_2O in the vial (about 2%).

Statistical analysis

For statistical analysis, the N_2O production was based on fungal dry weight (dw). N_2O production was log^{10}-transformed to achieve normality and homogeneity of variance of the residuals. Significant effects of fungal species and electron acceptors on fungal biomass (dw), N_2O production and oxygen exchange were tested using two-factorial ANOVAs. We made a pairwise comparison with Tukey's HSD test for interactions between species and electron acceptors influencing the N_2O production. For all ANOVAs, *T. hamatum* and *C. funicola* were excluded because electron acceptor effects could only be analyzed for species producing N_2O from NO_2^- as well as from NO_3^- treatments with triplicates. The significance of electron acceptor on N_2O production of all fungal species was tested with a t-test. The significance level (α) for ANOVAs was 0.05. We used linear regression to estimate the relationship between oxygen exchange and N_2O production. Statistical analysis was performed using R (R Core Team 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Results

Evidence of fungal growth

Fungal growth in the medium (turbid, globose or mixture of both) differed among species (Table 1). *Cy. lichenicola* grew unstructured and caused a turbidity in the medium. The culture was examined under a microscope to check for bacterial contamination. No bacterial contamination was visible. After seven days of anaerobic growth, the biomass of fungi with NO_3^- was much higher for *F. oxysporum* and *F.*

solani fsp. *pisi* than with NO_2^-, and thus differed significantly between electron acceptors ($P < 0.001$) and among species ($P < 0.001$).

N_2O production

All fungal species produced N_2O (Table 1), either solely with NO_2^- (*C. funicola*) or with both electron acceptors NO_2^- and NO_3^-. Compared to NO_3^- with an N_2O production between 6 and 47 nmol N_2O g dw^{-1} h^{-1}, NO_2^- as electron acceptor led to significantly greater N_2O production ($P < 0.001$): between 129 and 6558 nmol N_2O g dw^{-1} h^{-1} (Table 1). The N_2O production differed significantly among species ($P = 0.003$). The paired comparison of N_2O production in dependence of fungal species with the different electron acceptors showed that the large N_2O production by *F. oxsporum* with NO_2^- was predominantly responsible for the significant species effect. However, excluding *F. oxysporum* because of its remarkably great N_2O production from the ANOVA showed that there was still an electron acceptor effect ($P < 0.001$) on N_2O production, but there was also a species effect ($P = 0.001$) and an influence of the electron acceptors on species in producing N_2O ($P = 0.002$). The t-test with all fungal species showed an electron acceptor effect on N_2O production as well ($P < 0.001$). *C. funicola*, the only fungus belonging to the order of *Sordariales*, showed the least N_2O production with NO_2^- (129 nmol N_2O g dw^{-1} h^{-1}) among all species (other species between 811 and 6558 nmol N_2O g dw^{-1} h^{-1}) and did not produce detectable amounts of N_2O with NO_3^- as electron acceptor. The calculated reaction progress based on produced N_2O (Eq. 1) of *F. oxysporum* with NO_2^- was 11% and for all other species it was < 2.3% (Table 1), showing that only a small fraction of the added electron acceptors was used for denitrification during the incubation period.

Oxygen exchange between water and intermediates

The N_2O produced by the fungal species after seven days was enriched in ^{18}O (Table 1) due to O exchange between ^{18}O-labeled H_2O and denitrification intermediates. The O exchange was significantly dependent on electron acceptors ($P < 0.001$) and fungal species ($P < 0.001$) and the electron acceptors influenced the species in their O exchange ($P < 0.001$). With NO_2^-, O exchange was higher than with NO_3^- as electron acceptor, with the exception of *F. solani* fsp. *pisi*, which had higher O exchange with

NO_3^- compared to the treatment with NO_2^-. In the presence of NO_2^-, *F. oxysporum* and *T. hamatum* had a calculated O incorporation of > 100%, suggesting complete O exchange. The same result was obtained for *F. solani* fsp. *pisi* with NO_3^-.

The O exchange from labeled H_2O in N_2O varied between 74 and 106% in the NO_2^- treatments of the five species belonging to the order *Hypocreales* (Table 1). The only *Sordariales* species had a lower O exchange of 41% in the NO_2^- treatment. In the NO_3^- treatment, the O exchange varied between 11 and 109% for the fungal species of *Hypocreales*. With both electron acceptors, O exchange correlated positively with N_2O production (Figure 1). The correlation was significant for both electron acceptors ($P < 0.001$ for NO_2^- and $P = 0.007$ for NO_3^-).

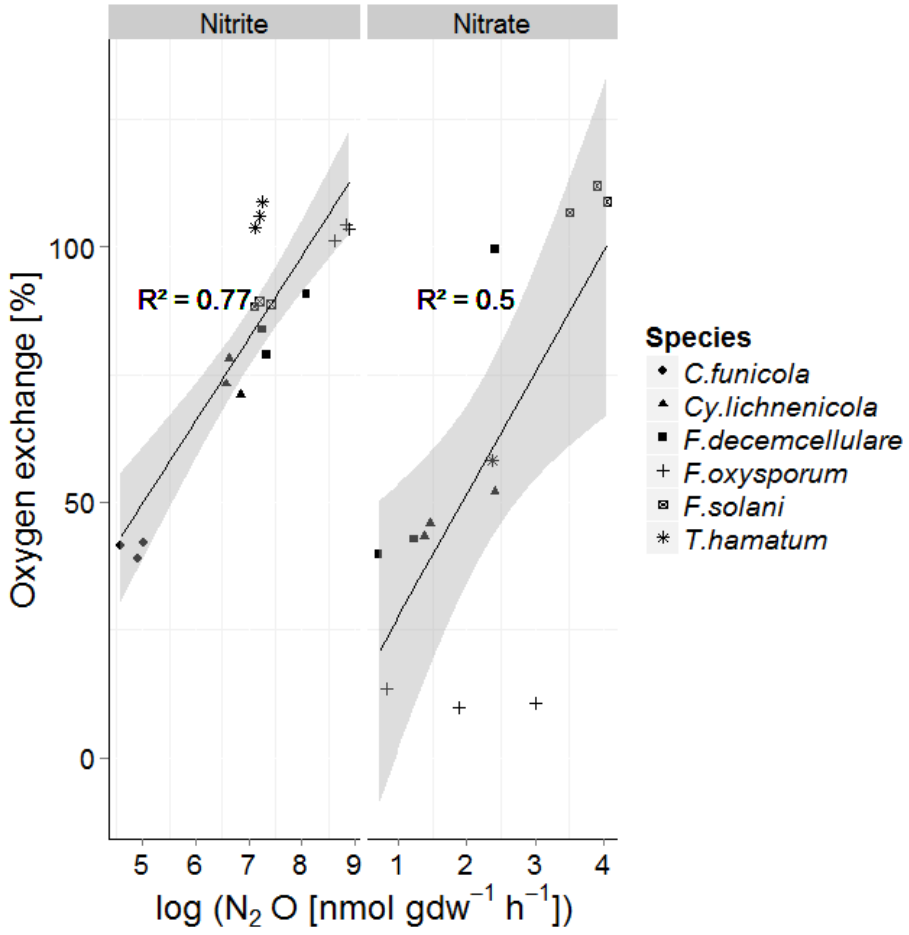

Figure 1: O exchange (%) between H_2O and intermediates during denitrification with NO_2^- and NO_3^- depending on the log-transformed N_2O production after seven days of incubation by six different fungal species with linear regression and 95% confidence interval (Regression for NO_2^- y= -30.828+ 15.559x; NO_3^- y= 3.998+ 17.783x).

Table 1: Results from incubations of six different fungal species (standard deviation in brackets, n = 3)

Species	Electron acceptor	Appearance of fungal culture[1]	Biomass[2] [g]	N_2O production [nmol $g^{-1}h^{-1}$]	^{18}O atom%excess [%]	O exchange [%]	Reaction progress [%]
Cy. lichnenicola	NO_2^-	t	0.023 (0.002)	811.28 (122.06)	0.22 (0.01)	74.1 (3.7)	1.06 (0.16)
F. decemcellulare	N	g	0.015 (0.002)	1425.60 (1038.89)	0.25 (0.02)	84.2 (6.4)	1.27 (0.86)
F. oxysporum	N	gt	0.029 (0.002)	6557.67 (891.06)	0.30 (0.005)	103.0 (1.6)	10.53 (1.43)
F. solani fsp. pisi	N	gt	0.029 (0.003)	1224.24 (241.77)	0.26 (0.001)	88.8 (0.5)	2.31 (0.39)
T. hamatum	N	g	0.006 (0.001)	1339.06 (86.48)	0.31 (0.01)	106.2 (2.5)	0.41 (0.03)
C. funicola	N	g	0.035 (0.004)	128.98 (27.79)	0.12 (0.005)	40.9 (1.6)	0.26 (0.05)
Cy. lichnenicola	N	t	0.020 (0.004)	6.44 (4.03)	0.21 (0.11)	47.1 (4.5)	0.01 (0.003)
F. decemcellulare	N	g	0.048 (0.009)	6.24 (5.57)	0.19 (0.10)	60.7 (33.6)	0.01 (0.01)
F. oxysporum	N	gt	0.048 (0.009)	16.56 (15.88)	0.04 (0.01)	11.3 (1.9)	0.01 (0.01)
F. solani fsp. pisi	N	gt	0.018 (0.004)	46.90 (12.43)	0.27 (0.09)	109.2 (2.7)	0.03 (0.01)
*T. hamatum**	N	g	0.009 (0.001)	10.7*	0.18*	58*	0.003*
C.funicola	N	g	0.014 (0.006)	n.d.	n.d.	n.d.	n.d.

n.d. = not detectable; *C. funicola* did not produce N_2O with NO_3^- as electron acceptor.

[1] The appearance of fungal cultures in the medium was turbid (t), globose (g) or a mixture of globose and turbid (gt).

[2] The average biomass (g dw) of each fungal species after the experiment was used to base the N_2O production on fungal biomass.

*Only one sample of *T. hamatum* with NO_3^- produced sufficient N_2O for isotopic analysis.

Discussion

N_2O production

Our studies with six pure fungal cultures showed that N_2O production varied among species. Further, N_2O production was larger with NO_2^- as electron acceptor than with NO_3^-. Comparable results were reported by Shoun et al. (1992). A higher rate of NO_2^- reduction compared to NO_3^- reduction could result from the attempt to reduce the toxicity of NO_2^- in the medium and could lead to the observed higher N_2O production rates with NO_2^- as electron acceptor. Cleemput and Samater (1995) conducted soil experiments with NO_2^- and found a high abundance of bacteria capable of NO_2^- reduction in areas of high NO_2^- concentrations, supporting the previously described toxicity avoidance. Previous studies by Shoun et al. (1992) with NO_3^- as electron acceptor detected N_2O production from *T. hamatum*, but not from the other fungal species we examined. In addition to Shoun *et al*. other studies also revealed that not all fungal species are capable of NO_3^- reduction, similar to our results for *C. funicola* (Bollag and Tung, 1972; Morozkina and Kurakov, 2007; Shoun et al., 1992; Takaya et al., 2003). Our results confirm that the fungal reduction of NO_3^- to NO_2^- proceeded at a low rate, whereas NO_2^- reduction to N_2O is much faster.

O exchange

The observed range of fungal O exchange (between 11% and full exchange) is consistent with results for several pure bacterial cultures (between 4 and 100%) (Aerssens et al., 1986; Casciotti et al., 2002; Garber and Hollocher, 1982; Knöller et al., 2011; Shearer and Kohl, 1988; Ye et al., 1991). In soil experiments conducted by Snider et al. (2013), Well and Flessa (2009) and Kool et al. (2009b) O exchange was always relatively large (> 65%). Snider *et al*. found O exchanges of 40 to 50% in stream sediments (Snider et al., 2013). Kool et al. (2009b) found almost complete O exchange with ^{18}O-labeled H_2O in several soils and concluded that the ^{18}O signature of N_2O was almost exclusively controlled by the ^{18}O signature of the H_2O. In line with this, Well and Flessa (2009) explained the relatively constant ^{18}O signature of N_2O produced by denitrification in two soils by an almost complete O exchange with soil water.

We found increasing O exchange with increasing N_2O production at a given NO_2^- supply. To interpret this observation, we propose a conceptual model of O exchange during fungal

denitrification based on the models for bacterial denitrification proposed byAerssens et al. (1986), Casciotti et al. (2007) and Snider et al. (2013) (Figure 2):

Under natural conditions, the isotopic signature of N_2O from fungal denitrification results from the isotopic enrichment factors of the reduction steps (ε_1, ε_2 and ε_3), the fractions of O exchange at the reduction step by Nir (x_1) and Nor (x_2), and isotopic enrichment factors of O exchange (ε_4 and ε_5) (Figure 2) (Casciotti et al., 2007; Snider et al., 2013). According to observations by Aerssens et al. (1986) x_1 is dependent on NO_2^- concentration. In our study, only the total O exchange with H_2O (sum of x_1 and x_2 in the conceptual model, Figure 2) was determined. In comparison to the high ^{18}O enrichment of the medium water and subsequent incorporation of enriched O into N_2O, the isotope effects are very small. However, when applying the model of Snider et al. (2013) (calculated with Eq. 5 of Snider et al. (2013) with enriched H_2O as precursor, the fractions of O exchange of this experiment and the isotope effects published by Casciotti et al. (2007) and Snider et al. (2013)., the bias of our tracer approach from the isotope effects ε_4 and ε_5 can be predicted. The impact of the isotope effects on $\delta^{18}O$-N_2O is largest, when full O exchange occurs at Nir. In that case the calculated O exchange can be overestimated by up to 2% if the isotope effect of O exchange is neglected as in our data analysis (Casciotti et al., 2007; Snider et al., 2013). This might influence the calculated values of O exchange and lead to values slightly above 100% as observed in few cases (see Table 1 and section “Calculations”).

The N_2O production rate reflects the abundance and activity of denitrification enzymes. Consequently, increasing N_2O production causes a stronger local depletion of added NO_2^-, leading to an enhanced O exchange. Our data are in agreement with the proposed inverse relation between NO_2^- and O exchange in this conceptual model (Aerssens et al., 1986). Even though only a small fraction of added NO_2^- was consumed in the entire system (Table 1), there may have been a strong local depletion in NO_2^-, especially in the globose-type cultures, causing an increased O exchange compared to the turbid cultures. In soil experiments Snider et al. found that O exchange was independent of N_2O production during denitrification and suggested that variations in O exchange between soils were dependent on the microbial soil community (Snider et al., 2009; Snider et al., 2013). Although the relation between N_2O production and O exchange is different from our pure culture study, this observation does not necessarily contradict our conceptual model for several reasons. First, depletion in NO_2^- concentration with higher N_2O production is not expected in soil incubations if NO_2^- is not added. Secondly, N_2O was emitted from intact microbial soil communities, containing a vast number of different organisms with possibly different rates of O exchange. Finally, the

proposed relation between O exchange and NO_2^- is effective at the scale of individual microbial cells and might thus be completely masked when looking at bulk soil processes including heterogenic spatial distribution of microbial species and N processes.

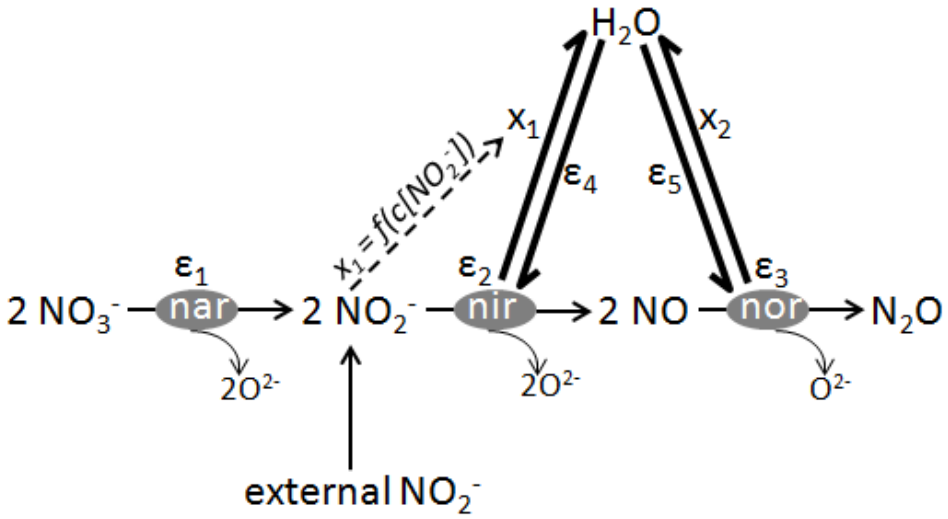

Figure 2: Conceptual model of intracellular O exchange and O isotope enrichment during fungal denitrification, adapted from previous concepts for bacterial denitrification after Casciotti et al. (2007) and Snider et al. (2013) (modified) and implementing controls of O exchange proposed by Aerssens et al. (1986) N_2O consumption is assumed absent. The isotopic enrichment factors (ε) are defined as the difference of the isotopic composition of the product and the substrate of a reaction (Snider et al., 2013). ε_1 and ε_2 describe the inter-molecular isotopic fractionation during NO_3^- reduction to NO_2^- or NO reduction to NO, respectively (Snider et al., 2013). Intra-molecular fractionation during NO_3^- or NO_2^- reduction due to preferred cleavage of $N^{16}O$ bonds results in ^{18}O-enriched NO_2^- or NO, respectively, and ^{18}O-depleted H_2O (Snider et al., 2013). ε_3 is the enrichment factor for the intermolecular isotopic effect during the reduction of 2 NO to N_2O, resulting in ^{18}O-enriched N_2O and ^{18}O-depleted H_2O (Snider et al., 2013). The fractions of the O exchange between denitrification intermediates and water during NO_2^- reduction by Nir and NO reduction by Nor are defined as x_1 and x_2, respectively. For x_1 the extent of O exchange is a function of the NO_2^- concentration $(c[NO_2^-])$, according to observations by Aerssens et al. (1986). for bacteria containing the heme cd-1 Nir type ($x_1 = f(c[NO_2^-])$ (indicated by dotted arrow) (Snider et al., 2013). External NO_2^- sources (from other N processes or experimentally added) and the rates of NO_2^- reduction by Nar and its consumption by Nir controls NO_2^- concentration and thus indirectly x_1. The isotope enrichment factors during O exchange at the Nir and Nor reduction steps are ε_4 and ε_5, respectively (Snider et al., 2013).

It is striking that *C. funicola*, the only species of the order *Sordariales*, exhibited several exceptional results. It did not produce N_2O in presence of NO_3^- and showed the smallest N_2O production as well as the smallest O exchange during denitrification with NO_2^-. The small O exchange might be specific for the enzyme structure of this order, a result that could be

comparable to differing O exchanges between the two bacterial Nir types (heme-cd 1 and copper) found by Ye et al. (1991). Further studies are needed to verify order-specific effects and also order-specific process controls of N_2O production as well as of O exchange.

What are the implications of the observed O exchange between H_2O and denitrification intermediates? The observed variability in O exchange among soil communities and pure bacterial and fungal cultures demonstrates the high relevance of this process when interpreting the isotopic fingerprint of soil-derived N_2O (Aerssens et al., 1986; Garber and Hollocher, 1982; Kool et al., 2011; Snider et al., 2009; Snider et al., 2013; Ye et al., 1991). Complete O exchange implies independence of the O signature of the precursor (NO_2^- or NO_3^-). In that case, $\delta^{18}O$-N_2O would only be controlled by the H_2O signature, isotopic fractionation of O exchange and the isotopic fractionation after O exchange during the NO_2^- reduction to N_2O (Kool et al., 2009b; Well et al., 2012). Our results suggest that O exchange by fungal denitrifers is similar to bacterial denitrifiers in two ways: (I) ranges are similar (<10 to 100%) and (II) almost all O exchange rates of our pure fungal cultures and of incubated soils (Kool et al., 2009b; Snider et al., 2013) were between 65 and 100%. These high O exchanges exclude the use of $\delta^{18}O$-values to identify N_2O precursors and pathways of N_2O formation without quantifying O exchanges and prevent the differentiation between fungal and bacterial sources of N_2O using ^{18}O signatures. Another consequence might be a small variability of $\delta^{18}O$-N_2O compared to $\delta^{18}O$-values of denitrification precursors. This would facilitate the estimation of N_2O reduction to N_2 using $\delta^{18}O$-values.

In view of the growing evidence on the role of fungal denitrification to N_2O fluxes the missing distinction between fungal and bacterial O exchange rates is relevant since it illustrates that variations in the fungal share of soil N_2O fluxes would not affect the extent of O incorporation from H_2O (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). Hence, the high O exchange during N_2O formation in soil as suggested by previous soil studies is probably independent of the fungal-to-bacterial ratio of the N_2O fluxes (Kool et al., 2009a; Kool et al., 2009b; Well and Flessa, 2009). However, since we could not clarify the mechanisms controlling fungal O exchange, more pure culture work is needed with defined manipulation of suspected controlling factors.

Conclusions

We found that O exchange between H_2O and intermediates of denitrification exists in pure fungal cultures. Variation in the extent of O exchange might be explained by the interaction between N_2O production rates and NO_2^- concentration. Further research is needed to estimate

fungal O exchange under soil conditions, e.g., by varying the ratio between NO_2^- and fungal biomass. Our data indicate that the identification of N_2O produced by fungi or bacteria based on $\delta^{18}O$-N_2O is not possible due to a similar range in O exchange by both organism groups. The suspected similarity of controlling factors during fungal and bacterial O exchange suggests that both microbial groups might yield an almost complete O exchange in soils, which would facilitate the estimation of N_2O reduction based on $\delta^{18}O$-values of emitted N_2O.

Acknowledgement

Susanne Behn is thanked for assistance with microbial work and Martina Heuer for isotopic analysis; the Max-Planck Institute for Terrestrial Microbiology in Marburg (Germany) for providing the fungal strains and for the possibility to work in their lab, and Roland Fuß for help with statistical analysis. We would like to thank Dina Führmann for editing the English. Further thanks to the two anonymous referees for improving our manuscript by helpful and constructive suggestions. This joint research project was financially supported by the State of Lower-Saxony and the Volkswagen Foundation, Hanover, Germany.

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Dual isotope and isotopomer signatures of nitrous oxide from fungal denitrification – a pure culture study

This chapter is a modified form of an article, which was in the reviewing process of the journal Rapid Communications in Mass Spectrometry when the thesis was submitted. After the submission of the thesis the article was published in a revised version (DOI:10.1002/rcm.6975):
Rohe, L., Anderson, T.-H., Braker, G., Flessa, H., Giesemann, A., Lewicka-Szczebak D., Wrage-Mönnig, N. and Well, R. (2014). Dual isotope and isotopomer signatures of nitrous oxide from fungal denitrification – a pure culture study

Abstract

RATIONALE: The contribution of fungal denitrification to the emission of the greenhouse gas nitrous oxide (N_2O) from soil has not yet been sufficiently investigated. The intramolecular ^{15}N site preference (SP) of N_2O could provide a tool to distinguish between N_2O produced by bacteria or fungi, since fungi showed much higher SP of N_2O in previous studies than bacteria.

METHODS: To further constrain isotopic evidence of fungal denitrification, we incubated six soil fungal strains under denitrifying conditions, either with the electron acceptor nitrate or nitrite, and analyzed the isotopic signature ($\delta^{18}O$, $\delta^{15}N_{bulk}$ and SP) of N_2O produced. Nitrogen and oxygen isotopic fractionation was calculated and oxygen isotope exchange associated with particular fungal enzymes was estimated.

RESULTS: Five fungi of the order *Hypocreales* produced N_2O with a SP of 31.7 ± 1.4 ‰ after seven days of anaerobic incubation independent of the electron acceptor, whereas one *Sordariales* species produced N_2O from NO_2^- only, with a SP of 19.7 ± 1.3 ‰. Smaller isotope effects of $^{15}N_{bulk}$ were associated with larger N_2O production. The $\delta^{18}O$ values were influenced by oxygen exchange between water and denitrification intermediates, which occurred predominantly at nitrite reductase.

CONCLUSIONS: Our results confirm that SP is a promising tool to differentiate between fungal and bacterial N_2O from denitrification. Modelling of ^{18}O fractionation processes indicated different mechanisms of the oxygen exchange for various fungal species studied. However, more information is needed about different groups of fungi as they may differ in fungal denitrification and SP.

KEYWORDS: Fungi, N_2O isotopologues, Isotopic fractionation, Oxygen exchange, Site preference

Introduction

Agricultural soils are the most important anthropogenic source of the greenhouse gas nitrous oxide (N_2O) and contribute to the rising N_2O concentration in the atmosphere (IPCC, 2013). Finding mitigation strategies for N_2O emissions is important, and for this purpose better knowledge of the controls of N_2O fluxes are urgently needed.

One of the main pathways producing N_2O is denitrification, the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and to gaseous dinitrogen (N_2) via the gaseous intermediates nitric oxide (NO) and N_2O under anaerobic conditions (Knowles, 1982). In the 1970s, it was shown that in addition to bacteria, fungi are capable of denitrification and N_2O production (Bollag and Tung, 1972). Since then a number of pure culture studies on fungal denitrification were published (Bollag and Tung, 1972; Shoun et al., 1992; Shoun and Tanimoto, 1991; Sutka et al., 2008). Of particular importance was the finding of Shoun et al. (1992), that most fungi lack the enzyme N_2O reductase (Nos), hence most fungi produce N_2O as the major end product of fungal denitrification. Referring to this, Sutka et al. (2008) hypothesized that in soils the lack of Nos enables fungi to potentially produce more N_2O than bacteria, given the assumption that both groups have identical or similar denitrification rates, and given that relative to bacteria, fungal biomass is much more abundant in most soils (up to 96% of biomass) (Braker and Conrad, 2011; Ruzicka et al., 2000).

Several laboratory studies with soils indicated that fungi compared to bacteria dominated N_2O production by denitrification (Blagodatskaya et al., 2010; Crenshaw et al., 2008; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). Commonly, antibiotic inhibitors of either fungal or bacterial growth are used to differentiate between N_2O produced by both microbial groups (Blagodatskaya et al., 2010; Crenshaw et al., 2008; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). However, there are organisms, which are not affected by the growth inhibitors and growth inhibitors do not affect active microorganisms that are not in growth stage. N_2O produced by this group may influence results of these experiments. Furthermore, the inhibition of growth may affect processes other than just the target processes. A direct method to differentiate between the production of N_2O by fungal and bacterial denitrification is not yet available. Studies to analyze the enzymes involved in denitrification identified only the NO reductase (Nor) of fungi (P450nor) as distinct from its bacterial counterpart, while all other enzymes involved in fungal denitrification were similar to the bacterial ones (Kim et al., 2009).

Measuring the dual isotopic composition of N_2O ($\delta^{15}N_{bulk}$ and $\delta^{18}O$) has become a useful tool for advancing our knowledge of N_2O production pathways and sources (Kool et al., 2011;

Ostrom and Ostrom, 2011; Wrage et al., 2005). Isotopic fractionation affects $\delta^{15}N_{bulk}$ and $\delta^{18}O$ values of N_2O. Hence, during denitrification theoretical fractionation results in the production of N_2O with decreased $\delta^{15}N_{bulk}$ and $\delta^{18}O$ values compared to the precursors while concurrent N_2O reduction results in residual N_2O that comprises comparatively increased $\delta^{15}N_{bulk}$ and $\delta^{18}O$ values compared to N_2O produced without influences of N_2O reduction (Ostrom and Ostrom, 2011; Toyoda et al., 2005). Oxygen (O) isotopes are additionally affected by O exchange between water (H_2O) and denitrification intermediates (Aerssens et al., 1986; Casciotti et al., 2002; Garber and Hollocher, 1982; Kool et al., 2009; Rohe et al., 2014; Ye et al., 1991). Consequently, isotopic fractionation and O exchange during denitrification affects the isotopic composition of N_2O and thus has to be taken into account when interpreting isotopic data of emitted N_2O. The intramolecular ^{15}N site preference of N_2O (SP), which is the difference between the $\delta^{15}N$ value of the central and terminal N-position of the asymmetric N_2O molecule, may be a prospective possibility to distinguish between N_2O produced by bacteria or fungi (Toyoda et al., 2005). The SP of N_2O produced by several bacterial strains ranged from -11 to 0 ‰ (Frame and Casciotti, 2010; Sutka et al., 2006), whereas the two fungal strains analyzed so far (*Fusarium oxysporum* and *Cylindrocarpon tonkinense*) produced N_2O with a SP of 37 ‰ (Sutka et al., 2008). Differences in SP between fungal and bacterial N_2O can be explained by enzyme structures, suggesting sequential binding of two NO molecules during N_2O formation in the fungal P450nor, whereas a parallel binding mechanism is assumed in bacterial Nors (Schmidt et al., 2004). During denitrification SP is independent of the isotopic composition of the N_2O precursors (Toyoda et al., 2002). but it might reflect the microbial source of N_2O. Hence, assuming that the current pure culture data are representative for most fungal and bacterial species under natural soil conditions, differences in SP might be used to quantify the contribution to N_2O emission from denitrification of microbial groups in a mixed soil community. If N_2O production pathways can be determined and clearly attributed to either nitrification or denitrification, and the contribution of the different microbial groups can be quantified, this would contribute to better understanding and prediction of soil N_2O fluxes and thus facilitate developing mitigation measures (Richardson et al., 2009).

However, up-to-date information on isotopic fractionation during N_2O production by fungal denitrifiers and the resulting SP of N_2O is scarce and more data is necessary to improve methods for a reliable quantification of bacterial and fungal contribution to N_2O emissions from the soil community. Therefore, to further constrain isotope effects of fungal denitrification, we incubated six fungal species including those used by Sutka et al. (2008)

under denitrifying conditions, either with NO_3^- or NO_2^- as electron acceptor, and analyzed the isotopologues of N_2O produced ($\delta^{18}O$, $\delta^{15}N_{bulk}$, SP).

Materials and Methods

Microorganisms

For isotopologue analysis of N_2O from fungal denitrification, we used six fungal species that are found in soils, described in Domsch et al. (1980) (Table 1). In experiments by Sutka et al. (2008), N_2O produced by *C. lichenicola* (synonymous name *C. tonkinense*, Nite Biological Research Center, Chiba, Japan) and *F. oxysporum* was measured to analyze the SP of N_2O from denitrification, but the authors used different strains (ATCC 42055 and ATCC 12581) thus enabling us to evaluate whether different strains from the same species produce identical SP of N_2O (Sutka et al., 2008). Accumulation of N_2O during incubations is assumed to represent N_2O production because N_2O reduction has been found to be negligible for fungi, with no detectable N_2 production by *F. oxysporum* and *F. decemcellulare*, while the other fungi produced only a small N_2 amount compared to bacteria capable of N_2O reduction (Shoun et al., 1992). Although Shoun et al. (1992) found indications by ^{15}N tracer application, that fungal codenitrification might have occurred, the contribution was minimal and thus we assume that codenitrification in our incubation played a negligible role.

Table 1: Fungal species in incubation experiments to determine isotopic composition of N_2O from denitrification.

Fungal species	Catalogue number[a]	Biological order
Chaetomium funicola	JCM 22733	*Sordariales*
Fusarium oxysporum	JCM 11502	*Hypocreales*
Trichoderma hamatum	JCM 1875	*Hypocreales*
Cylindrocarpon lichenicola	NBRC 30561	*Hypocreales*
Fusarium solani fsp. *pisi*	NBRC 9425	*Hypocreales*
Fusarium decemcellulare	NBRC 31657	*Hypocreales*

[a]JCM = Japan Collection of Microorganisms, Saitama, Japan and NBRC = Nite Biological Research Center, Chiba, Japan

Experimental procedure

We conducted experiments to determine dual isotope and isotopomer effects during N_2O production by fungal denitrification using pure culture incubation procedures according to the protocol of previous studies (Rohe et al., 2014; Shoun et al., 1992): The sterile fungal medium for the preparatory culture (100 mL) contained 1% glucose, 0.2% peptone, 0.02% $MgSO_4 \cdot 7\ H_2O$, 2 ppm $CaCl_2 \cdot 6\ H_2O$, 2 ppm $FeSO_4 \cdot 7\ H_2O$ and 0.01 mol potassium phosphate (pH 7.4) (Shoun et al., 1992).

We used two Setups each differing in the volumes used with three fungal species. Initially, three fungi (Setup A) were incubated in a relatively large amount of medium (1 L flasks) to ensure enough N_2O production for gas analysis. Because all three fungi produced far more N_2O than needed, further incubations were conducted with smaller volumes (Setup B) to save preparation time and resources. In Setup A, the stem cultures of *C. lichenicola*, *F. solani* fsp. *pisi* and *F. decemcellulare* were transferred to 500 mL medium and incubated aerobically at 22 °C in the dark in a rotary shaker (100 rpm) in a 1 L Erlenmeyer flask with cotton wool plugs for three to seven days (depending on the growth increase of the cells). Of this pre-culture, 60 mL were transferred in a 1 L flask with screw caps and a butyl stopper containing fresh medium (240 mL each). In Setup B, the stem cultures of *C. funicola*, *F. oxysporum* and *T. hamatum* were transferred to 100 mL medium and incubated aerobically at 22 °C in the dark in a rotary shaker (100 rpm) in a 250 mL Erlenmeyer flask with cotton wool plugs for three to seven days (depending on the growth increase of the cells). Of this pre-culture, 10 mL aliquots were transferred into a 120 mL crimp seal flask with a butyl stopper containing fresh medium (40 mL each).

Two treatments were established with either 10 mmol KNO_3 or 5 mmol $NaNO_2$ as electron acceptors. All treatments were conducted in triplicate. The headspace of the flasks was purged with filter-sterilized N_2 for 10 min while the flasks were stirred manually to expel dissolved O to establish anaerobic conditions. The cultures were incubated in the dark (22 °C) in a rotary shaker (120 rpm). Gas samples were collected (2x14 mL) after seven days of incubation and transferred with a syringe to evacuated sampling vials (12 mL Exetainer®, Labco Ltd., Lampeter, UK). Due to the suggested absence of N_2O reduction we expect consistent SP with time. The supply of electron acceptors NO_3^- and NO_2^- was much higher than their expected consumption and thus we assume no influence of substrate limitation or isotopic enrichment of substrates on isotopic signatures during the incubation.

To test for chemical production of N_2O, triplicates of 50 mL fresh medium containing either 10 mmol KNO_3^- or 5 mmol $NaNO_2^-$ were incubated, without inoculating with fungal pure

cultures, using the same protocol as above for seven days. No bacterial or fungal growth could be observed by visual inspection after seven days of incubation, and the controls showed N_2O production only in the presence of NO_2^- as electron acceptor (<0.01 nmol h^{-1}), while no N_2O production was detectable with NO_3^-.

To assess the biomass of each flask, the complete medium was centrifuged (5236 x g for 30 min) at the end of incubation and the pellet was washed twice with distilled H_2O. The supernatant was decanted and the cells were oven dried at 105 °C for 48 hours to determine the dry weight of the fungal biomass. As the fungal biomass differed among species, N_2O production was related to dry weight (dw) of the fungal biomass.

Analysis of gas samples

Headspace samples were analyzed by gas chromatography (GC, Agilent 7890A, Agilent, Böblingen, Germany) for N_2O concentration. The detection limit of GC analysis of our incubation was 0.01 nmol h^{-1} with a measurement precision of 1%. Dissolved N_2O in the medium was calculated by Henry's law (Davidson and Firestone, 1988). In the following, the N_2O production implies gas produced in the headspace as well as the calculated dissolved N_2O. Afterwards, samples were analyzed for N_2O isotopologues by isotope ratio mass spectrometry (IRMS, Thermo Fisher Scientific, Bremen, Germany) using a pre-concentrator (PreCon, Thermo–Finnigan, Bremen, Germany) interfaced to Delta V isotope ratio mass spectrometer (Brand, 1995; Köster et al., 2013; Toyoda and Yoshida, 1999). The typical analytical accuracy (1σ) was 0.1 ‰ and 0.2 ‰ and 1.5 ‰ for $\delta^{15}N_{bulk}$, $\delta^{18}O$ and SP, respectively.

Isotope analysis of NO_3^-, NO_2^- and H_2O

We used the bacterial denitrifier method according to Casciotti et al. (2002) to determine $\delta^{18}O$ and $\delta^{15}N_{bulk}$ values of NO_3^- or NO_2^- in the media. The working standards for this method were USGS34, USGS35 and IAEA-No3. The NO_2^- had a $\delta^{18}O$ of -22.4 ± 0.5 ‰ (vs. SMOW) and a $\delta^{15}N_{bulk}$ of -22.7 ± 0.1 ‰ (vs. air-N_2) and NO_3^- had a $\delta^{18}O$ of 22.0 ± 0.3 ‰ (vs. SMOW) and a $\delta^{15}N_{bulk}$ of 1.4 ± 0.3 ‰ (vs. air-N_2). The isotopic composition of the H_2O in the fungal medium with *Cy. lichenicola*, *F. decemcellulare* and *F. solani* fsp. *pisi* was analyzed using a thermal combustion elemental analyzer (TC/EA-ConFlo III, Thermo Fisher,

Bremen) coupled to IRMS (Delta plus, Thermo-Finnigan, Bremen) giving -9.9 ± 0.1 ‰ for $\delta^{18}O$ (Centre for Stable Isotope Research and Analysis, University of Göttingen, Germany). The isotopic composition of H_2O in the fungal medium with *F. oxysporum*, *T. hamatum* and *C. funicola* was analyzed using a cavity ring-down laser spectrometer (model L 1115-I, Picarro, Santa Clara, USA) giving -8.9 ± 0.2 ‰ for $\delta^{18}O$.

Calculations

The estimation of the isotope fractionation during a unidirectional reaction can be expressed as the kinetic fractionation factor α (Kendall, 1998; Mariotti et al., 1982), which is defined as

$$\alpha = \frac{R_P}{R_S} \quad \text{(Eq. 1)}$$

with R_P and R_S describing the isotope ratios of the product and the substrate, respectively.

The isotope enrichment factor $\varepsilon_{P/S}$ is defined as

$$\varepsilon_{P/S}\ (‰) = 1000 \times (\alpha\text{-}1) \quad \text{(Eq. 2)}$$

The reaction progress of fungal denitrification can be expressed as

$$Reaction\ progress\ (\%) = \frac{final\ N_2O\text{-}N}{initial\ NOx\text{-}N} * 100 \quad \text{(Eq. 3)}$$

with *final N_2O-N* describing the N_2O-N production and the *initial NO_x-N* describing the precursor amount added.

The reaction progress in our incubations was small, i.e., only a small portion of the initial N substrates was consumed (Table 2). Thus, we could assume that the change in the isotopic composition of the precursors (NO_2^- and NO_3^-) of N_2O would be negligible. Therefore, $\varepsilon_{P/S}$ can be approximated as follows, assuming that isotopic signature of the residual substrates is unaffected by fractionation

$$\varepsilon_{P/S}\ (‰) \approx \delta p - \delta s = \Delta\delta \quad \text{(Eq. 4)}$$

with δ_p for the isotopic signature of the product (N_2O) and δ_s for the isotopic signature of the substrate (NO_2^- or NO_3^-), respectively.

The $\delta^{18}O$ value of N_2O ($\delta^{18}O$-N_2O) produced by denitrification is affected by isotopic fractionation and by O exchange between denitrification intermediates and H_2O, which has to be taken into account, when interpreting the $\delta^{18}O$-N_2O values (Kool et al., 2009; Rohe et al., 2014). Therefore, the $\Delta\delta^{18}O$ value cannot be determined in a straight-forward way.

Statistical analysis

The N_2O production was calculated per dry weight (dw) of fungal biomass and $\log_{10}$-transformed to achieve normality and homogeneity of variance of the residuals. The negative $\Delta\delta^{15}N_{bulk}$ values had to be converted into positive numbers by multiplying by (-1) to be able to $\log_{10}$-transform the $\Delta\delta^{15}N_{bulk}$ for the same reasons as for N_2O production. We conducted three ANOVAs to test the effect of fungal species, the structure of fungal cultures and electron acceptors on N_2O production, SP and $\Delta\delta^{15}N_{bulk}$. For all ANOVAs, *C. funicola* had to be excluded, because it only produced N_2O with NO_2^- and therefore an electron acceptor effect cannot be determined. Nevertheless, we tested if SP of N_2O with NO_2^- as electron acceptor differed among all six fungal species including *C. funicola* with an ANOVA. A pairwise comparison with Tukey's HSD test was done to find differences between species influencing the SP of N_2O produced. The significance level α was 0.05. We calculated a linear regression of the calculated $\Delta\delta^{15}N_{bulk}$ and the $\log_{10}$-transformed N_2O production after seven days of incubation (with NO_3^- and NO_2^-) by six different fungal species with a 95% confidence interval. For statistical analyses, we used the program R (R Core Team, 2013).

Results

Evidence of fungal growth

The visible growth structure differed among species. While all other species showed globose growth (with or without turbidity), *Cy. lichenicola* grew unstructured and caused turbidity in the medium (Table 2). We examined this fungus and its growth medium microscopically to test for bacterial contamination. No contamination was detectable, only fungal mycelium was present.

N_2O production

Every fungal species produced N_2O in the presence of NO_3^- and NO_2^-, except for *C. funicola,* which produced N_2O solely with NO_2^- (Table 2). Production rates of N_2O per biomass were significantly higher with NO_2^- than with NO_3^- ($P < 0.001$) and differed significantly between the fungal structure ($P = 0.001$), but were independent of fungal species ($P = 0.124$). The progress of the reaction in Setup B was always $\leq 0.4\%$ (Table 2), so that only a small portion of the added electron acceptor (NO_3^-, NO_2^-) was consumed by denitrification. Setup A reached a reaction progress between 2 and 8% for NO_2^- and 0.01 to 0.06% for NO_3^- (Table 2).

SP of N_2O

Among all fungi, *C. funicola* showed the significantly smallest SP of 19.7 ‰. All other fungi produced similar SP (31.7 ± 1.4 ‰) (Table 2). SP was independent of species ($P = 0.716$), electron acceptors ($P = 0.289$) and structure of fungal species ($P = 0.778$) (ANOVA without *C. funicula*). The post hoc tests following the ANOVA including *C. funicula* ($P < 0.001$) to test if SP of N_2O differed between species showed that only SP of N_2O produced by *C. funicula* differed from all other species, while SP of N_2O produced by the other species did not differ between them.

$\delta^{18}O$ and $\delta^{15}N_{bulk}$ of N_2O

Both $\delta^{18}O$ and $\delta^{15}N_{bulk}$ values of N_2O produced were far more variable ($\delta^{15}N_{bulk}$ = -29.5 to -51.7 ‰, $\delta^{18}O$ = 11.8 to 55.3 ‰) than SP (Table 2). Compared to the relatively constant SP values of five *Hypocreales* fungi, their $\delta^{15}N_{bulk}$ values were highly variable ($\delta^{15}N_{bulk}$ = -39.8 ± 7.7 ‰ with NO_2^- and -36.5 ± 6.7 ‰ with NO_3^-). Furthermore, the $\delta^{18}O$ values varied widely among *Hypocreales* species with NO_3^- as electron acceptor ($\delta^{18}O$ = 43.6 ± 10.2 ‰) in contrast to treatments with NO_2^- ($\delta^{18}O$ = 36.5 ± 2.0 ‰). Only *C. funicola*, the only *Sordariales* species, produced SP (19.7 ± 1.3 ‰) and $\delta^{18}O$ (11.8 ± 0.3 ‰) values deviating from the *Hypocreales* values, while $\delta^{15}N_{bulk}$ values were within the range of the *Hypocreales* (-39.9 ± 0.4 ‰). The $\Delta\delta^{15}N_{bulk}$ was highly variable (-46 to -9 ‰) and it was independent of fungal species ($P = 0.229$). It was remarkable that the absolute values of $\Delta\delta^{15}N_{bulk}$ of N_2O were significantly higher in treatments with NO_2^- than in treatments with NO_3^- ($P < 0.001$) and were significantly dependent on the structure of fungal species ($P < 0.001$).Our results show the following relation between the visual structure of fungi and $\Delta\delta^{15}N_{bulk}$ (see Table 2): For both electron acceptors, the largest $\Delta\delta^{15}N_{bulk}$ was observed from the globose (g) forming fungi (g) (-17.5 to -29.3 ‰ with NO_2^- and -42.9 to -45.5 ‰ with NO_3), and the lowest $\Delta\delta^{15}N_{bulk}$ from turbid (t) grown fungus (*Cy. lichenicola*, -8.8 ‰ with NO_2^- and -30.8 ‰ with NO_3^-). The $\Delta\delta^{15}N_{bulk}$ values for the mixed growth type with globose formation in turbid medium (gt) were between those of the other growth forms (-13.8 to -14.0 ‰ with NO_2^- and -31.6 to -38.0 ‰ with NO_3^-). Furthermore the absolute value of $\Delta\delta^{15}N_{bulk}$ decreased significantly with increasing N_2O production ($P < 0.001$; Figure 1).

Table 2: Fungal growth, N_2O production and isotopic composition of N_2O (^{15}N site preference, $\delta^{18}O$, $\delta^{15}N_{bulk}$) in incubation studies with six different fungal species and with addition of nitrate and nitrite as electron acceptor for denitrification (standard deviation in brackets, n = 3).

Species	Electron acceptor	fungal growth[a]	Biomass[b] [g]	N_2O production[c] [nmol N_2O g^{-1} h^{-1}]	^{15}N site preference [‰]	$\delta^{18}O$ [‰]	$\delta^{15}N_{bulk}$ [‰]	Reaction progress[d] [%]	$\Delta\delta^{15}N_{bulk}$[e]
Cy. lichnenicola	NO_2^-	t	0.22 (0.16)	8800 (2498)	30.19 (0.89)	37.62 (1.68)	-31.20 (4.78)	7.69 (2.16)	-8.8 (4.8)
F. decemcellulare	NO_2^-	g	0.11 (0.09)	7709 (943)	31.76 (0.82)	36.14 (0.80)	-51.70 (2.58)	2.08 (0.70)	-29.3 (2.6)
F. solani fsp. *pisi*	NO_2^-	gt	0.06 (<0.01)	13865 (118)	32.00 (0.42)	38.40 (0.32)	-36.20 (0.05)	8.23 (0.06)	-13.3 (0.1)
F. oxysporum	NO_2^-	gt	0.04 (<0.01)	2441 (1255)	31.15 (2.36)	33.35 (0.59)	-36.36 (3.00)	0.328 (0.169)	-14.0 (3.0)
T. hamatum	NO_2^-	g	0.02 (<0.01)	1561 (128)	31.01 (0.27)	36.90 (0.70)	-43.47 (0.09)	0.420 (0.034)	-21.7 (0.1)
C. funicola	NO_2^-	g	0.01 (<0.01)	52 (10)	19.66 (1.27)	11.83 (0.28)	-39.90 (0.40)	0.005 (0.001)	-17.5 (0.4)
Cy. lichnenicola	NO_3^-	t	0.11 (0.02)	140 (96)	32.35 (1.64)	45.67 (1.17)	-29.52 (1.83)	0.06 (0.04)	-30.9 (1.8)
F. decemcellulare	NO_3^-	g	0.11 (0.03)	47 (26)	31.93 (2.77)	38.20 (8.65)	-44.22 (3.82)	0.01 (0.01)	-45.6 (3.8)
F. solani fsp. *pisi*	NO_3^-	gt	0.08 (<0.01)	155 (163)	32.00 (1.72)	43.46 (6.16)	-30.27 (4.98)	0.04 (0.04)	-31.6 (5.0)
F. oxysporum	NO_3^-	gt	0.04 (0.01)	162 (100)	32.59 (2.19)	55.32 (13.33)	-36.68 (1.54)	0.001 (<0.001)	-38.0 (1.5)
T. hamatum	NO_3^-	g	0.06 (0.02)	129 (185)	31.18 (0.14)	35.23 (1.40)	-41.56 (2.93)	0.002 (0.003)	-42.9 (2.9)
C. funicola	NO_3^-	g	0.01 (<0.01)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]n.d. = not detectable; *C. funicola* did not produce N_2O with NO_3^- as electron acceptor.

[b]The appearance of fungal cultures in the medium was turbid (t), globose (g) or a mixture of globose and turbid (gt).

[c]The average biomass (g dw) of each fungal species after the experiment was used to relate the N_2O production to fungal biomass.

[d]Reaction progress is calculated by the relative quotient of N_2O-N production and the precursor N-amount added (see Eq. 3).

[e]$\Delta\delta^{15}N_{bulk}$ describes the difference of the isotopic N signature of the product (N_2O) and the isotopic N signature of the substrate (NO_2^- or NO_3^-), respectively (see Eq. 4).

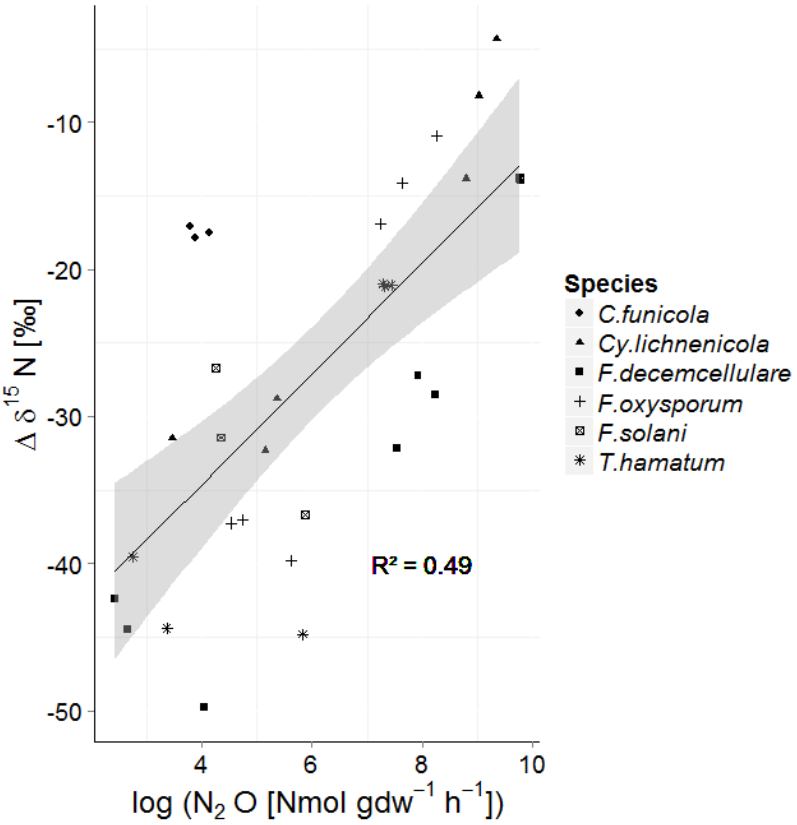

Figure 1: $\Delta\delta^{15}N$ [‰] depending on the $\log_{10}$-transformed N_2O production rate after seven days of incubation (with nitrate and nitrite) by six different fungal species with linear regression and 95% confidence interval (Regression: y = -49.7 + 3.8x).

Discussion

N_2O production and SP of N_2O

With respect to the potential use of SP of N_2O to identify fungal denitrification, the questions are (1.) whether SP of produced N_2O depends only on the enzyme type (Schmidt et al., 2004), (2.) if there is an additional species effect for a certain enzyme type, and (3.) if N_2O production dynamics are relevant:

1. We analyzed the SP of N_2O produced by four additional fungal species as compared to the two fungal species examined by Sutka et al. (2008). Variation of SP of N_2O produced by *F. oxysporum* and *Cy. lichenicola* with NO_2^- of our experiment was within the individual measurements by Sutka et al. (2008) (cf. Table 2 and Table 1 in Sutka et al. (2008)), although the mean SP of N_2O of both species of the present study was about 6 ‰ lower compared to the declared mean SP values of Sutka et al. (2008). All six fungal species studied here produced N_2O with a substantially higher SP (19.7 to 32.6 ‰) than reported for bacteria (-11 to 0 ‰) (Frame and Casciotti, 2010; Sutka et al., 2006). However, SP of *C. funicola*

compared to the other five fungi was in between the fungal SP (33 to 37 ‰ potentially with P450nor) and the bacterial SP (0 to -11 ‰ with cnor) (Frame and Casciotti, 2010; Sutka et al., 2008; Sutka et al., 2006). As SP values of N_2O are independent of the substrate (Sutka et al., 2006; Toyoda et al., 2002), enzyme activity might be a control of SP produced. The sequential binding of NO could be dependent on the enzyme activity and thus could result in different fractionation during the addition of the first or the second NO. Another controlling factor resulting in lower SP of N_2O by *C. funicola* compared to the other five fungal species might point to the occurrence of a different Nor than the typical P450nor for fungi. However, so far there is no information about fungal Nors other than P450nor, which was well examined for *F. oxysporum* and *C. tonkinense* and to our knowledge only for species of the order *Hypocreales* (Shoun et al., 2012; Shoun and Tanimoto, 1991). There is no information available on the Nor type of *C. funicola.*

2. If it could be confirmed that the Nor type of *C. funicula* differs from specific P450nor and that all *Hypocreales* have the same enzyme, we could conclude that the similarities of SP among *Hypocreales* indicate that there is no significant species effect and SP might be controlled by the enzyme type. But this conclusion would be invalid if *C. funicula* turned out to have P450nor. Further studies are needed to examine the enzymatic structure for fungal species under examination.

3. Neither the N_2O production rate nor the availability of two different electron acceptors affected the SP of the N_2O produced, which agrees with results of previous studies with bacteria (Toyoda et al., 2002). We did not directly test the impact of NO_3^- or NO_2^- concentration on SP. Because reaction progress was always relatively small, the $\delta^{15}N_{bulk}$ of the electron acceptors should not have changed much during the incubation period. However, we cannot rule out local depletion of NO_3^- or NO_2^- due to the structure of fungal species (e.g., in the globose type cultures; see also next section for detailed discussion). But if such differences in local depletion existed among *Hypocreales* species, apparently they did not significantly affect SP values of N_2O.

What is still missing is the knowledge about the archaeal contribution to denitrification (Santoro et al., 2011; Thomson et al., 2012). Information so far showed that the archaeal denitrification pathway is similar to bacterial denitrification (Hayatsu et al., 2008). To the best of our knowledge there is no information available about the isotopic fingerprint of N_2O originating from archaeal denitrification.

N fractionation effects derived from $\delta^{15}N_{bulk}$ of N_2O

The $\Delta\delta^{15}N$ values of N_2O (difference of δ_p and δ_s, Eq. 4) we determined for fungal N_2O production from NO_2^- (-8.8 to -29.3 ‰) as well as from NO_3^- (-30.8 to -45.5 ‰) were in the range of data from two fungal strains reported in a previous study by Sutka *et al*. giving -6.6 to -74.7 ‰ with NO_2^- as electron acceptor (Sutka et al., 2008). Snider et al. (2009) summarized previous literature data of ^{15}N fractionation effects for N_2O produced during bacterial denitrification in a similar range between -10 and -39 ‰. Isotope fractionation effects of N observed in pure culture experiments seem to overlap for fungal and bacterial denitrification as well as for nitrification (Ostrom and Ostrom, 2011). This is clearly confirmed by our results from fungal pure cultures, which cover the wide range of N fractionation factors known from bacterial denitrification and nitrification (Barford et al., 1999; Ostrom and Ostrom, 2011; Perez et al., 2006; Sutka et al., 2008; Yoshida, 1988). Along with this, soil incubation under denitrifying conditions resulted in similar N fractionation factors from -10 to -54 ‰ (summarized by Snider et al., 2009; Well and Flessa, 2009). Hence, $\delta^{15}N_{bulk}$ values of the produced N_2O ($\delta^{15}N_{bulk}$-N_2O) alone cannot be used to differentiate between microbial sources or pathways of N_2O production (Ostrom and Ostrom, 2011), but a closer look at N isotope effects controlling $\delta^{15}N_{bulk}$-N_2O might improve our understanding of the predominant process steps influencing the isotopic fractionation during denitrification (Ostrom and Ostrom, 2011; Sutka et al., 2008).

Net isotope effects (η), the sum of isotopic fractionations associated with the individual reduction steps of denitrification, can be described by the equation of Farquhar et al. (1982), which was adapted to N_2O production during fungal denitrification by Ostrom and Ostrom (see Eq. 12 in Ostrom and Ostrom (2011)). To interpret $\delta^{15}N_{bulk}$-N_2O we assume that η_{15N} is represented by the $\Delta\delta^{15}N$ values (Eq. 4, Figure 1, Table 2). In line with Sutka et al. (2008), our results (e.g., for *F. oxysporum*) showed that larger reaction rates (= large N_2O production) were associated with smaller $\Delta\delta^{15}N$ values compared to samples with smaller N_2O production (Figure 1). At high reaction rates, the enzyme activity and diffusion out of the cell is relatively large compared to diffusion into the cell (Ostrom and Ostrom, 2011). In this case $\Delta\delta^{15}N$ is mainly controlled by the fractionation of diffusion, whereas higher fractionation factors and lower N_2O productions indicate a dependence on the enzymatic fractionation (Ostrom and Ostrom, 2011; Well and Flessa, 2008).

Also, our results show a significant relation between the visual structure of fungi and $\Delta\delta^{15}N$ for both electron acceptors (see Table 2). Here the most negative and lowest negative $\Delta\delta^{15}N$ was for the globose (g) forming and turbid (t) grown fungi, respectively, while

$\Delta\delta^{15}N$ was in between for the mixed growth type (gt). We therefore suspect that the different growth types might have caused differences in the apparent isotope effects, which are reflected by the bulk isotopic values observed in our incubation vessels. Apparent isotope effects can be substantially lower compared to actual net isotope effects (η) in active micro-sites or cells due to spatial heterogeneity of processes including dispersion of substrates, reduction processes in isolated micro-niches and heterogeneity of reaction rates (Well et al., 2012). But we have no obvious explanation why apparent isotope effects should be larger in globose structures since complete consumption or high enrichment in isolated micro niches would lead to the opposite of our observations and isotope effect of diffusion into the globose grown fungi should be negligible (Granger et al., 2008; Mariotti et al., 1988). Hence, the reason for the observed growth pattern effect needs further clarification to get a better understanding about fungal fractionation during N_2O production, although the observed differences in growth pattern will be irrelevant under natural conditions in soil.

$\delta^{18}O$ of N_2O

The $\delta^{18}O$-N_2O was far more variable ($\delta^{18}O$ = 11.83 to 55.3 ‰) compared to SP of N_2O (Table 2) and was affected by isotopic fractionation during subsequent reduction steps (similarly as described above for N fractionation) as well as by O exchange between H_2O and denitrification intermediates (Rohe et al., 2014). This makes interpretation of the $\delta^{18}O$-N_2O more complicated than that of $\delta^{15}N_{bulk}$-N_2O. Therefore we estimated the O isotopic fractionation and O exchange during several steps of denitrification using a conceptual model (Rohe et al., 2014) adapted from previous concepts for bacterial denitrification after Casciotti et al. (2007), implementing controls of O exchange proposed by Aerssens et al. (1986) and using fractionation models developed by Snider *et al*. (Figure 2) (Aerssens et al., 1986; Snider et al., 2010; Snider et al., 2012, 2013). The fractionation model is based on the assumption that every reduction step by NO_3^- reductase (Nar), NO_2^- reductase (Nir) and Nor during denitrification is characterized by specific branching effects, i.e., kinetic isotopic fractionation during enzymatic reactions (ε_1) and equilibrium effects due to O exchange between H_2O and denitrification intermediates (ε_2) (Casciotti et al., 2007). The fraction of O exchange during each reduction step is defined as f_{Nar}, f_{Nir} and f_{Nor}, respectively. Based on this we can calculate the $\delta^{18}O$ ($\delta^{18}O_f$) of the particular intermediate or final product (NO_2^-, NO or N_2O):

$$\delta^{18}O_f = (1 - f_{ex})\,(\delta^{18}O_i + \varepsilon_1) + f_{ex}\,(\varepsilon_2 + \delta^{18}O_{water}) \qquad \text{(Eq. 6)}$$

with f_{ex} describing the fraction of O exchange between denitrification intermediates and H_2O during the particular reduction step (f_{Nar}, f_{Nir} *or* f_{Nor}), $\delta^{18}O_i$ representing $\delta^{18}O$ of the initial precursors (NO_3^-, NO_2^- and NO), and $\delta^{18}O_{water}$ representing $\delta^{18}O$ value of the H_2O.

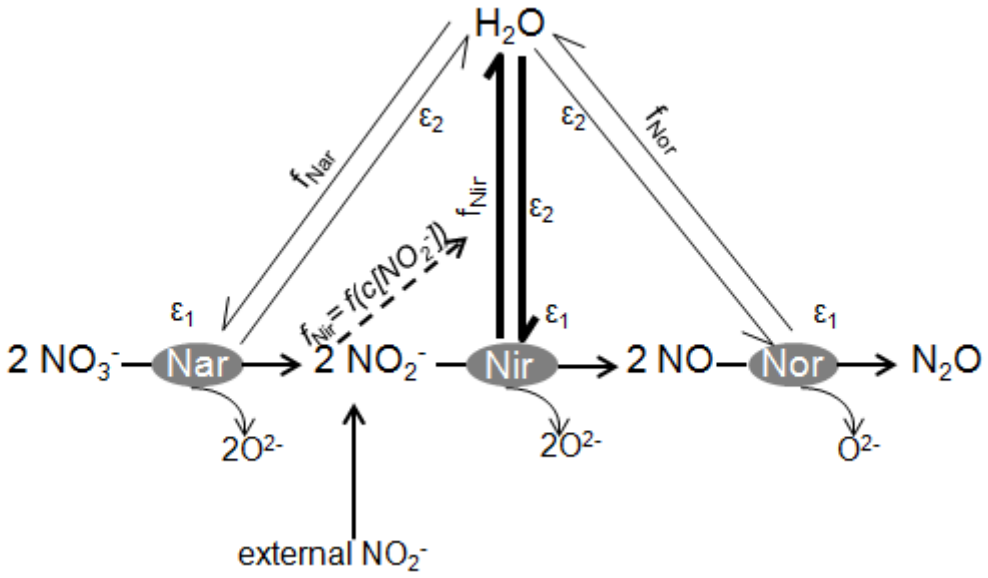

Figure 2: Conceptual model of fungal denitrification adopted from a model by Snider et al. (2013), Casciotti et al. (2007) and Aerssens et al. (1986), after Rohe et al. (2014), modified. The bold arrow shows the enzymatic step of the presumably main oxygen exchange, whereas thin arrows show the step with presumably less oxygen exchange.

To assess f_{Nar}, f_{Nir} and f_{Nor} with this model, the values for the extent of the total O exchange had to be known and were taken from Rohe et al. (2014). In that study, the O exchange between H_2O and denitrification intermediates was determined with ^{18}O-labeled H_2O for all six fungal species also examined in this study. Total O exchange between denitrification intermediates and water varied between 0.41 and full exchange with a standard deviation between 0.01 and 0.06, whereas with nitrate variation was between 0.11 and full exchange with a standard deviation between 0.02 and 0.34 (Rohe et al., 2014). We assume the same extent of O exchange for fungi in both this and the previous experiment. However, the experiment using ^{18}O-labeled H_2O provides only information about the total extent of O exchange, i.e., how many ^{18}O atoms were exchanged between enriched medium H_2O and the final product during the whole reaction sequence. Since this O exchange may take place due to the three following enzymatic reaction steps: Nar, Nir and Nor (Figure. 2), the total O exchange (t_{ex}) can be defined as follows:

$$t_{ex} = f_{Nor} + f_{Nir}(1 - f_{Nor}) + f_{Nar}(1 - f_{Nir})(1 - f_{Nor}) \qquad \text{(Eq.7)}$$

The time point when the O exchange occurs is crucial for the O isotopic signature of the final N_2O: If the majority of this O exchange appears in the initial reduction steps (i.e., Nar or Nir reduction steps) the branching effect occurring later will mainly determine the final $\delta^{18}O\text{-}N_2O$, resulting in higher values due to isotopic fractionation. Conversely, if O exchange occurs during the final reduction step by Nor, branching effects during preceding steps are partly or fully erased and the final $\delta^{18}O\text{-}N_2O$ signature will be governed by O exchange effects including the equilibrium effect and the $\delta^{18}O$ of H_2O. Therefore, we deciphered the mechanism of O exchange with H_2O by applying model calculations based on Eq. 6 (Table 3 for NO_2^- and Table 4 for NO_3^-) which were based on the measured $\delta^{18}O\text{-}N_2O$ values. The initial $\delta^{18}O$ values of O precursors were: -22.7 ‰ for NO_2^-; 22.0 ‰ for NO_3^-; -9.9 ‰ for H_2O in the medium of *C. lychenicola, F. solani* fsp. *pisi, F. decemcellulare* , and -8.9 ‰ for that of *C. funicola, F. oxysporum* and *T. hamatum*. We assumed the values for isotope effects according to Casciotti et al. (2007): the branching effect (ε_1) of Nir and Nor each with 30 ‰ and the equilibrium effect (ε_2) of Nir and Nor with 14 ‰. To assess f_{Nir} and f_{Nor} when NO_2^- was added as electron acceptor, we calculated three scenarios (Table 3): I. we supposed O exchange only takes place at Nor (f_{Nir} = 0); or II. only at Nir (f_{Nor} = 0), or III. we fitted measured and modeled $\delta^{18}O\text{-}N_2O$ values by adjusting O exchange during Nir and Nor steps.

Table 3: Results of modeling oxygen exchange with water during nitrite reduction to N_2O using Eq. 6. Three scenarios were calculated to assess the fractions of oxygen exchange at Nir (f_{Nir}) and oxygen exchange at Nor (f_{Nor}): I. we supposed oxygen exchange only takes place at Nor (f_{Nir} = 0), or II. only at Nir (f_{Nor} = 0) or III. measured and modeled $\delta^{18}O$-N_2O values were fitted by adjusting f_{Nir} and f_{Nor}. Calculated $\delta^{18}O$ values of NO and N_2O are shown and the goodness of fit is given as difference of measured and calculated $\delta^{18}O$-N_2O (Difference meas.- calc. $\delta^{18}O$-N_2O). Calculations are based on measured $\delta^{18}O$ values of NO and N_2O precursors (22.7 ‰ for NO_2^-; -9.9 ‰ for medium H_2O of *C. lychenicola, F. solani* fsp. *pisi*, *F. decemcellulare,* and -8.9 ‰ for medium H_2O of *C. funicola, F. oxysporum* and *T. hamatum*) and on isotope effects according to Casciotti et al. (2007) (branching effect ε_1 of Nir and Nor each with 30 ‰ and equilibrium effect ε_2 of Nir and Nor with 14 ‰). Moreover, f_{Nir} and f_{Nor} assumed in the model scenarios as shown were set to fulfill Eq. 7 using measured values of total oxygen exchange (mean values with standards in brackets) (Rohe et al., 2014).

		Scenario I, f_{Nir} = 0				**Scenario II, f_{Nor} = 0**				**Scenario III[b]**				
fungal species	Measured total oxygen exchange[a]	Calc. $\delta^{18}O$-NO [‰]	f_{Nor}	Calc. $\delta^{18}O$-N_2O [‰]	Difference meas.- calc. $\delta^{18}O$-N_2O [‰]	f_{Nir}	Calc. $\delta^{18}O$-NO [‰]	Calc. $\delta^{18}O$-N_2O [‰]	Difference meas.- calc. $\delta^{18}O$-N_2O [‰]	f_{Nir}	Calc. $\delta^{18}O$-NO [‰]	f_{Nor}	Calc. $\delta^{18}O$-N_2O [‰]	Difference meas.- calc. $\delta^{18}O$-N_2O [‰]
Cy. lichenicola	0.74 (0.04)	7.3	0.74	12.7	24.9	0.74	4.9	34.9	2.7	0.74	4.9	0.00	34.9	2.7
F. solani fsp. *pisi*	0.89 (0.01)	7.3	0.89	7.8	30.6	0.89	4.5	34.5	3.9	0.89	4.5	0.00	34.5	3.9
F. decemcellulare	0.84 (0.06)	7.3	0.84	9.3	26.8	0.84	4.6	34.6	1.5	0.84	4.6	0.00	34.6	1.5
C. funicola	0.41 (0.02)	7.3	0.41	24.1	-12.3	0.41	6.4	36.4	-24.6	0.00	-12.7	0.41	12.3	-0.5
F. oxysporum	1.00 (0.02)	7.3	1.00	5.1	28.3	1.00	5.1	35.1	-1.8	1.00	5.1	0.00	35.1	-1.8
T. hamatum	1.00 (0.03)	7.3	1.00	5.1	31.8	1.00	5.1	35.1	1.8	1.00	5.1	0.00	35.1	1.8

[a]Data of total O exchange during denitrification (NO_2^- to N_2O reduction) were taken from an ^{18}O tracer experiment by Rohe et al. (2014), where the same fungal species were used for incubation studies.

[b]Scenario III: Branching effect (ε_1) of Nir was adjusted to 10 ‰ for calculation of *C. funicola.*

Our results for treatments with NO_2^- showed that $\delta^{18}O\text{-}N_2O$ cannot be explained by branching effects during enzymatic steps of N_2O production alone and further confirmed the impact of O exchange. The results of our three scenarios (Table 3) suggest that O exchange occurred mostly at Nir. In Scenario I (without O exchange during NO_2^- reduction) the calculated ^{18}O values differed largely from measured values, showing that $\delta^{18}O\text{-}N_2O$ cannot be dominated by O exchange during NO reduction. In contrast to this, in Scenario II, with the assumption that the O exchange only takes place during NO_2^- reduction, the difference between calculated and measured $\delta^{18}O\text{-}N_2O$ was much smaller. In Scenario III (allowing O exchange during NO_2^- and NO reduction) all five fungi of *Hypocreales* gave the best fit with O exchange only at Nir. The fungus *C. funicola* (only *Sordariales*) yielded a better fit with Scenario I than Scenario II, indicating some O exchange at the Nor step. However, to obtain a good fit with Scenario III, it must be assumed that the branching effect of Nir is smaller (ε_1 = 10 ‰) than assumed for the *Hypocreales* species (30 ‰). Thus, the isotopic fractionation of the biological order *Sordariales* needs to be verified by future studies. So far, the existence of P450nor in the fungal denitrifying systems has to our knowledge only been studied and detected in species of *Hypocreales* (Shoun et al., 1992). In further studies, the P450nor occurrence in fungal denitrifying systems from various orders, including the *Sordariales*, should be analyzed.

The fractionation mechanism for NO_3^- treatments is even more complex because we deal with a possible additional O exchange at Nar (Figure 2). For model simplification, we assumed no branching effect during the nitrate-to-nitrite reduction step, since this branching isotope effect due to the intra-molecular $^{18}O/^{16}O$ fractionation (positive ε) is compensated by the intermolecular isotope effect resulting in preferential reduction of ^{18}O-depleted NO_3^- (negative ε) (Casciotti and McIlvin, 2007; Snider et al., 2013). Similarly as for NO_2^- treatments, we analyzed three scenarios assuming that O exchange at Nor = 0 as shown by assessment of data from the NO_2^- treatments in *Hypocreales* species: I. we supposed O exchange only takes place at Nir (f_{Nar} = 0 and f_{Nor} = 0); II. O exchange occurs only at Nar (f_{Nir} = 0 and f_{Nor} = 0), and III. we fitted measured and modeled $\delta^{18}O\text{-}N_2O$ values by adjusting O exchange during Nar and Nir steps (f_{Nor} = 0) and also by adjusting the magnitude of the branching effect. Our results for treatments with NO_3^- showed that the isotopic signature of ^{18}O in N_2O is as well as for NO_2^- treatments affected by O exchange. Results of our three scenarios (Table 4) indicate that O exchange might mostly occur by Nir but possibly with different branching effects (ε_1) than postulated by Casciotti et al. (2007). In Scenario I (without O exchange during NO_3^- reduction or NO reduction) the calculated $\delta^{18}O$ values are

mostly higher than the measured values for all fungi with the exception of *F. solani* fsp. *pisi*. In Scenario II, with the assumption that the O exchange only takes place at Nar, the difference between calculated and measured $\delta^{18}O$ values is even higher than in Scenario I. From these first two scenarios it is particularly noticeable that *F. solani* fsp. *pisi* shows a very distinct O fractionation mechanism than the other fungi. Moreover, during the previous experiment determining total O exchange (Rohe et al., 2014) this was also the only fungus showing higher O exchange with NO_3^- than with NO_2^-, which suggests that the additional O exchange must have occurred at Nar. Hence, in Scenario III for this fungus we accepted the total O exchange at both enzymatic steps Nar and Nir (according to the Eq. 7) and adjusted the magnitude of the f_{Nir} to get the best agreement between measured and calculated values. For the other four fungi of *Hypocreales* in Scenario III, Nir was the enzyme assumed to be solely responsible for O exchange, since the calculated values in Scenario I were closer to the actually measured values. This is in accordance with the previous experiment determining total O exchange,(Rohe et al., 2014) where for those fungi lower O exchange was found for NO_3^- treatments suggesting no additional O exchange at Nar. However, the values calculated in Scenario I are still very different from the actually measured values. This discrepancy can only be reduced by lowering the assumed isotopic fractionation associated with the branching effect. This was done in the Scenario III, and the best agreement between measured and calculated values was obtained for a branching effect of 19 ‰ (Table 4). Apparently, $\delta^{18}O$-N_2O values of the NO_3^- treatments were dependent on the branching effects, which presumably are different to NO_2^- reduction. Lower branching effects during NO_3^- reduction could be caused by lower reaction rates compared to NO_2^- reduction since the branching is a net isotope effect depending on the balance between diffusive and enzymatic effects (see discussion of $\delta^{15}N_{bulk}$ above).

For NO_3^-, calculation with Eq. 6 did not have the same precision as compared to NO_2^- treatments. This is because O exchange determined in our previous experiment (Rohe et al., 2014), and $\delta^{18}O$-N_2O measured in the present study, were far more variable in NO_3^- treatments. However, varying the total assumed O exchange did not affect the indication of the enzyme mostly contributing to O exchange.

Table 4: Results of modeling oxygen exchange with water during nitrate reduction to N_2O using Eq. 6. Three scenarios were calculated to assess the fractions of oxygen exchange at Nar (f_{Nar}), Nir (f_{Nir}) and oxygen exchange at Nor (f_{Nor}): I. we supposed oxygen exchange only takes place at Nir (f_{Nar} = 0 and f_{Nor} = 0), or II. only at Nar (f_{Nir} = 0 and f_{Nor} = 0) or III. measured and modeled $\delta^{18}O$-N_2O values were fitted by adjusting f_{Nar}, f_{Nir} and f_{Nor}. Calculated $\delta^{18}O$ values of NO_2^- and N_2O are shown and the goodness of fit is given as difference of measured and calculated $\delta^{18}O$-N_2O (Difference meas.- calc. $\delta^{18}O$-N_2O). Calculations are based on measured $\delta^{18}O$ values of NO and N_2O precursors (22.0 ‰ for NO_3^-; -9.9 ‰ for medium H_2O of *C. lychenicola, F. solani* fsp. *pisi, F. decemcellulare,* and -8.9 ‰ for medium H_2O of *C. funicola, F. oxysporum* and *T. hamatum*) and on isotope effects according to Casciotti et al. (2007) (branching effect ε_1 of Nar with 0 ‰, Nir and Nor each with 30 ‰ and equilibrium effect ε_2 of Nar, Nir and Nor with 14 ‰). Moreover, f_{Nar}, f_{Nir} and f_{Nor} assumed in the model scenarios as shown were set to fulfill Eq. 7 using measured values of total oxygen exchange (mean values with standards in brackets) (Rohe et al., 2014).

		Scenario I, f_{Nar} = 0, f_{Nor} = 0				**Scenario II, f_{Nir} = 0, f_{Nor} = 0**				**Scenario III[2]**				
fungal species	Measured total oxygen exchange[1]	Calc. $\delta^{18}O$-NO_2^- [‰]	f_{Nir}	Calc. $\delta^{18}O$-N_2O [‰]	Difference meas.- calc. $\delta^{18}O$-N_2O [‰]	f_{Nar}	Calc. $\delta^{18}O$-NO_2^- [‰]	Calc. $\delta^{18}O$-N_2O [‰]	Difference meas.- calc. $\delta^{18}O$-N_2O [‰]	f_{Nar}	Calc. $\delta^{18}O$-NO_2^- [‰]	f_{Nir}	Calc. $\delta^{18}O$-N_2O [‰]	Difference meas.- calc. $\delta^{18}O$-N_2O [‰]
Cy. lichenicola	0.47 (0.04)	22.0	0.47	59.5	-23.8	0.47	13.6	73.6	-37.9	0.00	22.0	0.47	42.6	-7.0
F. solani fsp. *pisi*	1.00 (0.03)	22.0	1.00	34.1	14.1	1.00	4.1	64.1	-15.9	0.98	4.5	0.50	49.3	-1.0
F. decemcellulare	0.61 (0.34)	22.0	0.61	52.9	-9.5	0.61	11.1	71.1	-27.7	0.00	22.0	0.61	37.6	5.9
F. oxysporum	0.11 (0.02)	22.0	0.11	76.7	-21.4	0.11	20.1	80.1	-24.8	0.00	22.0	0.11	56.4	-1.1
T. hamatum	0.77 (0.30)	22.0	0.77	45.8	-10.5	0.77	8.9	68.9	-33.7	0.00	22.0	0.77	32.3	3.0

[1]Data of total O exchange during denitrification (NO_2^- to N_2O reduction) were taken from an ^{18}O tracer experiment by Rohe et al. (2014), where the same fungal species were used for incubation studies.

[2]Scenario III: Branching effects (ε_1) of Nir and Nor were adjusted to 19 ‰ for calculation for *Cy. lichenicola, F. decemcellare, F. oxysporum and T. hamatum;* for *F. solani* fsp. *pisi* no change was made (30 ‰ as in scenario I and II was left).

Conclusions

We extended the database on the SP fingerprint of N_2O from pure cultures of fungal denitrifiers from previously two, to now six, fungal species. We confirmed that N_2O produced by fungi of the order *Hypocreales* exhibits high and relatively constant SP of N_2O irrespective of the N source (NO_3^- or NO_2^-) or species, whereas *C. funicola* of the order *Sordariales* showed SP N_2O between the bacterial and *Hypcreales* SP clusters. The fungus *C. funicola* was clearly different from the five *Hypocreales* species regarding the N_2O production, isotopomers and O exchange, which we determined in a previous experiment (Rohe et al., 2014). To our knowledge information about differences in physiology (incl. enzymes involved in denitrification) between both fungal orders is lacking. Until now P450nor was identified only in *Hypocreales* species. Our results from *Hypocreales* species confirm the possibility to use SP of N_2O to differentiate between N_2O from denitrification by bacterial and by certain fungal groups. However, this cannot be extended to fungal denitrification in general without knowing the significance of fungal orders other than *Hypocreales* to soil N_2O fluxes.

The control of $\delta^{15}N_{bulk}$ of the fungal pure cultures is similar to bacteria and whole soil communities with net N isotope effects increasing with N_2O production rates. This further confirms that $\delta^{15}N_{bulk}$ is a poor estimator of microbial pathways.

The control of $\delta^{18}O\text{-}N_2O$ of fungal N_2O largely depends on O exchange between H_2O and denitrification intermediates, which is similar to bacteria. Our data provide first evidence on the share of individual reduction steps where O exchange occurs in fungal pure cultures, suggesting that O exchange is predominantly associated with Nir for *Hypocreales* and with Nor for *Sordariales*. These findings help to better understand variations in $\delta^{18}O\text{-}N_2O$ of soil emitted N_2O and thus improve estimation of N_2O process dynamics based on N_2O isotopologues.

We confirm SP as a promising approach to differentiate between N_2O produced by fungal and bacterial denitrification, but also identified the need to check the relevance of denitrifying fungi with lower SP in future studies as well as SP of fungal N_2O from intact microbial soil communities.

Acknowledgements

Susanne Behn is thanked for assistance with microbial work and Martina Heuer for isotopic analysis; the Max-Planck Institute for Terrestrial Microbiology in Marburg (Germany) for providing the fungal strains and the possibility to work in their lab and Roland Fuß for help with statistical analysis. We would like to thank Dina Führmann for editing the English.

This joint research project was financially supported by the State of Lower-Saxony and the Volkswagen Foundation, Hanover, Germany.

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Comparing modified substrate induced respiration with selective inhibition (SIRIN) and N_2O isotope methods to estimate N_2O production of fungal denitrification in three arable soils

This chapter is currently prepared to be submitted.
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Abstract

RATIONALE: Pure culture studies gave evidence on the ability of soil fungi to produce N_2O during denitrification. Soil studies with selective inhibition indicated a dominance of fungal compared to bacterial N_2O production in soil, which recently drew more attention to fungal denitrification. Analyzing the isotopic composition of N_2O, especially the ^{15}N site preference (SP) of N_2O, showed that N_2O from pure bacterial or fungal cultures differed in SP values, which might enable it to quantify fungal N_2O based on the isotopic endmember signatures of N_2O produced by fungi and bacteria.

METHODS: Three different soils were incubated under denitrifying conditions using a modification of the substrate induced respiration with selective inhibition (SIRIN) to analyze N_2O evolved from selective organism groups. N_2O reduction was quantified by ^{15}N tracer application. The effect of N_2O reduction on N_2O production and the isotopic signature of N_2O were determined by parallel varieties with and without acetylene to block the N_2O reduction. RESULTS: In this study a low contribution of up to 22% of fungal denitrification was observed with an isotope endmember mixing approach (IEM). Quantifying the fungal fraction with modified SIRIN was only possible in one soil. This soil showed a fungal fraction of about 28%, which was similar to the results obtained by IEM.

DISCUSSION: This study was the first attempt to quantify the fungal contribution on N_2O production during denitrification by simultaneous application of two approaches, i.e. selective inhibition of microbial growth and SP of N_2O. There were indications that fungi played only a minor role in N_2O production from soils. Nevertheless, reliable methods, which are validated to quantify fungal contribution on N_2O production from soil are still lacking.

Keywords: selective growth inhibition, ^{15}N site preference, fungal denitrification, C_2H_2

Introduction

The greenhouse gas nitrous oxide (N_2O) contributes to global warming and to the depletion of the ozone layer in the stratosphere (IPCC, 2013). The highest anthropogenic N_2O emissions originate from agricultural soils and are mainly produced during microbial nitrification, nitrifier dentirification and denitrification (Bremner, 1997; Firestone and Davidson, 1989; IPCC, 2013; Wrage et al., 2005). In order to find mitigation strategies for N_2O emissions from arable soils, it is important to understand N_2O sources and sinks and thus knowledge about the production pathways and the microorganisms involved has to be improved. For a long time, it was believed that solely bacteria are involved in N_2O formation during denitrification (Firestone and Davidson, 1989); however, also several fungi are capable of denitrification (Bollag and Tung, 1972; Shoun et al., 1992). Denitrification describes the reduction of nitrate (NO_3^-) to dinitrogen (N_2), with the intermediates nitrite (NO_2^-), nitric oxide (NO) and N_2O (Knowles, 1982). While this entire reaction chain including the ability to reduce N_2O to N_2 is found among bacterial denitrifiers, most fungi lack N_2O reductase (Nos) (Shoun et al., 1992). To which part different microbial groups contribute to N_2O emissions from soil is not yet sufficiently investigated. In general fungi dominate the biomass in soil (up to 96%) compared to bacteria and thus fungi could potentially play a dominant role in N_2O production (Braker and Conrad, 2011; Ruzicka et al., 2000). Furthermore, a respiratory fungal-to-bacterial (F:B) ratio of 4 is typical for arable soils (Anderson and Domsch, 1975; Blagodatskaya and Anderson, 1998). The high fungal abundance in soil and the fact that N_2O is the major end product of fungal denitrification lead to the assumption that the potential activity of fungal N_2O production in soil may exceed the bacterial production, provided that both microbial groups have the same specific N_2O production (Shoun et al., 1992; Sutka et al., 2008). However, until now reliable methods for distinguishing between fungal and bacterial N_2O emissions from an indigenous soil community are lacking.

Soil incubation experiments could serve to differentiate between N_2O produced by fungi and bacteria during denitrification by the application of two antibiotics: streptomycin and cycloheximide, which inhibit bacterial or fungal growth, respectively, by inhibition of the protein biosynthesis and this method is known as substrate induced respiration with selective inhibition (SIRIN) (Anderson and Domsch, 1975; Blagodatskaya et al., 2010; Crenshaw et al., 2008; Laughlin and Stevens, 2002; Long et al., 2013). A few studies used a modification of this method for N_2O analysis and found a greater decrease of N_2O production with fungal than with bacterial growth inhibition (e.g. 89 vs. 23% decrease (Laughlin and Stevens, 2002)), indicating that fungi might dominate N_2O production (Blagodatskaya et al., 2010; Crenshaw

et al., 2008; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). Additionally to denitrification, Tanimoto et al. (1992) found some fungi capable of codenitrification, where one N atom from NO_2^- is combined with an N atom from compounds like azide or ammonium (NH_4^+) for N_2O production (Garber and Hollocher, 1982; Shoun et al., 1992; Spott et al., 2011; Tanimoto et al., 1992). Incubation experiments with a grassland soil under anaerobic conditions using stable isotope tracing to differentiate between sources of N_2O indicated a contribution of codenitrification of about 92% of N_2O produced (Laughlin and Stevens, 2002). This again stresses the large potential N_2O production by fungi.

Analyzing the isotopic composition of N_2O might be a promising tool to distinguish between N_2O from bacterial and fungal denitrification. Especially, the isotopomer ratios of N_2O in pure culture studies showed differences in N_2O from bacterial and fungal denitrification (Frame and Casciotti, 2010; Rohe et al., 2014a; Sutka et al., 2008; Sutka et al., 2006) and might be suitable to distinguish between N_2O produced by bacteria or fungi under denitrifying conditions. Isotopomer ratios of N_2O can be expressed as ^{15}N site preference (SP), i.e. the difference between $\delta^{15}N$ of the central and terminal N-position of the asymmetric N_2O molecule (Toyoda and Yoshida, 1999). The SP of N_2O from six pure fungal cultures was between 20 and 37 ‰ (Rohe et al., 2014a; Sutka et al., 2008), whereas several bacteria produced N_2O with a SP between -11 and 0 ‰ during denitrification (Frame and Casciotti, 2010; Sutka et al., 2006). However, SP of N_2O produced by pure bacterial cultures during nitrification is approximately 33 ‰ and interferes with SP of N_2O from fungal denitrification (Rohe et al., 2014a; Sutka et al., 2008; Sutka et al., 2006). This demonstrates the difficulty to use SP of N_2O as an indicator for different organism groups contributing to N_2O production from soil, where different pathways may co-occur. Although SP values of N_2O are independent of isotopic signatures of the precursors, $\delta^{15}N$ and $\delta^{18}O$ values of produced N_2O result from the isotopic signature of the precursor and isotopic fractionation during the N_2O production (Frame and Casciotti, 2010; Toyoda et al., 2005). Interpretation of $\delta^{18}O$ of N_2O is even more complex, because O exchange during denitrification between water and denitrification intermediates alters the final $\delta^{18}O$ value (Aerssens et al., 1986; Garber and Hollocher, 1982; Kool et al., 2007; Rohe et al., 2014b). Moreover, $\delta^{15}N$, $\delta^{18}O$ and SP values of N_2O produced in the course of denitrification are affected by isotopic fractionation due to N_2O reduction. During the N_2O reduction step, the $^{14}N^{16}O$ bond is preferentially broken compared to $^{14}N^{18}O$ or $^{15}N^{16}O$, resulting in residual N_2O, which is relatively isotopically enriched in ^{15}N and ^{18}O and shows higher SP values of N_2O compared to SP of N_2O from denitrification without the reduction step (Ostrom et al., 2007; Popp et al., 2002).

Consequently, the N_2O reduction inhibition method with acetylene (C_2H_2) (Groffman et al., 2006; Yoshinari and Knowles, 1976) should result in lower SP values of N_2O produced in laboratory studies (Lewicka-Szczebak et al., 2014). Quantification of N_2O reduction to N_2 during denitrification is possible by analysing $^{15}N_2$ fluxes in ^{15}N tracing experiments (Lewicka-Szczebak et al., 2014; Well et al., 2006).

Based on the above cited ranges for the isotopomer endmembers of fungal and bacterial denitrification, and assuming that only fungi and bacteria are responsible for N_2O production the fraction of fungal N_2O can be calculated from SP of N_2O produced in soil (SP_{prod}), provided there is no N_2O reduction occurring, which is altering SP of emitted N_2O (Ostrom and Ostrom, 2011; Ostrom et al., 2010). Inhibiting N_2O reduction with C_2H_2 during anaerobic incubation of soils is a means to determine SP_{prod} (Lewicka-Szczebak et al., 2014; Well and Flessa, 2009). Hence C_2H_2 inhibition might be suitable to quantify SP_{prod} in soils exhibiting significant N_2O reduction without inhibiting the N_2O reduction and thus allow quantification of fungal N_2O fluxes based on SP_{prod}.

The study aims at determining the fungal contribution on N_2O production from denitrification in three arable soils. The second aim is to compare the fungal contribution on N_2O production determined from a modified SIRIN and the isotope endmember mixing approach and thus assess factors of potential bias of both methods. The third aim is to determine SP of N_2O fluxes from fungal soil communities and thus to evaluate the transferability of the pure culture range of the fungal SP endmember.

Materials and Methods

Soil samples

As experiments were conducted with three soils, but one soil from two different sampling times, we named the experiments: Soil sampling of the loamy sand was done in December 2012 (Experiment 1), of the sand in January 2013 (Experiment 2), of the silt loam in December 2012 (Experiment 3), and of loamy sand in June 2011 (Experiment 4). For further characteristics of the soils, see Table 1. Soil samples of the upper 30 cm were collected in plastic bags with cotton wool stoppers and stored at 6 °C for maximally two months. To get information about the initial soil status, total contents of C and N in soil samples were analyzed by dry combustion of grinded samples (LECO TruSpec, Germany). The soil pH was measured in 0.01 M $CaCl_2$. The mineral nitrogen content (Nmin) of soil samples was determined before and after fertilization by extracting NO_3^- and NH_4^+ with 0.01 M calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$) according to ISO 14255 and analyzing NO_3^- and NH_4^+

concentrations in the extracts with a Continuous-Flow-Analyzer (SKALAR, Germany). The $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^- and NO_2^- in soil extracts (with 0.01 M calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$)) were analyzed by the bacterial denitrifier method (Casciotti et al., 2002). Respiratory biomass of the three soils was analyzed with substrate induced respiration (SIR) according to Anderson and Domsch (1978) and the respiratory F:B ratio was analyzed with substrate induced respiration with selective inhibition (SIRIN) in summer 2010 by a computer-generated selectivity analysis: "SIR-SBA 4.00" (Heinemeyer, copyright MasCo Analytik, Hildesheim, Germany) (SIRIN; Anderson and Domsch, 1975). The scheme of glucose and growth inhibitor combinations is listed below in section "Methodological approach".

Methodological approach

SIRIN pre-experiment:

To determine the fungal impact on N_2O emissions from soil we conducted a pre-experiment, in order to get information about optimal substrate and inhibitor concentrations for substrate induced growth inhibition. The method of substrate induced respiration (SIR) (Anderson and Domsch, 1978) was used to get information about the amount of respiratory biomass in soil. To this end, we added different concentrations of glucose (0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 mg g^{-1} dry weight (dw) soil) to find the optimal glucose concentration (c_{opt}(glucose)), which is the glucose concentration that causes maximum initial respiration rates (Anderson and Domsch, 1978). C_{opt}(glucose)) was 1.0 mg g^{-1} for Experiment 2 (sand) and 1.5 mg g^{-1} for Experiments 1, 3 and 4 (loamy sand and silt loam). Glucose served as substrate to initiate microbial growth. Then, for determining the respiratory F:B ratios the selective inhibition method (SIRIN) of Anderson and Domsch (1975) was applied.

We conducted SIRIN according to Anderson and Domsch (1975). Selectivity of the inhibitor combinations of streptomycin (bacterial respiratory inhibitor) and cycloheximide (fungal respiratory inhibitor) were tested with the following concentrations, 0.75, 1.0, 1.5 mg gdw^{-1}, respectively. The optimal concentration of inhibition of fungal respiratory was 0.75 mg gdw^{-1} soil cycloheximide (c_{opt}(cycloheximide)) and for bacterial respiratory inhibition 1.0 mg gdw^{-1} soil streptomycin (c_{opt}(streptomycin)).

Table 1: Soil characteristics of three arable soils from Germany used for incubation experiments (standard deviation in brackets).

Soil texture	Location	C content [%]	N content [%]	NH_4^+-N [mg L^{-1}]	NO_3^--N [mg L^{-1}]	pH ($CaCl_2$)	$\delta^{15}N$-NO_3^- [‰]	$\delta^{18}O$-NO_3^- [‰]	Resp. F:B ratio	Resp. Biomass [µg C gdw^{-1} soil]
Loamy sand	Braunschweig[a]	1.43 (<0.01)	0.10 (<0.01)	0.04	1.25	5.67	3.98	-4.82	2.6	234
Sand	Wennebostel[b]	2.31 (0.04)	0.14 (<0.01)	0.02	0.56	5.54	0.73	-2.68	2.6	161
Silt loam	Göttingen[c]	1.62 (0.02)	0.13 (<0.01)	n.d.[d]	2.05	7.38	4.18	2.32	4.9	389

[a]Thünen Institute, Braunschweig, Germany

[b]private agricultural field North of Hannover, water protection area Fuhrberger Feld, Germany

[c]Reinshof Experimental Farm, Georg-August-University, Göttingen, Germany

[d]not detectable (i.e. below detection limit of 0.005 mg L^{-1} NH_4^+-N)

Soil incubation with selective inhibition

The experimental design included two factors, (i.) microbial inhibition by fungal and/or bacterial inhibitors and (ii.) activity of N_2O reductase analyzed either by inhibition with C_2H_2 or quantification by ^{15}N tracing. To address factor (i.), the SIRIN method for determination of the respiratory F:B ratio based on CO_2 emission was modified to determine N_2O production by microbial groups. However, in contrast to previous studies by Laughlin and Stevens (2002), McLain and Martens (2006), Blagodatskaya et al. (2010) and Long et al. (2013) we did not pre-incubate the soil with the growth inhibitors, as this could result in changes of the microbial community (e.g. preferential growth of selected organisms). We intended to disturb microbial communities as little as possible.

The soil was sieved (2 mm) and pre-incubated at 22 °C for five to seven days in the dark with cotton wool stoppers to allow respiration and aerobic conditions in soil bags. Four microbial inhibitor treatments (each in triplicate) with c_{opt}(glucose) for each soil were established:

A Control, without growth inhibitors

B With streptomycin sulfate ($C_{42}H_{84}N_{14}O_{36}S_3$) to inhibit bacterial growth

C With cycloheximide ($C_{15}H_{23}NO_4$) to inhibit fungal growth

D With streptomycin and cycloheximide, to inhibit bacterial and fungal growth

The soil was moistened with distilled water to 80% water filled pore space (WFPS) and simultaneously fertilized with NO_3^- (varieties *natural* and C_2H_2 with 50 mg N kg^{-1} KNO_3 in Experiment 1, 2 and 3 and with 60 mg N kg^{-1} $NaNO_3$ in Experiment 4 and *traced* variety with 50 mg N kg^{-1} ^{15}N-KNO_3 in Experiment 1, 2 and 3 and 60 mg N kg^{-1} ^{15}N-KNO_3 in Experiment 4 with a ^{15}N-labeling of 50 atom% (at%)). For each treatment we incubated 100 g dw soil in 850 mL preserving jars (J. WECK GmbH u. Co KG, Wehr, Germany) with gas inlet and outlet equipped with three port luer lock stopcocks. A mixture of c_{opt}(glucose) and talcum (5 mg talcum gdw^{-1}) according to Anderson and Domsch (1978) was added to soil of treatment A and together with the growth inhibitors to the soil of treatments B, C and D. All treatments were mixed for 90 seconds with a handheld electric mixer. The soil density was adjusted to a target soil density of 1.6 g cm^{-3} in Experiment 1, 2 and 4 and of 1.3 g cm^{-3} in Experiment 3. To ultimately achieve denitrifying conditions and avoid catalytic NO decomposition in the C_2H_2 variety (Nadeem et al., 2013), the headspace of the closed jars was flushed with N_2. The manual sample collection of 14 mL gas in duplicates with a plastic syringe was performed after two, four and eight (Experiment 4) or six, eight and ten hours (Experiment 1, 2 and 3) of incubation time, respectively. The removed gas was replaced by the same amount of N_2.

To address factor (ii.), all microbial inhibitor treatments were conducted in three N_2O reductase varieties, i.e.: with ^{15}N-NO_3 fertilizer (variety "*traced*") to quantify N_2O reduction to N_2, with natural abundance NO_3^- and 10 kPa C_2H_2 in the headspace (variety "C_2H_2") to block N_2O reductase, and with natural abundance NO_3^- but without blocking N_2O reductase, i.e. no C_2H_2 added (variety "*natural*"). In total, we had three soils, three varieties with four treatments each and conducted every treatment with three replicates.

Gas analysis

Gas samples were analyzed for N_2O and CO_2 concentrations ($c(N_2O)$ and $c(CO_2)$) with a gas chromatograph (GC, Agilent 7890A, Agilent, Böblingen, Germany). The detection limit of N_2O was 0.04 ng N h^{-1} with a measurement precision of 1% and for CO_2 the detection limit was 4 ng C h^{-1} with a measurement precision of 0.5%. As a control, N_2 and O_2 concentrations in the samples were analyzed with GC to ensure anaerobic conditions during the incubation for N_2O production from denitrification.

The N_2O isotopologues of the gas samples of varieties *natural* and C_2H_2 were analyzed on a pre-concentrator (PreCon, Thermo–Finnigan, Bremen, Germany) interfaced with a GC (Trace Gas Ultra, Thermo Scientific, Bremen, Germany) and a Delta V isotope ratio mass spectrometer (IRMS, Thermo Fisher Scientific, Bremen, Germany) (Brand, 1995; Köster et al., 2013; Toyoda and Yoshida, 1999). The analytical precision was 0.1 ‰, 0.2 ‰ and 1.5 ‰ for $\delta^{15}N_{bulk}$, $\delta^{18}O$ and SP of N_2O, respectively.

The N_2O samples of variety *traced* were analyzed for the 29/28 and 30/28 ratios of N_2 according to Lewicka-Szczebak et al. (2013) using a modified GasBench II preparation system coupled to a MAT 253 isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany).

Inhibitor effects

For interpretation of N_2O or CO_2 production, the validity of the experimental results with respect to fungal and bacterial N_2O fluxes was checked using a flux balance comparing the sum of bacterial and fungal inhibition effects (treatments B and C) to the dual inhibition effect (treatment D):

$$D = A - [(A - B) + (A - C)] \quad \text{(Eq. 1)}$$

With *A*, *B*, *C* and *D* representing the N_2O production rates of the last sampling time of treatment *A*, *B*, *C* and *D*, respectively. Assuming that in the other three treatments (A, B and

C) non-inhibitable N_2O production was equal to treatment D, N_2O produced by bacteria or fungi may show the following relation between the four treatments:

$$(A - D) = (B - D) + (C - D) \quad \text{(Eq. 2)}$$

The fungal fraction contributing on N_2O production during denitrification with microbial inhibition (F_{FDmi}) can be calculated, when N_2O production of treatment D is significantly lower than N_2O production of treatments A, B and C by:

$$F_{FDmi} = \frac{(A-C)}{(A-D)} \quad \text{(Eq. 3)}$$

Isotopomer endmember mixing approach (IEM)

The fungal fraction (F_{FD}) contributing to N_2O production from denitrification in soil samples was calculated according to the isotope mixing model proposed by Ostrom et al. (2010), that was established for calculating the bacterial fraction (F_{BD}) on N_2O production. Assuming that bacteria (*OS*) and fungi (*FD*) are the only microorganisms responsible for denitrification in soil, the SP of produced N_2O (SP_{prod}) results from:

$$SP_{Prod} = F_{FD} * SP_{FD} + SP_{OS} * F_{OS} \quad \text{(Eq. 4)}$$

where F_{FD} and F_{OS} represent the fraction of N_2O produced by fungi and other N_2O sources than fungal denitrification, respectively, and SP_{FD} and SP_{OS} are the respective SP endmember values of N_2O (Ostrom and Ostrom, 2011; Ostrom et al., 2010). This calculation was based on the assumption that the sum of F_{OS} and F_{FD} equals 1 and that N_2O reduction to N_2 is negligible. Mean SP_{FD} was assumed to be 35 ‰ (Rohe et al., 2014a; Sutka et al., 2008) and SP_{OS} was assumed to be -11 and 0 ‰ (Frame and Casciotti, 2010; Sutka et al., 2006). For this IEM approach, only results from variety C_2H_2 could be used, as microorganisms of this variety produce N_2O that is not affected by reduction to N_2.

The opportunity to calculate the fungal fraction (*calc.* F_{FD}) contributing to N_2O production during denitrification was to put the measured SP of N_2O from treatment A of variety C_2H_2 as SP_{Prod} into Eq. 4.

N_2O product ratio (N_2O / (N_2 + N_2O)) of denitrification

The variety *traced* served to assess N_2O reduction during denitrification in each experiment. The $N_2O/(N_2+N_2O)$ product ratio of denitrification as given by the variety *traced (product ratio$_{15N}$*) was calculated as:

$$product\ ratio_{15N} = \frac{^{15}N-N_2O}{^{15}N-N_2+^{15}N-N_2O} \qquad \text{(Eq. 5)}$$

with ^{15}N-N_2O and ^{15}N-N_2 representing the concentrations of N_2O and N_2 derived from the ^{15}N-labeled fertilizer pool. To check the effectiveness of C_2H_2 to block the N_2O reduction, *product ratio$_{15N}$* was compared with *product ratio$_{C2H2}$*, where the latter can be calculated from N_2O production rates of varieties *natural* and C_2H_2:

$$product\ ratio_{C2H2} = \frac{N_2O_{nat}}{N_2O_{C2H2}} \qquad \text{(Eq. 6)}$$

with N_2O_{nat} and N_2O_{C2H2} representing the N_2O produced in varieties *natural* and C_2H_2, respectively.

If *product ratio$_{15N}$* and *product ratio$_{C2H2}$* were in agreement, a complete blockage of N_2O reduction could be assumed. This enabled to estimate reduction effects on the isotopic signatures of N_2O by comparing the isotopic values of N_2O produced without N_2O reduction effects of variety C_2H_2 (δ0 values) with isotopic values of N_2O of variety *natural*.

Sources of N_2O produced

Assuming that denitrification is the only process producing N_2O in the incubation experiment, the expected ^{15}N enrichment in N_2O produced (^{15}N-N_2Oexp) was given by

$$^{15}N - N_2Oexp\ [at\%] = \frac{(N_{soil}\ x\ ^{15}Nnat) + (N_{fert}\ x\ ^{15}Nfert)}{N_{bulk}} \qquad \text{(Eq. 7)}$$

with N_{soil}, N_{fert} and N_{bulk} describing the amount of N [mg] in unfertilized soil samples, fertilizer and fertilized soil samples, respectively and $^{15}Nnat$ and $^{15}Nfert$ is standing for ^{15}N enrichment under natural conditions (0.3663 at%) and in fertilizer (50 at%), respectively. Comparison of measured and expected ^{15}N enrichment in N_2O gave information about the contribution of processes other than denitrification contributing on N_2O production processes.

Statistical Analysis

We conducted several three-way analyses of variance (ANOVA) to test significant effects of soil, experimental variety and treatment on N_2O production, CO_2 production, and SP, $\delta^{15}N_{bulk}$ and $\delta^{18}O$ values of N_2O. The pairwise comparison with Tukey's HSD test was made to find differences between soils, varieties and treatments influencing N_2O production, CO_2

production, and SP, $\delta^{15}N_{bulk}$ and $\delta^{18}O$ values of N_2O. Significant effects of soils and treatments on *product ratio*$_{C2H2}$ and *product ratio*$_{15N}$ were tested by two-way ANOVA, while differences between soils and treatments influencing the product ratios were tested with pairwise comparison with Tukey's HSD test. Effects of varieties *natural* and *traced* on N_2O and CO_2 production were tested by ANOVA. For this ANOVA the N_2O production rate had to be $\log_{10}$-transformed to achieve homogeneity of variance and normality. The significance level α was 0.1 for every ANOVA. For some ANOVA treatments were excluded, when replicates were $n < 3$. The N_2O or CO_2 production rates of variety C_2H_2 were followed over three sampling times by regression. For statistical analysis, we used the program R (R Core Team, 2013).

Results

N_2O accumulation rates

N_2O and CO_2 accumulation rates of all treatments were similar in magnitude in almost all cases and mostly indistinguishable (Table 2). CO_2 accumulation rates were determined to get additionally information about the denitrifying process. N_2O accumulation rates exhibited increasing trends with ongoing incubation time for every soil with high variations within the treatments. Contrary to that, CO_2 accumulation rates showed decreasing trends (Figure 1, exemplary shown for data of variety C_2H_2). Calculations of inhibitor effects were based on average N_2O and CO_2 accumulation rates of the entire incubation period, i.e. 8 hours of incubation time for Experiment 1, 2 and 3 and 10 hours for Experiment 4.

N_2O and CO_2 accumulation rates of all treatments with C_2H_2 differed significantly among soils ($P < 0.001$) and N_2O accumulation rates differed as well significantly among treatments ($P < 0.001$). Highest N_2O production rate about 5.5 to 6.1 µg N $kg^{-1}h^{-1}$ was produced in Experiment 1 and 3, while in Experiment 2 and 4 N_2O production rates were lower after 10 or 8 hours of incubation (2.6 and 2.7 µg N $kg^{-1}h^{-1}$), respectively. N_2O and CO_2 accumulation rates were significantly higher in variety C_2H_2 than in variety *natural* of Experiment 1, 3 and 4 ($P = 0.002$, $P < 0.010$ and $P < 0.010$ for N_2O accumulation rate and $P = 0.027$, $P < 0.010$ and $P = 0.008$ for CO_2 accumulation rate, respectively) (Table 2), while *natural* and C_2H_2 of Experiment 2 did not differ in N_2O and CO_2 accumulation rates ($P = 0.402$ and $P = 0.288$, respectively).

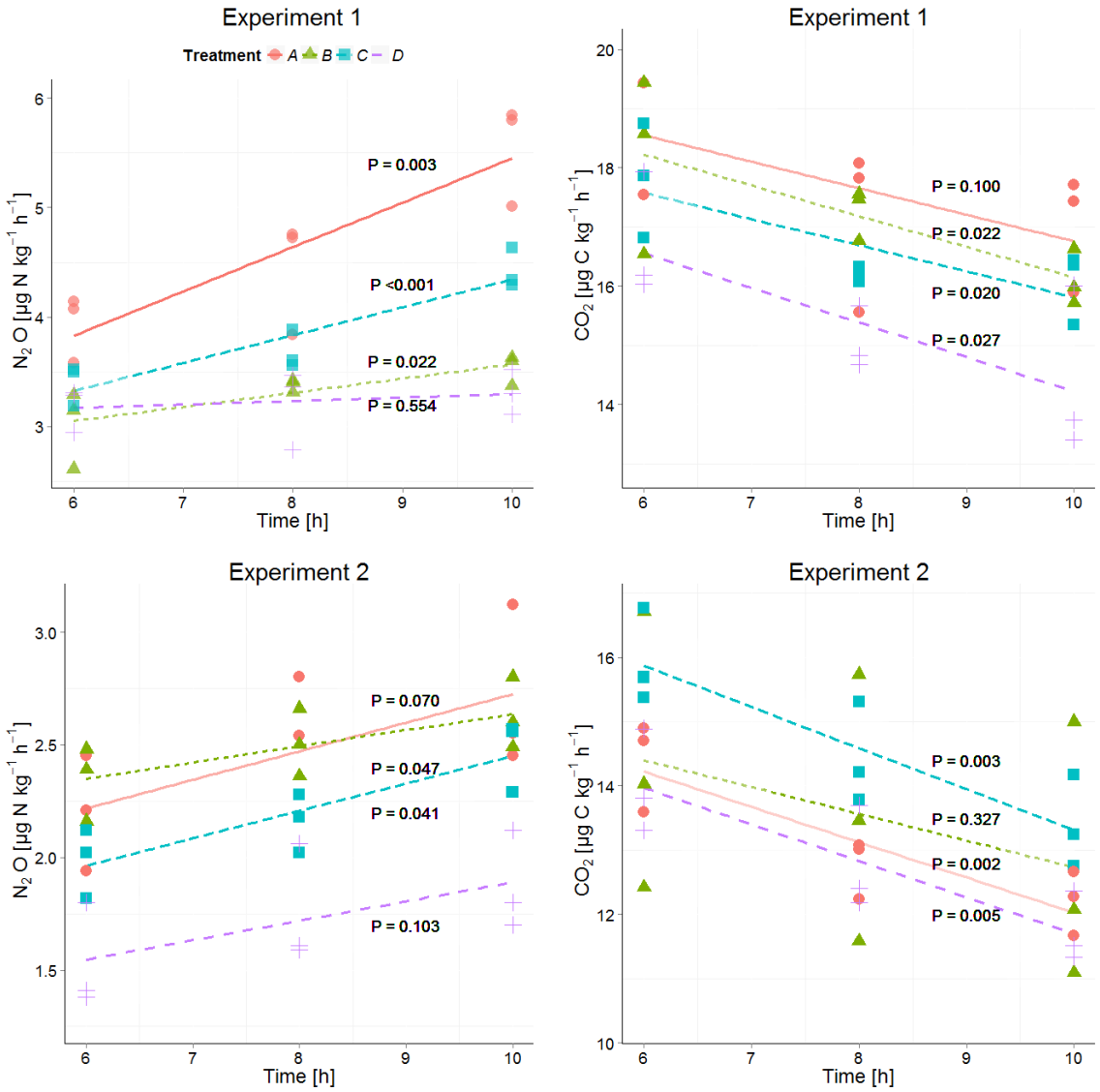

Figure 1: Time series of average N_2O and CO_2 accumulation rates during incubation of variety C_2H_2 at the three sample collection times of each soil (Experiment 1 - 4) for treatments A without growth inhibitors, B with bacterial growth inhibition, C with fungal growth inhibition, and D with bacterial and fungal growth inhibition; *P*-values for linear regressions (significance level $\alpha \leq 0.05$). For all significant regression R^2-values were ≥ 0.46 and in the case of non-significance R^2-values were ≤ 0.40. n.d.: There was no detectable N_2O production Experiment 4 at the first sampling time after 2 hours.
(Figure is continued on next page)

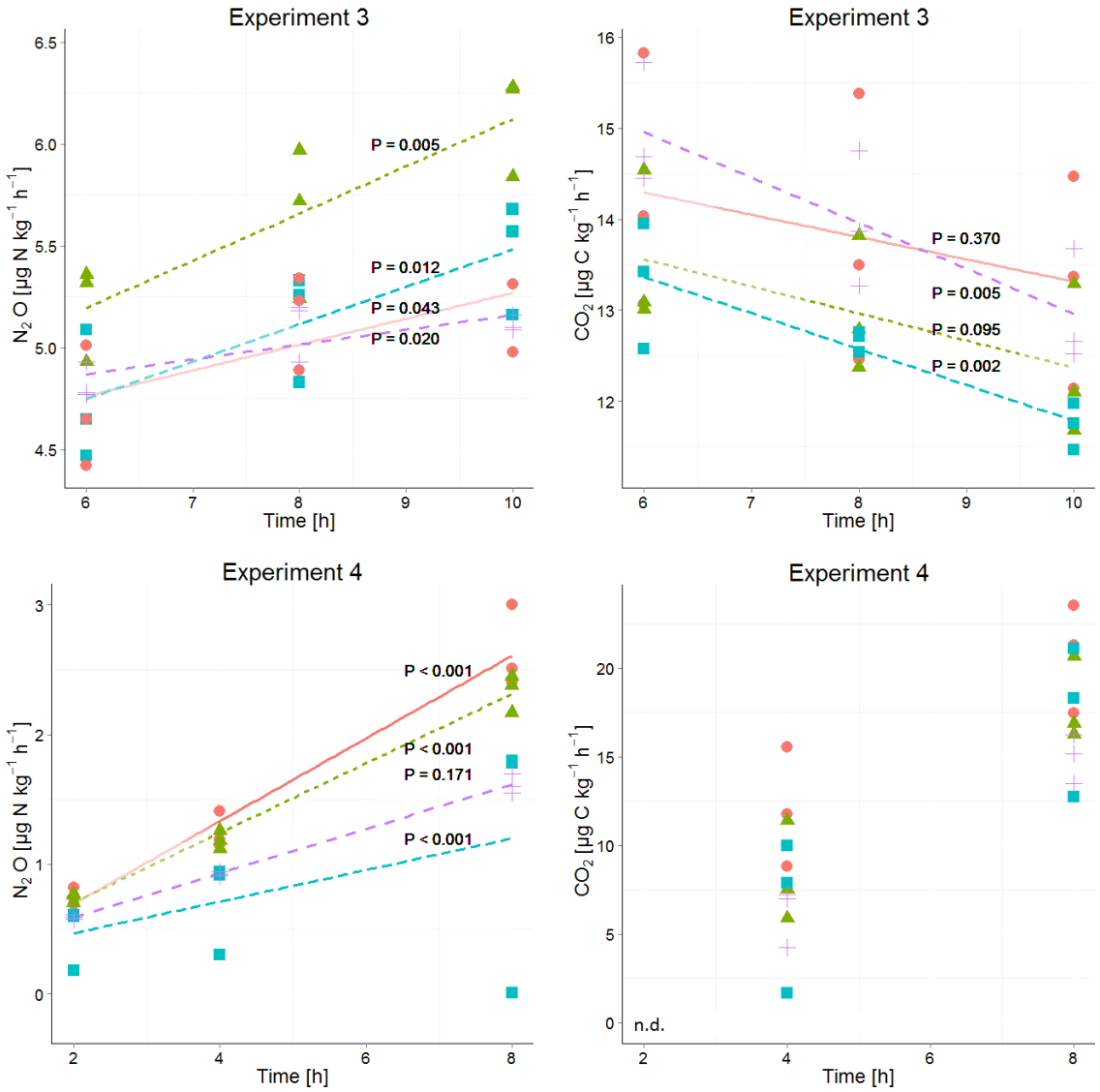

Figure 1 continued.

Without blockage of N_2O reductase, N_2O accumulation rates of the treatment A (variety *natural*) varied significantly among experiments with means between 1.6 and 3.6 µg N kg^{-1} h^{-1} ($P \leq 0.001$) (Table 2). In Experiment 1 N_2O accumulation was significantly higher (2.7 µg N kg^{-1} h^{-1}) compared to Experiment 4 (1.6 µg N kg^{-1} h^{-1}) ($P = 0.028$) in variety *natural*. The inhibitor application of each variety revealed in most cases that treatment A (without growth inhibitors) produced most N_2O, followed by either treatment B (bacterial growth inhibitor; more N_2O compared to treatment C in Experiments 2, 3 and 4) or treatments C (fungal growth inhibitor; more N_2O compared to treatment B in Experiment 1). In *natural, traced* and C_2H_2 varieties, non-inhibitable organisms (treatment D) showed lowest N_2O accumulation rates (except of variety *natural* of Experiment 1, varieties *natural* and traced of Experiment 3 and variety *traced* of Experiment 4). Microbial inhibitor treatments differed significantly in N_2O accumulation rates within the three *N_2O reductase varieties* (C_2H_2) of

each experiment (always $P \leq 0.040$), with the exception of inhibitor treatments of varieties *natural* and *traced* of experiments 4 ($P = 0.154$ and $P = 0.154$, respectively). Significant deviations of treatments without (A) or full inhibition (D) were found in the following cases (Table 2): N_2O accumulation of treatment A was significantly higher compared to the other three treatments of Experiment 1 (C_2H_2 and *natural*), Experiment 2 (C_2H_2) and Experiment 3 (C_2H_2); treatment D was significantly lower compared to the other three treatments in Experiment 2 (*natural*) and compared to treatments A and C of Experiment 1 (C_2H_2). Comparing variants *natural* and *traced*, N_2O and CO_2 accumulation rates did not differ ($P = 0.991$ for N_2O accumulation rate and $P = 0.490$ for CO_2 accumulation rate, respectively), confirming that ^{15}N-labeling did not affect N_2O and CO_2 processes.

Table 2: Average CO_2 and N_2O accumulation rates and N_2O isotopologue values of N_2O of the last sample collection with and without C_2H_2 application in the headspace (varieties *natural* and *C_2H_2*) of each soil (Experiment 1 - 4) for treatments A without, B with bacterial, C with fungal, and D with bacterial and fungal growth inhibition, respectively (standard deviation in brackets, *n = 3).*

Treatment/ variety	**mean N_2O [µg N kg^{-1} h^{-1}]**	**mean CO_2 [µg C kg^{-1} h^{-1}]**	**$\delta^{18}O$-N_2O [‰]**	**$\delta^{15}N_{bulk}$-N_2O [‰]**	**^{15}N-SP-N_2O [‰]**
Experiment 1 (Loamy sand, winter 2012)					
A *natural*	2.7 (0.4)a	12.3 (1.7)a	13.1 (0.2)a	-21.9 (1.7)a	1.6 (0.8)a
B *natural*	1.8 (0.2)b	12.8 (1.6)a	13.0 (<0.1)*	-24.2 (0.7)*	-1.3 (0.2)*
C *natural*	2.0 (0.1)b	11.2 (0.5)a	14.6 (0.4)a	-20.0 (0.8)a	-1.6 (0.5)a
D *natural*	2.1 (0.3)b	13.7 (0.4)a	15.2 (0.5)*	-20.2 (1.8)*	-0.3 (0.5)*
A *C_2H_2*	5.5 (0.5)a	17.0 (1.0)a	8.5 (0.1)a	-22.1 (0.3)a	-0.4 (0.3)a
B *C_2H_2*	3.5 (0.1)b	16.1 (0.5)a	7.5 (0.1)a	-26.1 (0.2)a	-1.2 (1.0)b
C *C_2H_2*	4.4 (0.2)c	14.0 (0.6)a	9.3 (0.2)a	-22.4 (0.4)a	-0.9 (0.4)b
D *C_2H_2*	3.3 (0.2)b	14.4 (1.4)a	7.8 (0.3)*	-24.2 (0.1)*	-2.3 (0.7)*
Experiment 2 (Sand, winter 2012)					
A *natural*	3.2 (0.4)a	13.1 (1.0)a	15.5 (1.8)a	-18.9 (2.6)a	-0.9 (2.5)a
B *natural*	2.4 (<0.1)b	12.1 (0.2)a	15.0 (1.3)a	-23.4 (2.5)a	-0.8 (<0.1)a
C *natural*	2.5 (0.2)b	12.0 (0.5)a	14.3 (0.1)a	-21.8 (0.2)a	-1.8 (0.2)a
D *natural*	2.0 (0.3)b	11.0 (0.2)a	13.4 (0.3)a	-24.5 (0.1)a	-1.2 (0.3)a
A *C_2H_2*	2.7 (0.4)a	12.7 (2.0)a	12.6 (0.3)a	-18.9 (4.6)a	-1.4 (0.3)a
B *C_2H_2*	2.6 (0.2)a	13.4 (0.7)a	12.3 (0.1)a	-24.6 (0.2)b	-2.0 (0.2)a
C *C_2H_2*	2.5 (0.2)a	12.2 (0.5)a	12.7 (0.1)*	-23.3 (0.2)*	-1.7 (0.4)*
D *C_2H_2*	1.9 (0.2)b	11.7 (0.6)a	12.2 (0.3)a	-26.0 (0.1)b	-1.5 (0.9)a
Experiment 3 (Silt loam, winter 2013)					
A *natural*	3.6 (0.2)a	12.3 (1.0)a	26.0 (0.5) a	-20.8 (0.5)a	-0.5 (0.4)a
B *natural*	3.3 (0.4)a	11.6 (1.8)a	24.1 (0.2)b	-22.0 (0.2)b	-0.1 (0.4)a
C *natural*	2.8 (0.1)a	10.6 (0.6)a	27.3 (0.1)b	-20.6 (0.3)a	0.6 (0.2)a
D *natural*	2.9 (0.4)a	11.2 (0.7)a	26.3 (0.3)a	-21.0 (0.1)a	-0.04 (0.18)a
A *C_2H_2*	6.1 (0.3)a	13.3 (1.2)a	15.2 (0.1)a	-25.6 (0.8)a	-2.8 (0.2)a
B *C_2H_2*	5.5 (0.3)b	12.4 (0.8)a	14.9 (0.2)a	-26.3 (<0.1)a	-3.5 (0.4)a
C *C_2H_2*	5.2 (0.2)b	11.7 (0.3)a	16.2 (<0.1)*	-25.2 (0.1)*	-4.0 (0.4)*
D *C_2H_2*	5.1 (<0.1)b	13.0 (0.6)a	16.0 (0.1)b	-25.1 (0.1)a	-4.3 (0.5)a
Experiment 4 (Loamy sand, summer 2011)					
A *natural*	1.6 (0.6)a	24.5 (1.4)a	25.7 (0.3)a	-30.6 (0.2)a	12.1 (1.6)a
B *natural*	1.7 (0.06)a	20.9 (0.2)b	28.0 (5.0)a	-32.3 (0.7)a	7.7 (1.4)b
C *natural*	1.2 (0.03)a	18.4 (1.9)b	29.3 (0.1)a	-30.0 (0.5)a	4.3 (1.0)c
D *natural*	1.2 (0.03)a	16.3 (1.2)b	28.9 (1.2)a	-31.8 (2.2)a	3.4 (2.0)c
A *C_2H_2*	2.6 (0.3)a	20.8 (3.1)a	13.5 (0.5)*	-34.7 (0.1)*	-1.0**
B *C_2H_2*	2.3 (0.2)a	17.9 (2.4)a	14.3 (1.7)a	-33.8 (0.9)a	-4.9 (0.9)a
C *C_2H_2*	1.2 (1.0)a	17.4 (4.2)a	19.0 (7.0)a	-33.1 (2.8)a	-1.7 (2.7)b
D *C_2H_2*	1.6 (0.1)a	15.0 (1.3)a	14.8 (0.5)a	-35.7 (0.2)a	-4.9 (0.7)c

Lowercase letters (a, b, c, d) denote significant differences ($P < 0.1$) between groups of treatments of a soil, if one group is significantly different from all other groups.
Asterisk indicate that only two samples (*) or one sample (**) of triplicates were analyzable.

Isotopologues of N_2O produced in different varieties and treatments

Variety C_2H_2

SP of N_2O of all treatments of variety C_2H_2 of all experiments were within a narrow range between -4.9 and -0.4 ‰ (Table 2), and differed only significantly among treatments of Experiment 4 ($P = 0.002$). In general there were only small differences between treatments: SP of N_2O of treatments A in variety C_2H_2 differed significantly ($P < 0.001$) between soils, with highest SP values of N_2O in Experiment 1 (-0.4 ‰) and lowest SP values of N_2O in Experiment 3 (-2.8 ‰). SP values of treatment D in variety C_2H_2 of all soils varied between -1.5 and -4.9 ‰, but only SP values of Experiment 2 differed significantly from SP values of the other Experiments ($P = 0.006$). For treatments B of variety C_2H_2, SP values differed only significantly between Experiment 1 and 4, 2 and 4, 1 and 3 (each $P = 0.002$). SP of N_2O from treatment C in variety C_2H_2 did not differ significantly ($P = 0.600$). For every soil we found significantly higher $\delta^{18}O$, $\delta^{15}N_{bulk}$ and SP values of N_2O in variety *natural* than in variety C_2H_2 ($P < 0.001$), except for Experiment 2, where $\delta^{15}N_{bulk}$ values of N_2O of variety *natural* were indistinguishable from variety C_2H_2 ($P = 0.400$). However, only in a few varieties there were significant differences in $\delta^{18}O$, $\delta^{15}N_{bulk}$ or SP values of N_2O between treatments with fungal and bacterial inhibition (B and C, respectively) (Table 2). As indicated in the above section, N_2O reduction blockage in varieties C_2H_2 was successful in most cases (Experiment 2, 3 and 4). SP values of N_2O are thus assumed to be valid estimates of $\delta 0$, i.e. SP of N_2O production, and can thus be used for applying the IEM.

Variety natural

SP of N_2O of all experiments and inhibitor treatments of variety *natural* were within a range of -1.8 to 12.1 ‰ (Table 2) and did not differ among inhibitor treatments ($P = 0.037$). SP in variety *natural* of Experiment 4 was particularly high (3.4 to 12.1 ‰) compared to the other experiments (1.6 to -1.6 ‰). As already stated above, in variety *natural* SP of N_2O was significantly higher than SP of N_2O in variety C_2H_2 (up to 2.4, 1.5, 4.6 and 4.1‰ in Experiment 1, 2, 3 and 4, respectively).

^{15}N tracer variety

The ^{15}N-labeling of N_2O (^{15}N-N_2O) or N_2 produced (^{15}N-N_2) gave information about the incorporated N from ^{15}N-labeled NO_3^- into N_2O or N_2 as well as N_2O reduction to N_2. Microorganisms in each treatment used the ^{15}N-labeled NO_3^- in variety *traced* (Table 3) and

expected $^{15}N\text{-}N_2O$ depended on the initial N abundance in NO_3^- of unfertilized soil (Eq. 7). Experiment 4 is the only one showing a large discrepancy between measured (about 30 at%) and calculated $^{15}N\text{-}N_2O_{exp}$ (49 at%) in N_2O, whereas the other experiments showed close agreement (Table 3).

Product ratios of denitrification and efficiency of N_2O reductase blockage by C_2H_2

Product ratio$_{C2H2}$ as well as *product ratio*$_{15N}$ of Experiment 2 were significantly higher than of the other experiments ($P \leq 0.001$) (Table 3). *Product ratio*$_{15N}$ of treatment B was significantly higher than of treatment C and D of Experiment 4 ($P = 0.032$), while all other treatments of other soils did not differ. *Product ratio*$_{C2H2}$ did not differ significantly between treatments ($P = 0.400$). In order to test the efficiency of blockage of N_2O reduction by C_2H_2 application, *product ratio*$_{C2H2}$ (Eq. 5) was compared with *product ratio*$_{15N}$ (Eq. 6). If varieties C_2H_2 were successful in complete blockage of N_2O reduction both calculated product ratios should result in similar values. In Experiment 1 *product ratio*$_{C2H2}$ was by far smaller than *product ratio*$_{15N}$, while both calculated product ratios were in similar ranges in the other three experiments and thus a successful blockage of N_2O reduction was assumed.

Table 3: Average CO_2 and N_2O accumulation rates of the last sample collection after 10 or 8 hours of variety *traced*, respectively, with ^{15}N labeling in N_2O (^{15}N-N_2O) and the calculated *product ratio*$_{15N}$ of variety *traced* and *product ratio*$_{C2H2}$ calculated from N_2O accumulation rates of variety *natural* and C_2H_2 of each soil (Experiment 1 - 4) for treatments A without, B with bacterial, C with fungal, and D with bacterial and fungal growth inhibition, respectively (standard deviation in brackets, *n = 3).*

Treatment	mean N_2O [µg N kg^{-1} h^{-1}]	mean CO_2 [µg N kg^{-1} h^{-1}]*	^{15}N-N_2O [at%]	^{15}N-N_2Oexp [at%][a]	Calc. total product ratio$_{15N}$[b*]	Calc. total product ratio$_{C2H2}$[c*]
Experiment 1 (Loamy Sand, 2012)						
A	2.6 (0.4)a	13.1 (1.7)	36.8 (0.1)	39	0.80 (0.02)	0.48 (0.07)
B	1.5 (0.3)a	11.5 (2.4)	36.4 (0.2)		0.76 (0.02)	0.48 (0.05)
C	1.9 (1.5)a	12.2 (1.1)	36.9 (<0.1)		0.72 (0.05)	0.45 (0.04)
D	1.5 (0.02)a	12.5 (0.5)	36.8 (0.1)		0.69 (0.02)	0.54 (0.05)
Experiment 2 (Sand, 2012)						
A	2.4 (0.01)a	12.9 (0.1)	43.2 (<0.1)	44	0.94 (0.01)	1.04 (0.10)
B	1.9 (0.03)a	11.6 (0.2)	43.0 (0.1)		0.94 (0.01)	0.81 (0.04)
C	2.4 (0.1)b	12.8 (0.6)	43.2 (0.1)		0.95 (0.01)	0.99 (0.09)
D	1.7 (0.1)a	12.0 (0.3)	42.7 (0.1)		0.93 (0.01)	0.98 (0.04)
Experiment 3 (Silt loam, 2013)						
A	2.9 (0.2)a	10.4 (0.5)	35.8 (<0.1)	34	0.62 (<0.01)	0.52 (0.04)
B	3.2 (0.2)a	12.0 (0.9)	35.5 (<0.1)		0.62 (0.01)	0.59 (0.02)
C	2.2 (0.3)a	9.8 (2.0)	35.5 (<0.1)		0.59 (0.02)	0.48 (0.04)
D	2.3 (0.1)a	9.9 (0.7)	35.3 (<0.1)		0.62 (0.01)	0.51 (0.04)
Experiment 4 (Loamy Sand, 2011)						
A	1.6 (0.6)a	31.1 (12.5)	31.1***	49	0.54 (0.05)	0.63 (0.10)
B	1.7 (0.06)a	23.2 (3.0)	26.5***		0.59 (0.03)	0.63 (0.17)
C	1.2 (0.03)a	17.9 (0.8)	30.1**		0.50 (0.01)	0.62 (0.02)
D	1.2 (0.03)a	17.1 (0.4)	33.5**		0.50 (0.01)	0.53 (0.12)

Different lowercase letters (a, b, c, d) denote significant ($P < 0.1$) differences between groups of treatments of a soil if one group is significantly different from all other groups.

Asterisk indicate that only two samples (**) or one sample (***) was analyzed.

[a] *^{15}N-N_2Oexp* [at%] was calculated from Eq. 7.

[b] *product ratio*$_{15N}$ = $N_2O/(N_2+N_2O)$ with N_2O or N_2 accumulation rates from variety *traced*; see Eq. 5

[c] *product ratio*$_{C2H2}$ = N_2O_{nat}/N_2O_{C2H2} with N_2O accumulation rate from varieties *natural* and *traced*; see Eq. 6, cf. Table 2

*No significant differences between treatments of each soil.

Fungal contribution to N_2O production from denitrification by microbial inhibitor approach (modified SIRIN)

When calculating F_{FDmi} contributing to N_2O production during denitrification N_2O accumulation rates of treatment D must be significantly lower compared to the other treatments and the flux balance according to Eq. 1 and 2 must be consistent. This was only the case in Experiment 2 of variety C_2H_2. The calculated F_{FDmi} (Eq. 3) contributing to N_2O production during denitrification was 0.28 ± 0.09. The respective flux of fungal N_2O was 0.24 ± 0.08 µg N kg^{-1} h^{-1}. For all other experiments calculation of F_{FDmi} was not possible.

Fungal contribution to N_2O production from denitrification by the isotope endmember mixing approach (IEM)

Calculation of the fungal fraction (*calc.* F_{FD}) contributing to N_2O production during denitrification by using Eq. 4 was possible by fitting F_{FD} and F_{OS} to achieve the measured SP values of N_2O in treatments A of variety C_2H_2 (Table 2). The fitted fungal fraction (*calc.* F_{FD}) contributing to N_2O production did not exceed approximately 0.22 (Table 4).

Table 4: Application of the isotopomer endmember mixing approach (IEM) (Eq. 4) with measured N_2O accumulation rates and SP of N_2O produced. Due to the bacterial range of SP values of N_2O produced the fraction of N_2O produced by fungi (F_{FD}) were calculated by Eq. 4.

Experiment	calc. F_{FD} ranges[a]	calc. F_{FD} ranges[b]
Experiment 2	0	0.21
Experiment 3	0	0.18
Experiment 4	0	0.22

[a]Eq. 4 with assuming SP of N_2O produced by bacteria was 0 ‰.
[b]Eq. 4 with assuming SP of N_2O produced by bacteria was -11 ‰.

SP of N_2O produced by the fungal soil community

Solving Eq. 4 for SP_{FD} enables to calculate SP of N_2O produced from the fungal soil community. Estimates for SP_{FD} and SP_{OS} from the results of the modified SIRIN (0.28 and 0.72, respectively, section "Fungal contribution to N_2O production from denitrification by microbial inhibitor approach (modified SIRIN)") and SP_{Prod} of N_2O (SP_{Prod} = -1.4 ‰) of the respective treatment A (Table 2) served to calculate SP of N_2O produced by fungi for Experiment 2. Assuming -11 or 0 ‰ for the bacterial SP endmember of N_2O (Frame and Casciotti, 2010; Sutka et al., 2006) resulted in SP_{FD} between -5 ‰ (SP_{BD} = -11 ‰) and 23 ‰ (SP_{BD} = 0 ‰).

Discussion

This was the first attempt to determine SP of N_2O produced by fungi or bacteria from soil communities using microbial growth inhibitors with a modification of SIRIN and comparing microbial inhibitor and an isotope endmember mixing (IEM) approaches to estimate fungal contribution to N_2O production from denitrification. Using IEM revealed that the fungal contribution to N_2O production was small (calc.$F_{FD} \leq 0.22$). One experiment with modified SIRIN allowed the calculation of the fungal fraction producing N_2O during denitrification and revealed a similar result to the IEM result of about 0.28. The strict application of the SIRIN method prescribes proof of selectivity of the inhibitors (i.e., streptomycin should not inhibit fungi and cycloheximide should not inhibit bacteria). The results obtained with respect to N_2O production by the fungal or bacterial fraction were rather unsatisfactory and led to unsolved questions, which are discussed in the following sections.

Experimental setup

Inhibitor effects, expressed by lower N_2O production with selective inhibitors (treatments B, C and D) compared to treatments without inhibitors (A), were only minor in the present study. Previous studies found much larger inhibitor effects by pre-incubating the soil with selective inhibitors (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013). The experimental design of our incubation setup was, however, without soil pre-incubation with selective inhibitors, because we aimed to minimize disturbance of soil microbial community and to work with the close to “naturally occurring” F:B ratio. This was contrary to previous studies (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013) and we suppose that pre-incubation with selective inhibitors changes the F:B ratio compared to the undisturbed soil considerably more than soil incubation without this pre-incubation step. Blagodatskaya et al. (2010) did not find more inhibitor efficiency of pre-incubation with streptomycin, but found greater inhibitor effects with pre-incubation with cycloheximide. This could indicate that microbial distribution changed after exposition to this inhibitor. Anderson and Domsch (1975) stated already that CO_2 production of initially active organisms can only be ensured up to six or eight hours of experimental duration and biomass activity is changed by both inhibitors (Sutka et al., 2008). It has to be noticed that pre-incubation in the other studies was without glucose, while the N_2O production was analyzed after the addition of glucose as substrate in previous studies and in the present study as well (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). Glucose initiates the growth of active organisms. Pre-incubation under denitrifying conditions is not

needed for microorganisms to produce denitrifying enzymes as pure cultures synthesized enzymes capable of denitrification within two to three hours (USEPA, 1993). We started gas sample collection after two or four hours, when organisms should have produced denitrifying enzymes and microbial growth of initially active organisms should have started. With on-going incubation time production rates of CO_2 decreased, probably because of experimental incubation conditions, for example increasing partial pressure or physiological changes due to staling factors.

The conventional practice of SIRIN implies determination of c_{opt}(glucose), c_{opt}(streptomycin) or c_{opt}(cycloheximide) with a "Ultragas 3" CO_2 analyzer (WösthoffCo., Bochum) (Anderson and Domsch, 1973) with continuous gas flow and we used this method to determine optimal concentrations for SIRIN and used these concentrations for the modified SIRIN approach as well. This procedure was also different from studies of other groups (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013), which ascertained optimal concentrations in static systems. We supposed that optimal concentrations for CO_2 respiration could work as well for denitrification, if both inhibitors are apt to inhibit the denitrification process as well. SIRIN had been tested with pure cultures and soils for CO_2 respiration (Anderson and Domsch, 1975), but information on N_2O producing processes, especially denitrification, are lacking so far and should be investigated in further studies.

Inhibitor effects

Even with both growth inhibitors (treatment D) N_2O production was high in all experiments, i.e., often not significantly lower than the other three treatments. Thus, we suppose similar contributions of non-inhibitable organisms in all other treatments (A, B and C). Non-inhibitable organisms could be, for example, bacteria or fungi that are not in growth stage or may be not affected by inhibitors. These organisms could be archaea as well, which are also known to be capable of denitrification (Hayatsu et al., 2008; Philippot et al., 2007). It is known, that archaea are not affected by streptomycin or cycloheximide (Seo and DeLaune, 2010). However, there were some tests to find adequate inhibitors for archaea (halobacteria) (Bonelo et al., 1984) and they found three of 20 antibiotics (erythromycin, chloramphenicol and haloquinone) to inhibit archaeal growth of eleven tested species. However, effects of archaeal occurrence in soil or secondary effects on fungi or bacteria were not tested in this study.

Is SIRIN without C_2H_2 suitable to examine the fungal contribution to N_2O production in soil?

In order to determine SP of produced N_2O without alteration by partial reduction to N_2, C_2H_2 was used to block the N_2O reduction during denitrification quantitatively. We found the expected effect of C_2H_2 application, i.e. higher N_2O production rates in variety C_2H_2 compared to variety *natural*. Calculated product ratios varied between 0.5 and 0.95 (*product ratio*$_{15N}$) in all soils, showing that N_2O reduction can have significant effects on measured N_2O production and isotopic values. The product ratio is controlled by the reaction rate or by the activity of enzymes capable of N_2O reduction (Nos) in the system. A product ratio of 0.95 (Experiment 2), for example, indicates that nearly 100% N_2O from denitrification remains in the system while only minimal amounts of N_2O are reduced to N_2. The calculated *product ratio*$_{C2H2}$ was within the same range as *product ratio*$_{15N}$ in Experiment 2, 3 and 4 (maximal 9% difference) which proves the effective blockage of N_2O reductase in variety C_2H_2. Only in Experiment 1 *product ratio*$_{15N}$ and *product ratio*$_{C2H2}$ differed about 34% with higher reduction in the *tracer* variety, which might be explained by potential error sources of the C_2H_2 method. Nadeem et al. (2013) found some artifacts with C_2H_2, which resulted in lower N_2O production rates due to NO oxidation accelerated by C_2H_2 application in the presence of very small oxygen (O) amounts ($\geq$ 0.19 mL L^{-1}). Moreover incomplete C_2H_2 diffusion into denitrifying aggregates might lead to incomplete N_2O reductase blockage (Groffman et al., 2006). For the other three experiments (2, 3 and 4) it can be supposed that the isotopic signature of N_2O of variety C_2H_2 showed isotopic signatures of produced N_2O without influences of N_2O reduction. By comparing varieties *natural* and C_2H_2, isotopologue values of all soils (except $\delta^{15}N_{bulk}$ values of Experiment 2) of variety *natural* were significantly higher than that of variety C_2H_2. The enrichment of residual N_2O in heavy isotopes is a typical isotope effect associated with N_2O reduction (Jinuntuya-Nortman et al., 2008; Lewicka-Szczebak et al., 2014; Well and Flessa, 2009). This explains why C_2H_2 application is essential for analyzing N_2O produced by different microbial organism groups from soil.

Due to the lack of N_2O reductase in most fungi (Shoun et al., 1992), we expected higher product ratios for treatments B with bacterial growth inhibition. However, we did not find significant differences in product ratios within the treatments of one soil and variety. Nevertheless, Experiments 1, 3 and 4 showed the tendency of higher product ratios for treatments B, where probably fungi dominated the microbial community, compared to treatment C, where probably bacteria dominated the microbial community. There was nearly no N_2O reduction in Experiment 2 independent of the treatment, which might be due to

microorganisms lacking N_2O reductase dominating the microbial community or ambient conditions, which lowered the reduction rates.

SP of N_2O produced by microbial community

The SP of N_2O produced of each soil indicated predominantly bacteria to be responsible for N_2O production from denitrification, providing that results of SP of N_2O from denitrification of pure bacterial cultures is transferable to bacteria of soil communities contributing to denitrification. Based on this assumption, there was no evidence of fungi contributing greatly to N_2O production during denitrification, although the IEM approach revealed a fungal contribution up to 22% on N_2O production during denitrification. For all treatments (varieties *natural* and C_2H_2) SP of N_2O was within the range of bacterial dentirification known from pure culture studies to be between -11 and 0 ‰ (Frame and Casciotti, 2010; Sutka et al., 2006), with exception of Experiment 4 (variety *natural*), where SP of N_2O of every treatment (SP = 3.4 to 12.1 ‰) was in between SP of N_2O known from bacterial and fungal dentirification. SP of treatments A differed between soils and this could result from different microbial communities in every soil either with differences in F:B ratios or different species occurring in soils. SP of N_2O of treatment A within variety C_2H_2 showed that the signature of produced N_2O was not affected by reduction effects and might give evidence of microbial community contributing to N_2O production regarding differences in SP of N_2O of pure bacterial or fungal culture studies (Frame and Casciotti, 2010; Rohe et al., 2014a; Sutka et al., 2008; Sutka et al., 2006). However, variations in SP of N_2O in treatments A of variety C_2H_2 are very small and do not give a clear evidence of any differences in microbial soil community producing N_2O. Lewicka-Szczebak et al. (2014) analyzed SP of N_2O produced during denitrification with blockage of N_2O reduction by C_2H_2 for the same soils as used in the present study for Experiment 1 and 3 and revealed SP values between -3.6 and -2.1 ‰, which is similar to the respective SP values of the present study from -4.9 to -0.4 ‰. This reinforces the conclusion that bacteria dominate N_2O production under denitrifying conditions in these both soils. However, there are studies using different soils, which found higher SP values of produced N_2O unaffected by the reduction of up to +6 ‰ (Koester et al., 2013; Lewicka-Szczebak et al., 2014) most probably as a result of higher contributions of fungi on N_2O production.

$\delta^{18}O$ of N_2O

The analysis of $\delta^{18}O$ of N_2O can give information about O exchange between water and denitrification intermediates by various microorganisms, like it is known for several pure bacterial and fungal cultures (Aerssens et al., 1986; Kool et al., 2007; Rohe et al., 2014b). The range of $\delta^{18}O$ in our study for variety C_2H_2 (7.5 to 19.0 ‰) is quite similar to the range found by Lewicka-Szczebak et al. (2014) for the same soils (4.8 to 16.3 ‰), where almost complete O exchange with soil water was documented. Hence, for this study most probably this O exchange was also very high. Moreover, there were no remarkably differences in $\delta^{18}O$ values of N_2O among treatments within one variety and soil and therefore we assume no differences in O exchange between the treatments.

Codenitrification

The influence of codenitrification, which is predominantly associated to fungi (Spott et al., 2011), is completely unknown in the present study, but it may have a high impact on N_2O production, since Laughlin and Stevens (2002) found N_2O production in their experiment derived to 92% by codenitrification and only 8% by denitrification. Until now there is no study focusing on SP of N_2O produced by codentrification. Codenitrification could have been a co-occuring process in Experiment 4. The ^{15}N enrichment of soil NO_3^- was about 60% higher than analyzed ^{15}N enrichment in N_2O, leading to the assumption that N_2O was produced not only by denitrification. When N in N_2O originates only from ^{15}N –labeled soil NO_3^- measured ^{15}N values of N_2O should show identical ^{15}N enrichment to the labeled soil NO_3^-. During codenitrification, when N in N_2O originates from labeled NO_3^- and also from another unlabeled and unknown N source, this results in dilution of the applied ^{15}N labeling of N_2O. In the other soils there was no indication of codenitrification being relevant for N_2O production. Thus, the SP of N_2O in Experiment 4 might be influenced by codenitrification. There is no knowledge about the isotopic signature of N_2O produced during codenitrification so far.

Calculating the fungal fraction contributing to N_2O production

Due to the inefficiency of microbial inhibition regarding the N_2O production in most cases calculation of F_{FDmi} contributing to N_2O production was only possible for Experiment 2. Comparing the modified SIRIN with the IEM approach revealed that the fungal fraction contribution to N_2O production was smaller (0.28 in modified SIRIN and 0.21 in IEM)

compared to bacterial fraction. Although we did not obtain a very clear picture of various microorganisms contributing to N_2O production due to the large uncertainties of the calculated fractions, both approaches revealed similar values for the fungal fraction contributing to denitrification.

Since in some soil studies the SP values of N_2O, after correction for the reduction effect on SP, showed significantly higher values (Koester et al., 2013; Lewicka-Szczebak et al., 2014), it can be supposed that based on the IEM approach various soils may largely differ in the microbial community that contributes to N_2O from denitrification. The three tested soils seemed to contain a microbial community where fungi have minor contribution to N_2O emissions from denitrification compared to bacteria.

The calculated fungal SP of N_2O (section "*SP of N_2O produced by the fungal soil community*") from fungal fraction contributing to N_2O was highly variable (between -5 and +23 ‰) and lower that the SP of N_2O known from pure cultures (20 - 37 ‰) (Rohe et al., 2014a; Sutka et al., 2008) and indicates a high uncertainty of the estimation according to Eq. 4. The IEM should be further investigated with soils, where presumable fungal contribute largely to N_2O production during denitrification (Koester et al., 2013; Laughlin and Stevens, 2002).

Conclusions

Although the present study with different selective growth inhibitors did not confirm the expected effect on N_2O production and SP of N_2O, it can be supposed that fungi played a minor role in N_2O production from denitrification in the studied soils. The IEM was promising, but was not fully validated, because the fungal fraction contributing to N_2O production could not be quantified with modified SIRIN. There might be several artefacts in the modified SIRIN, where further studies should focus on, e.g. on effects of bacterial consumption of N_2O produced by fungi in the presence of bacterial growth inhibitors. The present study could show that consideration of N_2O reduction in further studies is inevitably necessary. Further studies should also determine the range of SP of N_2O produced by fungi as well as the effect of specific inhibitors on microbial groups producing N_2O during denitrification.

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General Discussions

Discussion

The present study aimed to answer the following questions concerning the fungal pathway of denitrification:

1. Is fungal denitrification associated with an O exchange between water and denitrification intermediates? What is the characteristic O isotopic signature of fungally produced N_2O?
2. Is the ^{15}N site preference (SP) of N_2O produced by fungal pure cultures characterized by higher values compared to SP of N_2O known from bacterial pure cultures?
3. Are microbial growth inhibitors suitable to quantify the fungal contribution to denitrification in soil and do fungi in soil produce N_2O with a SP range comparable to SP of N_2O from pure cultures?

Two pure culture studies extended the recent knowledge about the isotopic signature of N_2O produced by fungi. In the ^{18}O-traced experiment, the incorporation of ^{18}O from labeled water into N_2O during denitrification was examined and revealed that fungi, similar to bacteria, showed a significant O exchange between denitrification intermediates and water. The extent of O exchange varied among species in a range between 11% and full exchange. O exchange differed with regard to the applied electron acceptor. Higher O exchange rates were observed during denitrification with nitrite compared to nitrate, which indicates the significance of the nitrite reductase in the process of O exchange and reinforced a hypothesis proposed by Aerssens et al. (1986). Lowest values of O exchange were shown by *C. funicola* (41%), the only *Sordariales.* The other five species under evaluation belonged to the *Hypocreales* and showed an O exchange between 74% and full exchange with nitrite as electron acceptor. Only one (*F. solani* fsp. *pisi*) of the six species tested showed higher O exchange between denitrification intermediates and water with nitrate as electron acceptor than with nitrite. This study proved that the O isotopic signature of N_2O cannot be used to differentiate between N_2O produced by fungal or bacterial denitrification. However, if O exchange during denitrification in soil might be close to 100%, the isotopic signature of produced N_2O after complete O exchange should be stable and well predictable if the O signature of the ambient water and the isotopic fractionation associated with O exchange is known.

The SP of N_2O produced by six fungal species was determined in a natural abundance experiment. A previous study by Sutka et al. (2008) showed very high SP values of produced N_2O for two of these fungal species (SP ≈ 37 ‰) and the values found in the current study

were in the same range. Furthermore, this study supports the assumption that SP values of N_2O from fungal denitrfiication are higher than those for N_2O produced by bacterial denitrification (Frame and Casciotti, 2010; Sutka et al., 2006; Toyoda et al., 2005) as the additional four fungal species under evaluation gave SP values between 19.7 and 32.6 ‰. Again only the fungus belonging to the order *Sordariales* showed deviating results from the other five species belonging to *Hypocreales*. Besides remarkably small O exchange during denitrification between denitrifying intermediates and the ambient water, also SP-values of N_2O produced were by far smaller (SP = 19.7 ‰) than from the five *Hypocreales* (SP = 30.2 to 32.6 ‰). These results indicate that fungal strains belonging to different genera reveal different isotopic O exchange between denitrification intermediates and water and SP values of N_2O. This study is the first to report on this phenomenon and indicates that further approaches to analyze the denitrifying process of fungi must take different genera into account. Furthermore, a greater number of fungal species should be considered in future studies, because the six species of the present study only reflect a minimal representation of all fungal species of a soil community.

The performance of comparable experiments with the same six fungal species under denitrifying incubation conditions with an ^{18}O tracer approach on the one hand, and a natural abundance approach on the other hand, enabled the estimation of the extent of O exchange catalyzed by the different enzymes during denitrification. For this investigation a fractionation model proposed by Snider et al. (2013) for bacterial denitrification was used to estimate enzymes associated with O exchange. Probably nitrite reductase contributes most to O exchange, whereas nitrate reductase and NO reductase have a minor contribution to the total O exchange of *Hypocreales*. Again only the *Sordariales* species showed exceptional results, as most O exchange was here assumed at NO reductase. This fractionation model gave only initial indications that specific enzymes are predominantly responsible for O exchange during fungal denitrification. Pure culture studies have to be developed to actually measure the exact extent of O exchange by single enzymes, e.g. with improved tracer approaches using ^{18}O labeled water and different electron acceptors (NO_3^-, NO_2^- or NO) for N_2O production with various fungal denitrifiers.

The results of the soil studies with selective microbial growth inhibitors indicated that isotopic signatures of N_2O from denitrification did not show fungal dominance of N_2O from denitrification in the three tested soils, despite such suggestions by several previous studies (Blagodatskaya et al., 2010; Laughlin and Stevens, 2002; Long et al., 2013; McLain and Martens, 2006). However, the used method of microbial inhibition did not reveal the expected

effect, i.e. a significant decrease in N_2O production with the growth inhibition of fungi, bacteria alone or both groups together compared to N_2O production from the not inhibited soil community. The calculation of reliable values for fungal contribution to N_2O production from denitrification based on the inhibitor approach was not possible for all soils tested. Simultaneous application of both microbial growth inhibitors, i.e. for fungi and bacteria, resulted only in a small reduction of N_2O production, indicating that organisms not affected by both antibiotics played an important role. Thus, this not inhibited N_2O production overlaid the N_2O production and isotopic fingerprint in treatments with selective growth inhibitors (for fungi or bacteria) as well. As neither knowledge about the microbial composition of this group, nor information of SP values of N_2O produced, was available, calculating SP of N_2O produced by the fungal soil community was impossible. However, assuming that bacteria and fungi contributed predominantly to denitrification and that both groups produced SP of N_2O known from pure culture studies, the isotopic analysis of N_2O revealed that N_2O was predominantly produced by bacteria. Different soil types could vary in the composition of microbial communities contributing to N_2O emissions, which could lead to the discrepancy in findings between the present and previous studies. Hence, further studies should include different soils differing in the microbial community.

The fungal species under evaluation represent only a small portion of fungal species present in soils and five of six species belonged to *Hypocreales*, while only one species was *Sordariales*. This again illustrates the insufficient research on fungal denitrification and for this reason, studies focusing on fungal denitrification should be expanded. The present study also revealed the difficulty of transferring knowledge about N_2O produced by pure cultures to N_2O produced in soils by either the total microbial community or in soils where either fungal or bacterial growth was inhibited. Future studies should also focus on the effectiveness of microbial inhibitors for denitrification. As bacteria and only a few fungi are known to further reduce N_2O to N_2 during denitrification (Shoun et al., 1992), the measured N_2O production as well as the isotopic signature of N_2O may be affected when N_2O reduction is carried out. The potential bias in estimating fungal N_2O using selective inhibition, which can be expected due to the lowering of N_2O reduction when inhibiting bacterial denitrifiers, was confirmed since SP of N_2O responded to acetylene, which is used to block N_2O reduction. This shows that N_2O reduction has to be taken into account when analyzing the isotopic signature of N_2O produced from the soil community. In the present study, the expected reduction of N_2O production after microbial inhibitor application in soil incubation experiments was not

achieved. Potential interactions between the applied acetylene and the microbial inhibitors, which could affect N_2O production, have not yet been investigated.

To sum up, the pure culture studies with fungi showed that the isotopic O signature of N_2O is not suitable for distinction between fungal or bacterial denitrification, whereas the SP of N_2O produced by fungi during denitrification might be a promising tool to differentiate between N_2O produced by bacteria or fungi. Knowledge about the contribution of fungi on N_2O production during denitrification in soil as well as a validated method to quantify the contributions to N_2O production from different microbial groups are still lacking. Further studies are necessary to improve the information about the amount of N_2O produced by fungi to advance models for predicting N_2O emissions from soil and to find mitigation strategies for N_2O emissions.

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Summary

The trace gas nitrous oxide (N_2O) contributes to climate change as well as to the depletion of the ozone layer in the stratosphere. Agricultural soils account for about 70% of the high anthropogenic N_2O emissions. Microbial processes in soil use, for instance fertilizer N to produce N_2O, are an important factor. An understanding of N_2O production pathways is imperative to evaluate reliable mitigation methods for N_2O emissions.

The present study focused on denitrification, which, besides nitrification and nitrifier denitrification, is one of the main N_2O production pathways in soils. Denitrification describes the reduction from nitrate (NO_3^-) to N_2, with nitrite (NO_2^-), nitrous monoxide (NO) and N_2O as intermediates. For a long time denitrification was attributed only to heterotrophic bacteria. In 1972, however, pure culture studies showed that fungi are also capable of denitrification, and two decades later most fungi were found to lack the N_2O reductase, resulting in N_2O being the main product of fungal denitrification instead of N_2. This could indicate that fungi might produce more N_2O compared to bacteria, providing that both groups have the same production rates. However, the contribution of different microbial groups to N_2O emissions from soil has not yet been sufficiently investigated.

Analysis of the isotopic signature of N_2O found this to be a promising tool to distinguish between N_2O produced by different microbial groups. Especially the site preference of ^{15}N in N_2O (SP = difference between $\delta^{15}N$ of the outer and central N atoms in N_2O) from denitrification revealed differences between pure bacterial cultures (SP = -11 to 0 ‰) and two studied pure fungal cultures (SP ~ 37‰). Although it is known that all enzymes involved in fungal denitrification, with the exception of the N_2O reductase, equals the enzymes of bacteria, most denitrification studies with pure cultures covered the bacterial pathway. The different N_2O reductases might be the reason for different SP of N_2O produced by bacteria or fungi. An O exchange between denitrification intermediates and water between 4 and 100% was found during bacterial denitrification, while there has been no study analyzing the existence of O exchange during fungal denitrification so far. If O exchange were not to occur during fungal denitrification, this could provide an additional ability to differentiate between N_2O produced by fungi or bacteria. The O isotopic signature of N_2O produced by fungi would significantly differ from that produced by bacteria.

The present study focused on three subjects. With an isotope tracer experiment with ^{18}O labeled water, the existence of O exchange between denitrification intermediates and water during denitrification was studied with six fungal species. The fungi showed an O exchange

of up to 100% and consequently a differentiation between fungal and bacterial denitrification with an O isotopic signature is impossible.

The second subject was verification of the high SP values of N_2O from fungal denitrification in four additionally tested species and consideration of whether it was reproducible for the two tested species known from literature. This study confirmed higher SP values of N_2O (SP = 19.7 to 31.7‰) compared to the SP of N_2O known from bacteria. Based on the results of the isotope tracer experiment and the O isotopic signature of N_2O under natural conditions, mechanisms of the O isotope fractionation were analyzed by applying values of fractionation effects known from the literature in an isotope fractionation model to estimate the involved enzymes on O exchange during denitrification. The O exchange of NO_2^- reductase was high compared to O exchange of NO_3^- and NO reductases.

The knowledge obtained from pure fungal culture studies was used in Subject Three to test the transferability to microbial communities in soils by using microbial inhibitors for bacteria or fungi in soil incubation experiments. A modification of substrate induced respiration with selective inhibition (SIRIN) was used to determine whether the specific SP values of N_2O known for bacteria and fungi are measurable after selective growth inhibition by specific antibiotic application. The expected effect of growth inhibition on SP of N_2O was not found. In most cases the SP of N_2O was in the range known from pure bacterial cultures and bacterial growth inhibition did not result in the expected shift of SP values. Consequently the SP values of this incubation experiment did not serve to associate the N_2O production in inhibited treatments to different microbial groups. It remained unclear if this was due to the modified SIRIN method or if transferability of differences in SP of N_2O known from fungi and bacteria on a microbial community in soil is possible. Future studies should approach the existing problems regarding the methods to identify fungal denitrification in soil.

Zusammenfassung

Das Spurengas Lachgas (N_2O) trägt zur Klimaerwärmung und Zerstörung der Ozonschicht in der Atmosphäre bei. Mit einem Anteil von ca. 70% sind landwirtschaftliche Böden weltweit Hauptverursacher der hohen anthropogenen N_2O Emissionen. N_2O entsteht in Böden durch verschiedene mikrobiologische Prozesse, bei denen N_2O unter anderem aus düngerbürtigem N gebildet wird. Die Entwicklung effektiver Minderungsmaßnahmen wird erst möglich, wenn ein Verständnis der N_2O Quellprozesse und ihrer Dynamik in Böden vorhanden ist.

In dieser Studie wurde die Denitrifikation als ein Quellprozess untersucht, der zusammen mit Nitrifikation und Nitrifizierer-Denitrifikation hauptsächlich für die N_2O Emissionen aus Böden verantwortlich ist. Die Denitrifikation beschreibt die Reduktion von Nitrat (NO_3^-) zu N_2, wobei Nitrit (NO_2^-), Stickstoffmonoxid (NO) und N_2O Zwischenprodukte dieses Reaktionsweges sind. Lange Zeit galten heterotrophe Bakterien als alleinige Verursacher von N_2O Emissionen aus der Denitrifikation. Im Jahr 1972 wurde allerdings in Versuchen mit Pilzreinkulturen nachgewiesen, dass auch Pilze in der Lage sind, N_2O über die Denitrifikation zu bilden. Zwei Jahrzehnte später wurde gezeigt, dass den meisten Pilzen das Enzym N_2O-Reduktase fehlt. Somit ist nicht N_2, sondern N_2O das hauptsächliche Endprodukt der pilzlichen Denitrifikation. Dies lässt vermuten, dass die Bildung von N_2O durch pilzliche Denitrifikation noch unterschätzt wird, vorausgesetzt Pilze und Bakterien haben ähnliche Prozessraten. Bisher wurde jedoch nicht ausgiebig erforscht, welchen Anteil die einzelnen mikrobiellen Gemeinschaften an der N_2O Bildung tatsächlich haben.

Zur Unterscheidung der N_2O Bildungsprozesse in Bezug auf die beteiligten Mikroorganismen stellt die Isotopenanalyse von N_2O eine vielversprechende Anwendung dar. Vor allem die ^{15}N-Positionspräferenz im N_2O (SP = site preference, d.h. die Differenz zwischen den $\delta^{15}N$-Werten der außenständigen und zentralen N-Atome im linearen N_2O-Molekül) aus der Denitrifikation zeigte starke Unterschiede zwischen Reinkulturen einiger Bakterien (SP = -11 bis 0 ‰) und zwei untersuchten Pilzen (SP ~ 37‰). Jedoch wurden Bakterienreinkulturen bisher ausgiebiger untersucht als Pilzreinkulturen, auch wenn bekannt ist, dass sich die beteiligten Enzyme bei der Denitrifikation, bis auf die NO-Reduktase, zwischen Bakterien und Pilzen nicht unterscheiden. Die verschiedenen NO-Reduktasen sind vermutlich die Ursache für die unterschiedlichen SP-Werte des von Pilzen und Bakterien produzierten N_2O. Des Weiteren wurde bei Bakterien ein Austausch der Sauerstoffatome von Zwischenprodukten der Denitrifikation und dem umgebenden Wasser gefunden, der zwischen 4 und 100% beträgt. Ob es einen solchen Sauerstoffaustausch auch bei Pilzen gibt, ist bisher jedoch unerforscht. Würde der Sauerstoffaustausch bei pilzlicher Denitrifikation nicht

erfolgen, ermöglichte dies neben der unterschiedlichen SP eine weitere Unterscheidung der Herkunft des N_2O. Der Sauerstoffaustausch würde signifikante Unterschiede in der O Isotopensignatur im N_2O pilzlicher bzw. bakterieller Herkunft verursachen.

In der vorliegenden Studie, die Aufschluss über die pilzliche N_2O Produktion aus der Denitrifikation geben soll, wurden drei Hauptthemen behandelt. In einem Isotopen-Tracerexperiment mit ^{18}O-angereichertem Wasser wurde untersucht, ob bei sechs Pilzreinkulturen ein Sauerstoffaustausch zwischen Wasser und Zwischenprodukten der Denitrifikation stattfindet. Die Pilzreinkulturen zeigten tatsächlich durch Inkorporation von ^{18}O aus Wasser in N_2O einen Sauerstoffaustausch. Auch Pilze können bis zu 100% des O während der Denitrifikation austauschen. Eine Unterscheidung zwischen der Denitrifikation durch Bakterien und Pilze anhand der Sauerstoffsignatur ist somit nicht möglich.

Das zweite Thema sollte Auskunft darüber geben, ob hohe SP-Werte des N_2O aus der Denitrifikation bei Pilzreinkulturen allgemeingültig sind. Neben den zwei bisher untersuchten wurden vier weitere Pilzreinkulturen inkubiert. Diese Studie zeigte für die getesteten Pilzarten ebenfalls höhere SP-Werte (SP = 19.7 bis 32.6‰) im Vergleich zum Wertebereich von Bakterienreinkulturen. Basierend auf den Ergebnissen zum Sauerstoffaustausch aus dem Isotopen-Tracerexperiment wurde für die jeweiligen sechs Pilze, anhand der im Rahmen dieses Versuchs ermittelten natürlichen Sauerstoffisotopensignaturen, Mechanismen zur O Isotopenfraktionierung untersucht. Dafür wurden, neben den Werten des Sauerstoffaustausches und der natürlichen O Isotopensignatur der Pilzreinkulturen, Werte für Fraktionierungseffekte aus der Literatur in einem Isotopenfraktionierungsmodell angewendet, um die Beteiligung der verschiedenen Enzyme, die während der Denitrifikation an dem Sauerstoffaustausch beteiligt sind, abzuschätzen. Im Vergleich zu den NO_3^-- und NO-Reduktasen wies die NO_2^--Reduktase einen maßgeblich höheren Sauerstoffaustausch auf.

Die Erkenntnisse aus den Experimenten mit den Pilzereinkulturen sollten im Rahmen des dritten Themas auf Ihre Übertragbarkeit auf die mikrobiellen Gemeinschaften in Böden untersucht werden, indem Bodeninkubationsversuche mit selektiver Hemmung der Organismengruppen (Pilze und Bakterien) durchgeführt wurden. Bei dieser Modifizierung der Methode zur Substrat-induzierten Respiration mit selektiver Hemmung (SIRIN) sollte untersucht werden, ob sich die spezifischen SP-Werte für Bakterien und Pilze nach selektiver Wachstumshemmung von Bodengemeinschaften durch spezifische Antibiotika nachweisen lassen. Die Ausprägung des Hemmungseffekts auf SP-Werte in den drei getesteten Böden entsprach nicht den Erwartungswerten, die sich aus den SP-Werten der Pilz- und Bakterienreinkulturen ergaben. Die ermittelten SP-Werte lagen in den meisten Fällen im

Bereich jener bakterieller Reinkulturen und eine Hemmung der Bakterien führte in keinem Fall zu der erwarteten Veränderungen der SP-Werte. Folglich konnten die SP-Werte dieser Versuche nicht dazu dienen, die N_2O Bildung in den gehemmten Varianten den verschiedenen Organismengruppen zu zuordnen. Ungeklärt blieb, ob dies durch fehlende Eignung der modifizierten SIRIN-Methode zu erklären ist, oder ob die an Reinkulturen beobachteten SP-Unterschiede zwischen Pilzen und Bakterien nicht auf mikrobielle Gemeinschaften der Versuchsböden übertragbar sind. Im Hinblick auf nach wie vor bestehende methodische Defizite bei der Untersuchung der Pilzdenitrifikation im Boden sollte dies in weitergehenden Studien geklärt werden.

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List of publications

2010

- KTBL conference, Bad Staffelstein, Germany (2010) (Emissionen landwirtschaftlich genutzter Böden): Anteil der pilzlichen und bakteriellen N_2O-Bildung in verschiedenen Böden -Konzept und Versuchsdesign- (poster presentation)

2011

- Annual Meeting of German Society of Soil Science (2011): Anteil von Pilzen und Bakterien an der N_2O-Bildung in verschiedenen Böden (oral presentation)
- Rohe, L., Well, R., Wrage, N., Anderson, T.-H-, Flessa, H. (2011). Anteil von Pilzen und Bakterien an der Lachgasbildung in verschiedenen Böden, Annual Meeting of German Society of Soil Science, non-peer-reviewed online-publication

2012

- Nitrogen Workshop, Wexford, Ireland (2012): Differentiation between fungi and bacteria as a source of N_2O formation in soil (poster presentation)
- 8th Isoecol, Brest, France (2012): Using site preference of N_2O to differentiate between fungal and bacterial N_2O formation in soil (poster presentation)
- Jesium, Leipzig, Germany (2012): Isotopomer ratios of N_2O produced during denitrification by fungal pure cultures and associated oxygen exchange with water (poster presentation)
- 17th Nitrogen Cycle Meeting, Oslo, Norway (2012): Isotopomer ratios of N_2O produced during denitrification by fungal pure cultures and associated oxygen exchange with water (poster presentation)

2013

- Lewicka-Szczebak, D., Well, R., Giesemann, A., Rohe, L., Wolf, U. (2013): An enhanced technique for automated determination of ^{15}N signatures of N_2, (N_2+N_2O) and N_2O in gas samples, Rapid Communications in Mass Spectrometry 27, 1548-1558.
- General assembly of European Geoscience Union, Vienna, Austria (2013): Isotope Effects and O Exchange with water during N_2O Production by Denitrifying Fungi (poster presentation)
- COST-SIBAE Meeting, Wroclaw, Poland (2013): Selective inhibition and Isotopomer Analysis of N_2O to Estimate the N_2O Formation by Fungal Denitrification in Soil (poster presentation)
- First Conference of Scientific Cooperation between Lower Saxony and Israel (2013): Isotopomer analysis of N_2O after selective inhibition to estimate fungal N_2O fomation during denitrification in soil (poster presentation)
- ASI-Jahrestagung (2013): Selective inhibition and isotopomer analysis of N_2O to estimate the N_2O formation by fungal denitrification in soil (poster presetation)

2014

- Rohe, L., Anderson, T.-H., Braker, B., Flessa, H., Giesemann, A., Wrage-Mönnig, N., Well, R., 2014. Fungal Oxygen Exchange between Denitrification Intermediates and Water. Rapid Communications in Mass Spectrometry 28, 377-384.

Danksagung

Das Promotionsprojekt war Teil des Projektes „Pathways of N_2O with special emphasis on the distinction between fungal and bacterial denitrification as affected by soil type, land use and water-content –quantification of N-rates and associated isotope effects“, das vom Land Niedersachsen und der VolkswagenStiftung finanziert wurde.

Als erstes möchte ich Prof. Dr. Nicole Wrage-Mönnig und Dr. Reinhard Well für die Betreuung des Projektes danken. Herzlichen Dank für eure Unterstützung, ohne die die Bearbeitung des Projektes nicht möglich gewesen wäre. Eure Hilfestellung und Erfahrung bei der Umsetzung der Projektziele hat mich immer wieder vorangetrieben. Vielen Dank auch für die Motivation, die ich aus Gesprächen mit Euch schöpfen konnte. Obwohl die Zeit im Arbeitsalltag meist knapp war, habt ihr euch immer Zeit genommen für meine Fragen oder Probleme. Vielen Dank!

Prof. Dr. Johannes Isselstein und Sabine Hippe danke ich für die freundliche Aufnahme in der Abteilung Graslandwissenschaften des DNPW der Universität Göttingen.

Ich freue mich, dass ich auch in einem zweiten Team so herzlichen aufgenommen wurde und bedanke mich daher beim Thünen-Institut für Agrarklimaschutz, besonders bei Prof. Dr. Heinz Flessa, für die Möglichkeit meine Doktorarbeit dort schreiben zu können.

Ein besonderer Dank gilt Frau Dr. Anderson für viele hilfreiche und unterstützende Gespräche über das Durchführen von Versuchen und Verfassen von Manuskripten. Ihre Erfahrung, die sie in die Bearbeitung einfließen lassen haben und ihre motivierenden Worte haben mir sehr geholfen.

Dr. Anette Giesemann danke ich für den einwöchigen Crashkurs zur Messung am Massenspektrometer und auch für zahlreiche Diskussionen über Probenmessungen, Ergebnisse und den Interpretationsmöglichkeiten!

Dr. Dominika Lewicka-Szczebak danke ich für viele Ideen zu Versuchsdurchführungen und viele anregende Diskussionen!

Natürlich wäre all die Arbeit ohne das tolle Laborteam nicht möglich gewesen. Daher danke ich dem technischen Personal für die Unterstützung im Labor, besonders Susanne Behn und Martina Heuer!

Bei Marianna Deppe, Greta Roth, Ulrike Wolf, Jasmin Miltz und Caroline Buchen bedanke ich mich für viele aufmunternde Gespräche und auch für viele Hilfestellungen.

Während meiner Doktorarbeit hatte ich zwei tolle Laboraufenthalte. Ich bedanke mich herzlich für eure Hilfe und die Einführung in die Arbeit mit Bakterien und Pilzen. Durch die beiden Laboraufenthalte konnte ich die Methoden, die ich bei den Versuchen angewendet habe erlernen und hatte dadurch die Möglichkeit auch im AK-Labor mit beiden Methoden zu arbeiten. Die Denitrifizierer-Methode wurde mir bei Dr. Jan Kaiser gezeigt. Vielen Dank für die spontane Möglichkeit zu euch zu kommen und für die viele Zeit, die ihr euch genommen habt, um mir die Methode zu zeigen! Many thanks to Laura Campisi who spent her precious time to teach us three phD students in the denitrifier method! Thanks to the School of Environmental Science of the University of East Anglia for giving me the opportunity to get practical experience in the denitrifier method. Das Arbeiten mit Pilzreinkulturen habe ich bei Dr. Gesche Braker am des Max-Plank-Instituts für terrestrische Mikrobiologie in Marburg kennen gelernt. Vielen Dank für deine Hilfe Pilzstämme zu bekommen und die Zeit, die du geopfert hast!

Dem Thünen-Institut für Biodiversität, besonders Prof. Dr. Tebbe, danke ich für die Bereitstellung von Arbeitsmaterial und für die Unterstützung bei der Umsetzung der Reinkulturversuche am TI. Für zahlreiche Gespräche mit hilfreichen Hinweisen, ohne die das mikrobiologische Arbeiten im Labor sehr schwer geworden wäre, danke ich Dr. Anja Dohrmann, Karin Trescher, Jana Usarek und Michael Hemkemeyer. Außerdem danke ich Prof. Dr. Stefan Schrader für die Bereitstellung des Autoklaven und anderen Labormaterialien.

Bei dem Versuchsgut Reinshof, der Versuchsstation Braunschweig vom FLI und der Familie Riechers bedanke ich mich für die Möglichkeit Bodenproben für die Inkubationsversuche zu bekommen.

Für Analysen von ^{15}N in N_2O und N_2 und ^{18}O im Wasser danke ich dem KOSI der Universität Göttingen, vor allem Dr. Jens Dyckmans, Reinhard Langel und Lars Szwec.

Schließlich bedanke ich mich bei meinen Eltern und meiner Schwester für die Unterstützung!