Comparing modified substrate induced respiration with selective inhibition (SIRIN) and N_2O isotope approaches to estimate fungal contribution to denitrification in three arable soils under anoxic conditions

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Abstract

Pure culture studies provide evidence of the ability of soil fungi to produce nitrous oxide (N_2O) during denitrification. Soil studies with selective inhibition indicated a possible dominance of fungal compared to bacterial N_2O production in soil, which drew more attention to fungal denitrification. Analyzing the isotopic composition of N_2O, especially its ^{15}N site preference (SP_{N2O}), showed that N_2O of pure bacterial or fungal cultures differed in SP_{N2O} values, which might enable the quantification of fungal N_2O based on the isotopic endmember signatures of N_2O produced by fungi and bacteria.

This study aimed to identify the fungal contribution to N_2O emissions and determine fungal SP_{N2O} from repacked soil samples incubated under anaerobic conditions. Three approaches were established (modified substrate induced respiration with selective inhibition (SIRIN) approach, endmember mixing approach (IEM) and the SP/$\delta^{18}O$ mapping approach (SP/$\delta^{18}O$ Map) to independently investigate the fungal fraction contributing to N_2O from denitrification. Three soils were incubated under anaerobic conditions to promote denitrification with four treatments of the SIRIN approach. While one treatment without microbial inhibition served as a control the other three treatments were amended with inhibitors to selectively inhibit bacterial, fungal or bacterial and fungal growth. These treatments were performed in three varieties. In one variety, the ^{15}N tracer technique was used to estimate the effect of N_2O reduction on N_2O produced, while two other varieties were performed under natural isotopic conditions but with and without acetylene.

All three approaches revealed a small fungal contribution to N_2O fluxes (f_{FD}) under anaerobic conditions in the soils tested. Quantifying the fungal fraction with modified SIRIN was not successful due large amounts of uninhibited N_2O production and pre-incubation effects. In only one soil, f_{FD} using modified SIRIN could be estimated and resulted in 28±9 %, which was possibly overestimated as results obtained by IEM and SP/$\delta^{18}O$ Map for this soil resulted in f_{FD} of below 15 and 20 %, respectively. As a consequence of the unsuccessful SIRIN approach, estimation of fungal SP_{N2O} values was impossible.

To our knowledge, this study was the first attempt to quantify the fungal contribution to anaerobic N_2O production by simultaneous application of three approaches, i.e. modified SIRIN, IEM and SP/$\delta^{18}O$ Map. While all successful methods coincided by suggesting a small or missing fungal contribution, further studies with stimulated fungal N_2O fluxes by added fungal C substrates and an improved modified SIRIN approach, including alternative inhibitors, are needed to better cross-validate the methods.

1. Introduction

The greenhouse gas nitrous oxide (N_2O) contributes to global warming and to the depletion of the ozone layer in the stratosphere (Crutzen, 1970; IPCC, 2013). The largest anthropogenic N_2O emissions originate from agricultural soils and are mainly produced during microbial nitrification, nitrifier denitrification and denitrification (Firestone and Davidson, 1989; Bremner, 1997; IPCC, 2013; Wrage-Mönnig et al., 2018). 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 improve knowledge about the production pathways and the microorganisms involved.

Denitrification is the stepwise 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; Shoun et al., 2012; Higgins et al., 2018). 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). Pure culture studies indicated, however, that although only some fungal species (e.g. *Fusarium* strains) are performing respiratory denitrification, these may produce substantial amounts of N_2O (Higgins et al., 2018; Keuschnig et al., 2020). N_2O produced by fungi may thus contribute largely to N_2O from denitrification in soil. Firstly, fungi dominate the biomass in soil (up to 96 %) compared to bacteria in general (Ruzicka et al., 2000; Braker and Conrad, 2011). A respiratory fungal-to-bacterial (F:B) ratio of 4 is typical for arable soils (Anderson and Domsch, 1975; Blagodatskaya and Anderson, 1998). Secondly, the fact that N_2O is the major end product of fungal denitrification led to the assumption that the potential activity of fungal N_2O production in soil may exceed that of bacteria, provided that both microbial groups have the same specific denitrification activity (Shoun et al., 1992; Sutka et al., 2008). Thirdly, co-denitrification was found to often co-occur with fungal denitrification (Shoun and Tanimoto, 1991; Tanimoto et al., 1992). During co-denitrification, a hybrid N_2O is formed using one N atom from NO_2^- and one N atom from compounds like azide or ammonium (NH_4^+) for N_2O production (Tanimoto et al., 1992; Shoun et al., 1992; Rohe et al., 2017; Spott et al., 2011). This pathway was found to contribute about 92 % to N_2O produced in an incubation experiment with a grassland soil under anaerobic conditions (Laughlin and Stevens, 2002). This again stresses the large potential N_2O production by fungi. Additionally, as shown in pure culture studies, not only co-denitrification, but also abiotic N_2O formation may co-occur with fungal denitrification (Phillips et al., 2016a; Phillips et al., 2016b; Rohe et al., 2017), which may contribute to N_2O production but potentially lead to overestimation of the importance of fungal N_2O production. However, pathway differentiation is still challenging.

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. This method is known as substrate induced respiration with selective inhibition (SIRIN) (Anderson and Domsch, 1975; Laughlin and Stevens, 2002;

Crenshaw et al., 2008; Blagodatskaya et al., 2010; 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, respectively (Laughlin and Stevens, 2002)), indicating that fungi might dominate N_2O production (Laughlin and Stevens, 2002; McLain and Martens, 2006; Crenshaw et al., 2008; Blagodatskaya et al., 2010; Long et al., 2013; Chen et al., 2014; Chen et al., 2015).

Analysing the isotopic composition of N_2O might also be a promising tool to distinguish between N_2O from bacterial and fungal denitrification and other pathways. Especially, the isotopomer ratios of N_2O (i.e. N_2O molecules with the same bulk ^{15}N isotopic enrichment but showing different positions of ^{15}N in the linear N_2O molecule (Ostrom and Ostrom, 2017)) in pure culture studies showed differences in N_2O of bacterial and fungal denitrification (Sutka et al., 2006; Sutka et al., 2008; Frame and Casciotti, 2010; Rohe et al., 2014a; Rohe et al., 2017). This might be suitable for distinguishing 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_{N2O}), 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_{N2O} values of N_2O of six pure fungal cultures was between 16 and 37 ‰ (Sutka et al., 2008; Rohe et al., 2014a; Maeda et al., 2015; Rohe et al., 2017), whereas several bacteria produced N_2O with SP_{N2O} values between -7.5 and +3.5 ‰ during denitrification (Toyoda et al., 2005; Sutka et al., 2006; Rohe et al., 2017). However, the SP_{N2O} value of N_2O produced by pure bacterial cultures during nitrification is approximately 33 ‰ and interferes with SP_{N2O} values of fungal denitrification (Sutka et al., 2006; Sutka et al., 2008; Rohe et al., 2014a). This demonstrates the difficulty to use SP_{N2O} values as an indicator for different organism groups contributing to N_2O production from soil, where different pathways may co-occur.

While it is generally assumed that SP_{N2O} values of N_2O produced by fungal pure cultures during denitrification is transferable to N_2O produced by fungal soil communities, this has not yet been proven. Until now, studies reporting possible ranges of fungal contributions to N_2O fluxes from soil were based on SP_{N2O} values of pure cultures (Köster et al., 2013b; Zou et al., 2014; Lewicka-Szczebak et al., 2017; Senbayram et al., 2018; Senbayram et al., 2020; Lewicka-Szczebak et al., 2014), but uncertainty of this approach arose from the fact that SP_{N2O} values between 16 and 37 ‰ have been reported (Sutka et al., 2008; Maeda et al., 2015; Rohe et al., 2017). It would thus be useful to constrain fungal SP_{N2O} values for a specific soil or soil type.

Although SP_{N2O} values are independent of isotopic signatures of the precursors, $\delta^{15}N$ and $\delta^{18}O$ values of produced N_2O ($\delta^{15}N^{bulk}_{N2O}$ and $\delta^{18}O_{N2O}$, respectively) result from the isotopic signature of the precursor and isotopic fractionation during N_2O production (Toyoda et al., 2005; Frame and Casciotti, 2010). Regarding $\delta^{18}O_{N2O}$, a complete exchange of oxygen (O) between NO_3^- and soil water can be assumed and consequently, one can use the $\delta^{18}O$ values of soil water for interpretation of $\delta^{18}O_{N2O}$ values (Lewicka-Szczebak et al., 2014; Kool et al., 2009; Snider et al., 2009). However, interpretation of $\delta^{18}O_{N2O}$ values from different microbial groups may be more complex due to incomplete O exchange: variations in the extent of O exchange between water and N oxides affect the final $\delta^{18}O_{N2O}$ value (Garber and Hollocher, 1982; Aerssens et al., 1986; Kool et al., 2007; Rohe et al., 2014b; Rohe et al., 2017). Recently, fungal and bacterial N_2O showed different ranges for $\delta^{18}O_{N2O}$ values and this isotopic signature may also be helpful in differentiation of these pathways (Lewicka-Szczebak et al., 2016).

Moreover, $\delta^{15}N^{bulk}_{N2O}$, $\delta^{18}O_{N2O}$ and SP_{N2O} values are in the course of denitrification affected by isotopic fractionation due to N_2O reduction. During N_2O reduction, 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 that is relatively isotopically enriched in ^{15}N and ^{18}O and shows

larger SP_{N2O} values compared to SP_{N2O} values of N_2O from denitrification without the reduction step (Popp et al., 2002; Ostrom et al., 2007). One possibility for quantifying N_2O reduction to N_2 during denitrification is the application of ^{15}N tracing experiments using ^{15}N enriched substrates and analysing $^{15}N_2$ fluxes (Well et al., 2006; Lewicka-Szczebak et al., 2014). Another possibility is based on N_2O isotopocules, which also enables to quantify pathways producing N_2O (i.e. N_2O with differing number or positions of N or O isotopes (Ostrom and Ostrom, 2017)). In this latter approach, i.e. the isotope mapping approach, isotope fractionation factors together with $\delta^{15}N$ values of precursors ($\delta^{15}N_{NOx}$) as well as $\delta^{15}N^{bulk}{}_{N2O}$ and SP_{N2O} values of N_2O produced were used (Toyoda et al., 2011). Recently, this isotope mapping approach was further developed (SP/$\delta^{18}O$ Map) using $\delta^{18}O_{N2O}$ and SP_{N2O} values and $\delta^{18}O$ values of precursors (Lewicka-Szczebak et al., 2014; Lewicka-Szczebak et al., 2017) by using different slopes of N_2O reduction and mixing lines in the $\delta^{18}O$ – SP isotope plot. This approach allows for differentiation of isotope effects due to N_2O reduction and admixture of fungal N_2O. Hence, N_2O reduction can be estimated together with the N_2O mixing due to application of two isotopic signatures of N_2O. Thus, the N_2O reduction to N_2 does not affect the outcome of the SP/$\delta^{18}O$ Map.

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 using the isotope endmember mixing approach (IEM) with SP_{N2O} values of N_2O produced in soil (SP_{prod}), provided N_2O reduction, which is altering SP_{N2O} values of emitted N_2O, does not occur (Ostrom et al., 2010; Ostrom and Ostrom, 2011). This can be ensured in laboratory experiments by inhibiting N_2O reduction to N_2 using acetylene (C_2H_2) during anaerobic incubation experiments (Yoshinari and Knowles, 1976; Groffman et al., 2006; Well and Flessa, 2009; Nadeem et al., 2013). Hence, C_2H_2 inhibition might be suitable to quantify SP_{prod} values in soils exhibiting significant N_2O reduction and would thus allow quantification of fungal N_2O fluxes based on SP_{prod} values.

So far, the described methods for distinguishing between fungal and bacterial N_2O emission have not been evaluated and compared in the same soil and their accuracy and possible bias remains unknown. We hypothesized that the fungal fraction contributing to N_2O from denitrification in different soils using a modified SIRIN approach and isotopic methods will be correlated but not match exactly due to limited inhibitability of microbial communities and variability in SP_{N2O} endmember values. Furthermore, successful application of the modified SIRIN approach with determined fungal fraction contributing to N_2O from denitrification will yield fungal SP_{N2O} endmember values within the range of values previously reported in the literature.

Therefore, this study aims at (i) determining the fungal contribution to N_2O production by denitrification under anoxic conditions and glucose addition using three arable soils and three approaches (modified SIRIN, IEM and the $SP/\delta^{18}O$ Map), and to assess their usefulness in soil studies and thus assess factors of potential bias of the methods and (ii) to estimate the SP_{N2O} values from fungal soil communities and thus to evaluate the transferability of the pure culture range of the fungal SP_{N2O} endmember values.

2. Materials and Methods

2.1 Soil samples

All experiments were conducted with three arable soils differing in texture, C_{org} content, C/N ratio and pH. Thus it was assumed that the soils harbour different denitrifying communities, i.e., different fractions of bacteria and fungi contributing to denitrification. One of the soils was sampled during a second season to evaluate if the

fungal fraction contributing to N_2O production is soil-specific or can be subject to seasonal change of microbial communities. As this soil was sampled at two different time points, we conducted four experiments and named the different experiments "*Soil 1*", "*Soil 2*", "*Soil 3*" and "*Soil 4*": *Soil 1* with loamy sand sampled in December 2012, *Soil 2* with sand sampled in January 2013, *Soil 3* with silt loam sampled in December 2012, and *Soil 4* with loamy sand sampled in June 2011 (Table 1).

Soil samples of the upper 30 cm were collected in plastic bags aerated via cotton wool stoppers and stored at 6 °C for maximally two months. To get information about the initial soil status, the mineral nitrogen content (N_{min}) 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 analysing NO_3^- and NH_4^+ concentrations in the extracts with a Continuous-Flow-Analyser (SKALAR, Germany) directly after sample collection. Other soil characteristics (C and N content, soil pH value, isotopic values of soil NO_3^- and NO_2^-) were analysed with samples of *Soil 1, Soil 2* and *Soil 3*. Total contents of C and N in soil samples were analysed by dry combustion of ground samples (LECO TruSpec, Germany). The soil pH was measured in 0.01 M $CaCl_2$. The $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^- and NO_2^- ($\delta^{15}N_{NOx}$ and $\delta^{18}O_{NOx}$, respectively) in soil extracts (with 0.01 M calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$)) were analysed by the bacterial denitrifier method (Casciotti et al., 2002).

To gain information on the respiratory biomass, the three soils were analysed for substrate induced respiration (SIR) according to Anderson and Domsch (1978) and the respiratory F:B ratio was analysed 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) (Anderson and Domsch, 1975). The scheme of glucose and growth inhibitor combinations is listed below in section "Methodical approach". The characteristics of the soils are listed in Table 1.

2.2 Methodical approach

The experimental setup with various measures is presented in the following sections and illustrated in Figure 1. Important terms used and its descriptions are listed in Supplementary Material, Table S1.

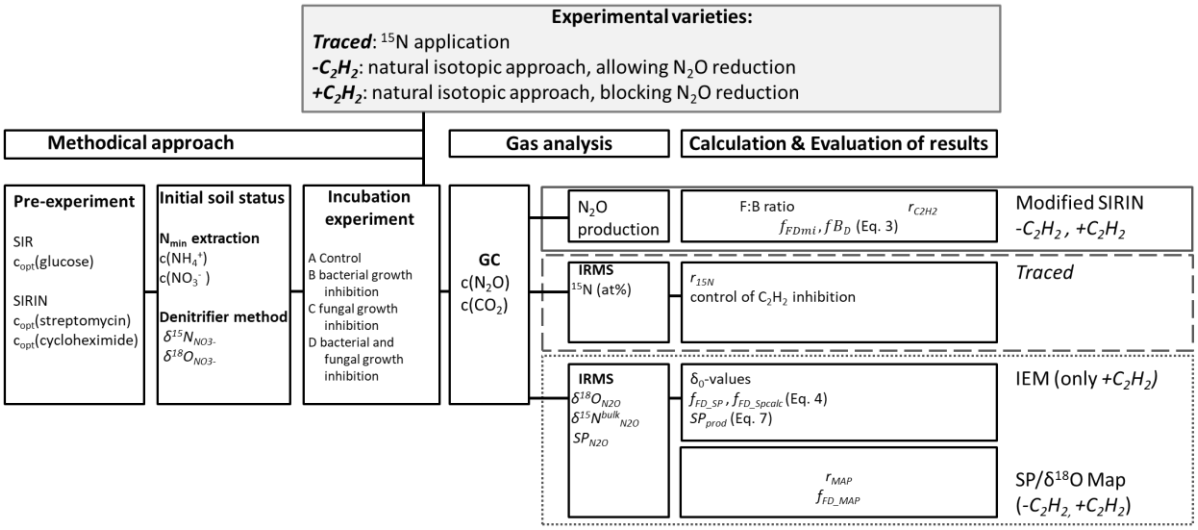


Figure 1: Schematic to represent the experimental setup of soil incubations with three varieties (*traced*, *-C_2H_2*, *+C_2H_2*). The methodical approach comprised a pre-experiment and the incubation experiment with a modified substrate induced respiration with selective inhibition (SIRIN) approach. Produced gas was analysed for its concentration ($c(CO_2)$ and $c(N_2O)$) using gas chromatography (GC) and N_2O was further analysed by isotope ratio mass spectrometry (IRMS). Please refer to the Material & Methods section for more information.

2.2.1 SIRIN pre-experiment

As in most studies applying the SIRIN method on N_2O emissions (e. g. Laughlin and Stevens, 2002; Chen et al., 2014; Ladan and Jacinthe, 2016), a pre-experiment was conducted with samples collected in 2010, in order to get information about optimal substrate and inhibitor concentrations for substrate induced respiration with growth inhibition. The pre-experiments of the present study were conducted in two steps as described in the original methods, i.e., CO_2 production under oxic conditions was analysed to estimate the substrate induced respiration by the SIR method (Anderson and Domsch, 1978) and the substrate induced respiration with selective inhibition by the SIRIN method (Anderson and Domsch, 1975) as follows.

In a first pre-experiment (Figure 1), the SIR method (Anderson and Domsch, 1978) was used to get information about the amount of respiratory biomass in soil under oxic conditions. In this pre-experiment glucose served as substrate to initiate microbial growth (Anderson and Domsch, 1975). 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 by analysing CO_2 production (Anderson and Domsch, 1978). C_{opt}(glucose)) was 1.0 mg g^{-1} for *Soil 2* (sand) and 1.5 mg g^{-1} for *Soils 1, 3* and *4* (loamy sand and silt loam).

In a second pre-experiment (Figure 1), the SIRIN method was used according to Anderson and Domsch (1975) for determining the respiratory F:B ratio. The c_{opt}(glucose) determined in the first pre-experiment was used, while selectivity of the inhibitor combinations of streptomycin (bacterial respiratory inhibitor) and cycloheximide (fungal respiratory inhibitor) were tested with three concentrations (0.75, 1.0, 1.5 mg g^{-1} dw, respectively). The optimal concentration for inhibition of fungal respiration was 0.75 mg g^{-1} dw soil cycloheximide (c_{opt}(cycloheximide)) and for bacterial respiratory inhibition 1.0 mg g^{-1} dw soil streptomycin (c_{opt}(streptomycin)). As in the first pre-experiment, CO_2 production under oxic conditions was analysed. The determined optimal concentrations of glucose, streptomycin and cycloheximide were used in the modified SIRIN approach, because we supposed that optimal concentrations for CO_2 respiration work as well for denitrification.

Table 1: Soil characteristics of three arable soils from Germany used for incubation experiments (*Soil*) (standard deviation in brackets). Except for NH_4^+ and NO_3^-, soil characteristics of loamy sand were only analysed once for samples collected in 2012.

Soil (Year)	Soil texture	Soil type (WRB)	Location	C content [%]	N content [%]	NH_4^+ [mg N kg^{-1}]	NO_3^- [mg N kg^{-1}]	pH ($CaCl_2$)	$\delta^{15}N_{NOx}$ [‰][e]	$\delta^{18}O_{NOx}$ [‰][e]	F:B[f]	Biomass[g] [µg C gdw^{-1} soil]
1 (2012)	Loamy sand	Haplic Luvisol	Braun-schweig[a]	1.43 (<0.01)	0.10 (<0.01)	0.4 (<0.1)	14.1 (2.1)	5.67	3.98	-4.82	2.6	234
4 (2011)				/	/	1.0 (0.4)	11.0 (0.3)	/	/	/	/	/
2 (2013)	Sand	Gleyic Podzol	Wenne-bostel[b]	2.31 (0.04)	0.14 (<0.01)	1.9 (0.2)	6.6 (0.2)	5.54	0.73	-2.68	2.6	161
3 (2013)	Silt loam	Haplic Luvisol	Götting-en[c]	1.62 (0.02)	0.13 (<0.01)	n.d.[d]	22.7 (<0.1)	7.38	4.18	2.32	4.9	389

[a] **Experimental Station of the Friedrich-Löffler 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 0.06 mg kg^{-1} NH_4^+-N)**
[e] **Isotopic values of natural soil NO_3^- using the denitrifier method (Casciotti et al., 2002).**
[f] **Respiratory fungal-to-bacterial (F:B) ratio analysed by SIRIN method (Anderson and Domsch, 1973, 1975).**
[g] **Respiratory biomass analysed by CO_2 production from SIR method (Anderson and Domsch, 1978).**

2.2.2 Soil incubation with selective inhibition to determine N_2O forming processes

The experimental design included two approaches, (i.) microbial inhibition by fungal and/or bacterial inhibitors and (ii.) activity of N_2O reductase analysed either by inhibition with C_2H_2 or quantification by ^{15}N tracing (Figure 1). To address the microbial inhibition approach (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

To address the other approach (ii.), all microbial inhibitor treatments were conducted in three 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 "-C_2H_2") (Figure 1). In total, there were 48 experimental treatments and 144 vessels (four *Soils* with four inhibitor treatments (A, B, C, D) and three varieties (*traced,* +C_2H_2 and -C_2H_2,), each in triplicate).

The soil was adjusted to 80 % water filled pore space (WFPS) with distilled water. Simultaneously, the soil was fertilized with NO_3^- (varieties -C_2H_2, +C_2H_2, and *traced*). The soil sample used with *Soil 4* was incubated prior to the other soils and was amended with 60 mg N kg^{-1} $NaNO_3$, while in agreement with other experiments conducted in our laboratory, 50 mg N kg^{-1} KNO_3 were used with *Soil 1, 2* and *3*. . In variety *traced,* NO_3^- with a ^{15}N enrichment of 50 atom% (at%) was used. 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 plastic stopcocks (Braun, Melsungen, Germany). According to the original SIRIN method (Anderson and Domsch, 1973, 1978) a mixture of c_{opt}(glucose) and carrier material talcum (5 mg talcum g dw^{-1}) was added to soil of treatment A and together with the growth inhibitors to the soil of treatments B, C and D. The soil and additives of each treatment were mixed for 90 seconds with a handheld electric mixer. During packing, the soil density was adjusted to an expected target soil density of 1.6 g cm^{-3} in *Soil 1, 2* and *4* and of 1.3 g cm^{-3} in *Soil 3* to imitate field conditions. To ultimately achieve denitrifying conditions in all treatments and to 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 to exchange the headspace 10 times. Directly following, 85 mL of the gas in the headspace in variety +C_2H_2 were exchanged by pure C_2H_2 resulting in 10 kPa C_2H_2 in the headspace. The manual sample collection of 14 mL gas in duplicates with a plastic syringe was performed after six, eight and ten hours (*Soil 1, 2* and *3*) or two, four and eight hours (*Soil 4*) of incubation time, respectively. The removed gas was replaced by the same amount of N_2.

2.3 Gas analysis

Gas samples were analysed for N_2O and CO_2 concentrations ($c(N_2O)$ and $c(CO_2)$) with gas chromatography (GC, Agilent 7890A, Agilent, Böblingen, Germany) (Figure 1). The analytical precision of measurements was derived from analysing laboratory standards of different concentrations (0.5-1,000 ppm N_2O and 340-10,000 ppm CO_2) and resulted in a measurement precision of 1 % for N_2O and 0.5 % for CO_2. The instrumental detection limit of N_2O was 4 µg N kg^{-1} h^{-1} and of CO_2 it was 137 µg C kg^{-1} h^{-1}. As a control, N_2 and O_2 concentrations in the samples were analysed with GC to ensure anaerobic conditions during the incubation for N_2O production from denitrification. CO_2 and N_2O production rates were calculated by averaging the measured N_2O production, i.e., between the time point of flushing with N_2 (t=0) and six, eight or ten hours (or two, four and eight hours with *Soil 4*).

The N_2O isotopic analysis of the gas samples of varieties $-C_2H_2$ and $+C_2H_2$ (Figure 1) were performed on a pre-concentrator (PreCon, Thermo–Finnigan, Bremen, Germany) interfaced with a GC (Trace Gas Ultra, Thermo Scientific, Bremen, Germany) and analysed by isotope ratio mass spectrometry (IRMS, Delta V, Thermo Fisher Scientific, Bremen, Germany) (Brand, 1995; Toyoda and Yoshida, 1999; Köster et al., 2013b). A laboratory standard N_2O gas was used for calibration, having $\delta^{15}N^{bulk}_{N2O}$, $\delta^{18}O_{N2O}$ and SP_{N2O} values of -1.06 ‰, 40.22 ‰, and -2.13 ‰, respectively, in three concentrations (5, 10 and 20 ppm). The analytical precision was 0.1 ‰, 0.2 ‰ and 1.5 ‰ for $\delta^{15}N^{bulk}_{N2O}$, $\delta^{18}O_{N2O}$ and SP_{N2O} values, respectively. H_2O and CO_2 were trapped with magnesium perchlorate and ascarite, respectively, to prevent any interference with N_2O analysis.

The gas samples of variety *traced* from *Soil 1, 2,* and *3* were analysed 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 IRMS (MAT 253, Thermo Scientific, Bremen, Germany). The gas samples of variety *traced* from *Soil 4* were analysed at the Centre for Stable Isotope Research and Analysis (University of Göttingen, Germany). The N_2 produced was analysed using an elemental analyser (Carlo Erba ANA 1500) that was coupled to dual inlet IRMS (Finnigan MAT 251) (Well et al., 1998; Well et al., 2006). Isotopic values of N_2O of *Soil 4* (variety *traced*) were analysed in the same lab using a pre-concentration unit coupled to IRMS (Precon-DeltaXP, Thermo Scientific, Bremen, Germany) (Well et al., 2006). Isotope ratios were used applying the non-random distribution approach to calculate the fraction of N_2 and N_2O originating from the ^{15}N-labelled N pool as well as the ^{15}N enrichment of that N pool (a_p) (Bergsma et al., 2001; Spott et al., 2006).

2.4 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 and fungi should show the following relation between the four treatments:

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

The fungal contribution to N_2O production during denitrification with microbial inhibition (f_{FDmi}) can be calculated, when N_2O production of treatment D is significantly smaller than N_2O production of treatments A, B and C by:

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

A detailed discussion of inhibitor effects and difficulties with organisms that were not inhibited or abiotic sources (treatment D) is presented in section 4.1.

2.5 Isotope methods

2.5.1 Isotope 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 (IEM) proposed by Ostrom et al. (2010), which was established for calculating the bacterial fraction (f_{BD}) of N_2O production. Assuming that bacteria (*BD*) and fungi (*FD*) are the only sources of N_2O in soil, the ^{15}N site preference values of produced N_2O (SP_{prod}) results from the SP_{N2O} mixing balance:

$$SP_{prod} = f_{FD} * SP_{FD} + f_{BD} * SP_{BD} \quad \text{(Eq. 4)}$$

where f_{FD} and f_{BD} represent the fraction of N_2O produced by fungi and other N_2O sources than fungal denitrification, respectively, and SP_{FD} and SP_{BD} are the respective SP_{N2O} endmember values (Ostrom et al., 2010; Ostrom and Ostrom, 2011). This calculation was based on the assumption that the sum of f_{BD} and f_{FD} equals 1 and that N_2O reduction to N_2 is negligible. The mean SP_{FD} value was assumed to be 33.6 ‰ (Sutka et al., 2008; Maeda et al., 2015; Rohe et al., 2014a; Rohe et al., 2017) and the SP_{BD} value from heterotrophic denitrification was assumed with minimum and maximum values from -7.5 to +3.7 ‰ (Yu et al., 2020). For this IEM approach, only results from variety $+C_2H_2$ could be used to calculate the fungal fraction contributing to N_2O production (f_{FD_SP}), as microorganisms of this variety produce N_2O that is not affected by reduction to N_2. The f_{FD_SP} contributing to N_2O production during denitrification was calculated using the measured SP_{N2O} value of variety $+C_2H_2$ as SP_{prod} value in Eq. 4 that was solved for f_{FD} (f_{FD} = 1-((SP_{prod}-SP_{FD})/(SP_{BD}-SP_{FD}))). By applying this equation, a range for f_{FD_SP} is received when using minimum and maximum SP_{BD} values.

2.5.2 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 product ratio of denitrification [N_2O/(N_2+N_2O)] as given by the variety *traced* (r_{15N}) was calculated as:

$$r_{15N} = \frac{^{15}N_{N2O}}{^{15}N_{N2} + ^{15}N_{N2O}} \quad \text{(Eq. 5)}$$

with $^{15}N_{N2O}$ and $^{15}N_{N2}$ representing N_2O and N_2 produced in the ^{15}N-labeled fertilizer pool. To check the effectiveness of C_2H_2 to block the N_2O reduction, r_{15N} was compared with r_{C2H2}, where the latter can be calculated from N_2O production rates of varieties $-C_2H_2$ and $+C_2H_2$:

$$r_{C2H2} = \frac{N_2O_{-C2H2}}{N_2O_{+C2H2}} \quad \text{(Eq. 6)}$$

with N_2O_{-C2H2} and N_2O_{+C2H2} representing the N_2O produced in varieties $-C_2H_2$ and $+C_2H_2$, respectively.

It was possible to assess the completeness of blockage of N_2O reduction by C_2H_2 with the experimental setup. If r_{15N} and r_{C2H2} were in agreement, a complete blockage of N_2O reduction could be assumed. This enabled us to

estimate reduction effects on the isotopic signatures of N_2O by comparing the $\delta 0$ values, i.e., isotopic values of N_2O produced without N_2O reduction effects of variety $+C_2H_2$, with isotopic values of N_2O of variety $-C_2H_2$. The information on the product ratio was used as an additional possibility to calculate the f_{FD} also for variety $-C_2H_2$. The Rayleigh-type model presented by Lewicka-Szczebak et al. (2017) and Senbayram et al. (2018) for similar closed-system incubations was used to calculate the ^{15}N site preference values of the originally produced N_2O of variety $-C_2H_2$ (SP_{prod}). SP values of emitted N_2O, i.e. after partial reduction of produced N_2O ($SP_{N2O\text{-}r}$), were corrected with the net isotope effect of N_2O reduction (ηr) and the r_{15N} as follows:

$$SP_{prod} = SP_{N2O-r} + \eta r \ln(r_{15N}) \quad \text{(Eq. 7)}$$

According to Yu et al. (2020) the ηr was assumed to be -6 ‰. Subsequently, Eq. 4 was used to calculate the f_{FD} by using SP_{prod} values of variety $-C_2H_2$ (f_{FD_SPcalc}) obtained from Eq. 7.

2.5.3 SP/δ^{18}O isotope mapping approach (SP/δ^{18}O Map)

The f_{FD} contributing to N_2O production from denitrification in soil samples was also estimated with the SP/δ^{18}O Map (f_{FD_MAP}) (Lewicka-Szczebak et al., 2017; Lewicka-Szczebak et al., 2020). This method allows for estimating both the f_{FD} and N_2O product ratio [$N_2O/(N_2+N_2O)$] (r_{Map}). For precise estimations, the $\delta^{18}O$ values of soil water ($\delta^{18}O_{H2O}$) applied in the experiments are needed and these values were not determined. However, since we have independent information on the N_2O product ratio from the *traced* variety (r_{15N}), we can calculate the possible $\delta^{18}O_{H2O}$ values of soil to get the nearest N_2O product ratios in natural and ^{15}N treatments. The fitting of $\delta^{18}O_{H2O}$ values was performed for mean, minimal und maximal values of SP_{BD} (-1.9, -7.5 and 3.7 ‰, respectively) and aimed at obtaining the minimal difference between r_{Map} and that measured in the *traced* variety, *i.e.*, the minimal value of $(r_{15N} - r_{Map})^2$ (according to least squares method) (for explanation of the product ratio see section 2.5.2). This further allows calculation of the possible ranges for f_{FD} for particular $\delta^{18}O_{H2O}$ fitted values (Table 4) based on the SP/δ^{18}O mapping approach (Lewicka-Szczebak et al., 2017; Lewicka-Szczebak et al., 2020). Namely, the fitted $\delta^{18}O_{H2O}$ values are applied to properly correct the $\delta^{18}O_{N2O}$ values of the mixing endmembers (*BD* and *FD*) which depend on the ambient water. Afterwards, the corrected values of mixing endmembers are applied to calculate the f_{FD} values. The calculations with this approach may be performed assuming two different scenarios of the interplay between N_2O mixing and reduction (Lewicka-Szczebak et al., 2017; Lewicka-Szczebak et al., 2020), but for this study both scenarios yielded almost identical results (maximal differences of 0.02 in N_2O product ratio and 2 % for f_{FD} were found), due to f_{BD} near 100 %. Hence, we only provide the results assuming the reduction of bacterial N_2O followed by mixing with fungal N_2O. In the following, all calculated fractions are presented in percent (%).

2.6 Other sources of N_2O

Assuming that denitrification was the only source of N_2O in the incubation experiment, the expected ^{15}N enrichment in N_2O produced ($^{15}N_{N2O\_exp}$) was given by

$$^{15}N_{N2O\_exp}\ [at\%] = \frac{(N_{soil} \times {}^{15}N_{nat}) + (N_{fert} \times {}^{15}N_{fert})}{N^{bulk}} \quad \text{(Eq. 8)}$$

with N_{soil}, N_{fert} and N^{bulk} describing the amount of N [mg] in unfertilized soil samples (Table 1), fertilizer and fertilized soil samples, respectively, and $^{15}N_{nat}$ and $^{15}N_{fert}$ is standing for ^{15}N enrichment under natural conditions (0.3663 at%) and in fertilizer (50 at%), respectively. Comparison of measured ^{15}N enrichment in N_2O and $^{15}N_{N2O\_exp}$ gave information about the contribution of processes other than denitrification to N_2O production.

2.7 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_{N2O}, $\delta^{15}N^{bulk}_{N2O}$ and $\delta^{18}O_{N2O}$ values. The pairwise comparison with Tukey's HSD test allowed to find differences between soils, varieties and treatments influencing N_2O production, CO_2 production, and isotopic values. Significant effects of soils and treatments on r_{C2H2} and r_{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 -C_2H_2 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 ANOVAs treatments were excluded, when replicates were $n < 3$. This was the case when only one or two samples out of three replicates could be analysed. This is denoted in the captions of tables (Table 2 and 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). Excel Solver tool was used to determine the $\delta^{18}O_{H2O}$ values in the application of SP/δ^{18}O Map calculations.

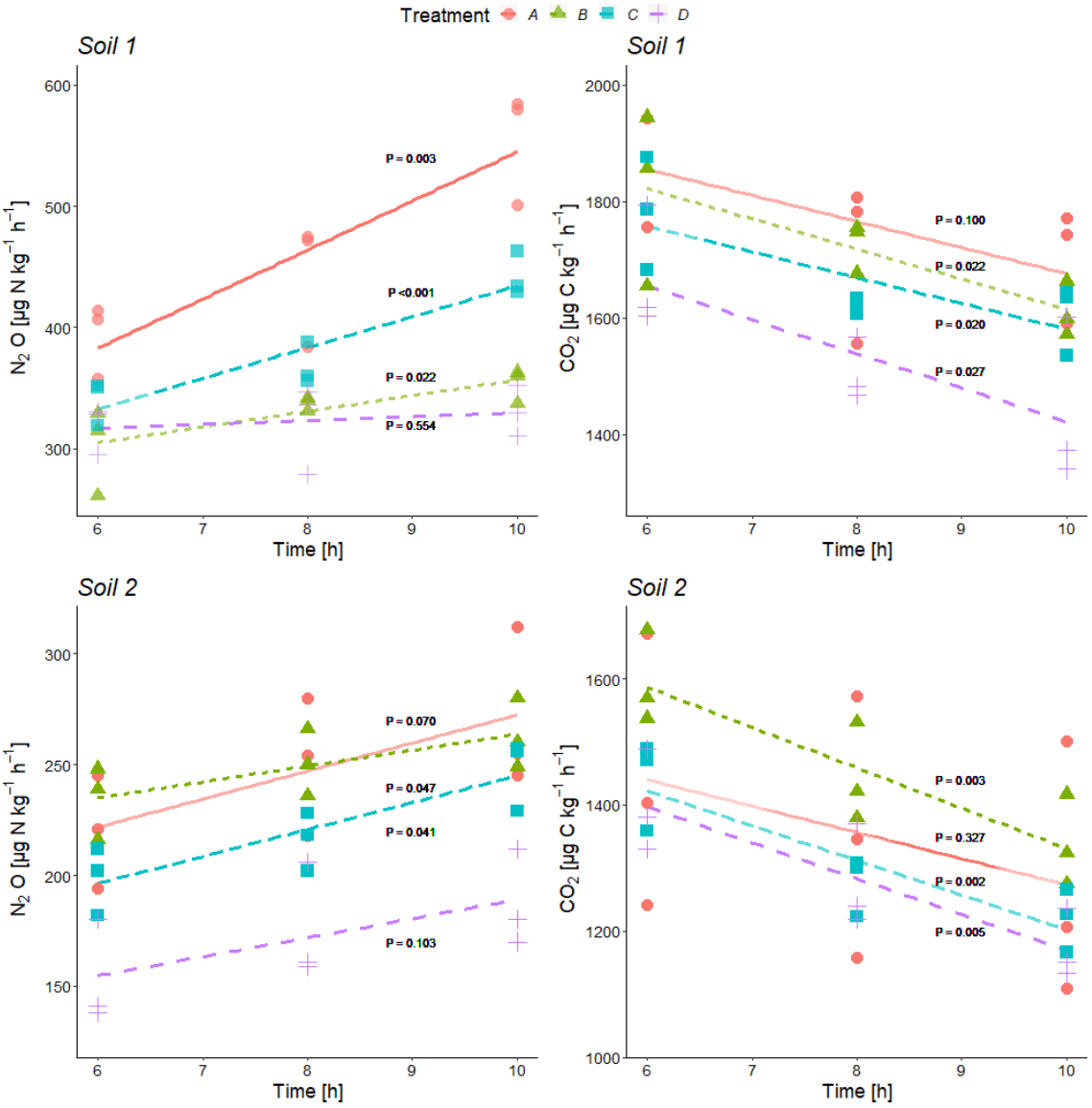
3. Results

3.1 N_2O production rates

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

N_2O and CO_2 production rates of all +C_2H_2 varieties differed significantly among soils ($P < 0.001$) and N_2O production rates differed also significantly among treatments ($P < 0.001$). Largest N_2O production rates of about 555 to 613 µg N kg^{-1} h^{-1} were obtained in *Soil 1* and *3*, respectively, while in *Soil 2* and *4* N_2O production rates were smaller (271 and 264 µg N $kg^{-1}h^{-1}$, respectively). N_2O and CO_2 production rates were significantly larger in variety +C_2H_2 than in variety -C_2H_2 of *Soil 1, 3* and *4* ($P = 0.002$, $P < 0.010$ and $P < 0.010$ for N_2O production rate and $P = 0.027$, $P < 0.010$ and $P = 0.008$ for CO_2 production rate, respectively) (Table 2), while -C_2H_2 and +C_2H_2 varieties of *Soil 2* did not differ in N_2O and CO_2 production rates *(*$P = 0.402$ and $P = 0.288$, respectively).

Figure 2: Time series of average N_2O and CO_2 production rates during incubation of variety $+C_2H_2$ at the three sample collection times of each soil (*Soil 1* to *4*) for treatment 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 regressions, 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 CO_2 production in *Soil 4* at the first sampling time after 2 hours. (Figure is continued on next page)



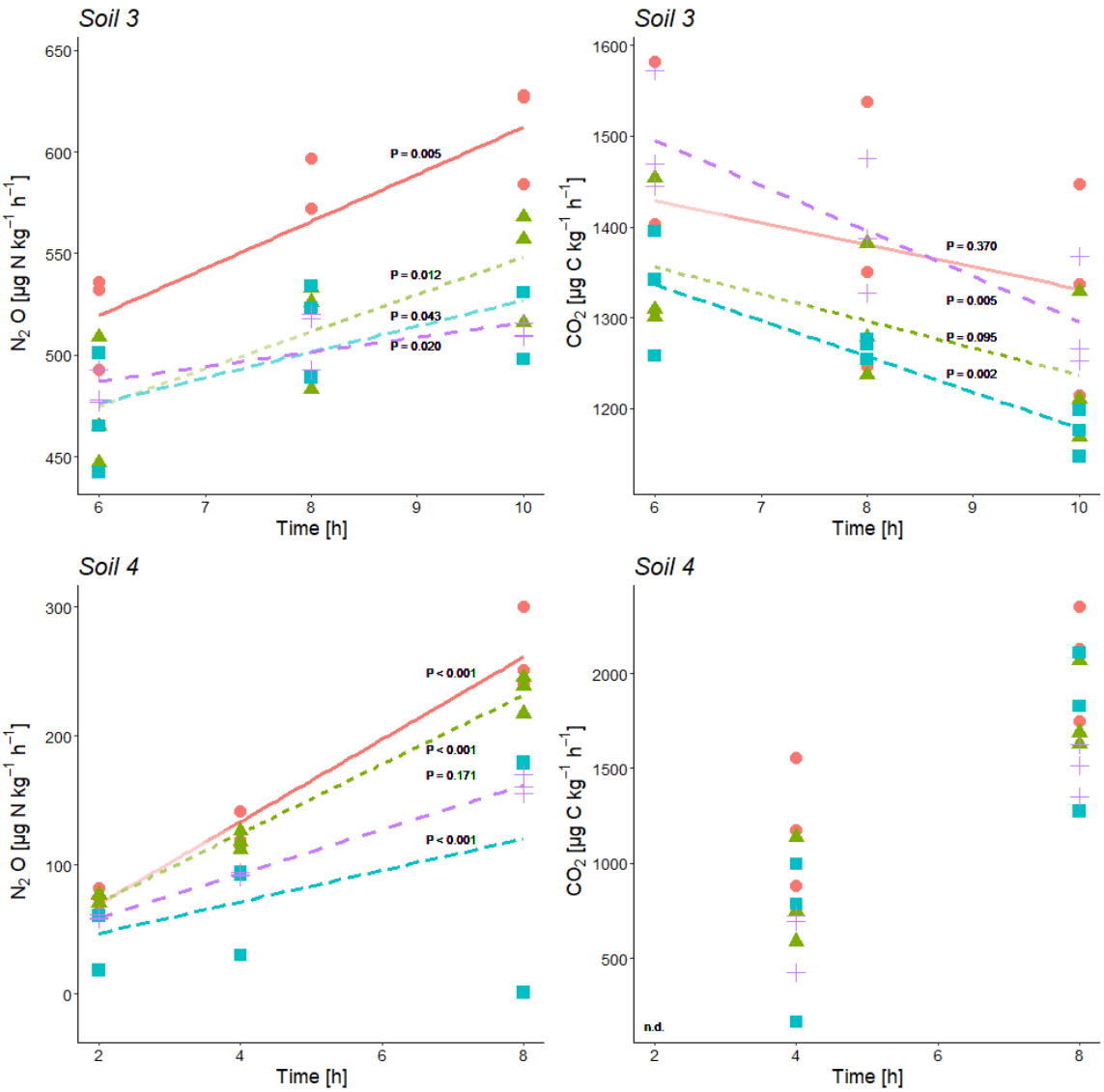


Figure 2 continued.

Without blockage of N_2O reductase (variety -C_2H_2), N_2O production rates of treatment A varied significantly among *Soils* with mean values between 175 and 355 µg N kg^{-1} h^{-1} ($P \leq 0.001$) (Table 2). In *Soil 1*, N_2O production rate was significantly larger (272 µg N kg^{-1} h^{-1}) than in *Soil 4* (175 µg N kg^{-1} h^{-1}) ($P = 0.028$) in variety -C_2H_2. In most cases of the three varieties (-C_2H_2, +C_2H_2, and *traced*) 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 *Soils 2*, *3* and *4*) or treatment C (fungal growth inhibitor; more N_2O compared to treatment B in *Soil* 1). Smallest N_2O production rates were in most cases found in treatment D (non-inhibitable N_2O production) (except for variety -C_2H_2 of *Soil 1*, varieties -C_2H_2 and traced of *Soil 3* and variety *traced* of *Soil 4*). Microbial inhibitor treatments differed significantly in N_2O fluxes of variety +C_2H_2 of each *Soil* (always $P \leq 0.040$), while this was not the case for inhibitor treatments of varieties -C_2H_2 and *traced* of *Soil 4* ($P = 0.154$ and $P = 0.154$, respectively). Significant deviations of treatments without (A) or with full inhibition (D) were found in the following cases (Table 2): N_2O production rate of treatment A was significantly larger compared to the other three treatments of *Soil 1* (+C_2H_2 and -C_2H_2), *Soil 2* (+C_2H_2) and *Soil 3* (+C_2H_2); treatment D was significantly smaller compared to the other three treatments in *Soil 2* (+C_2H_2) only and compared to treatments A and C in

435 *Soil 1* (+C_2H_2). A detailed discussion of inhibitor effects and difficulties with organisms that were not inhibited or abiotic sources is presented in section 4.1. Comparing varieties -C_2H_2 and *traced*, N_2O and CO_2 rates did not differ ($P = 0.991$ for N_2O production rate and $P = 0.490$ for CO_2 production rate, respectively), confirming that ^{15}N-labeling did not affect N_2O and CO_2 processes.

Table 2: Average CO_2 and N_2O production rates and N_2O isotopic values of N_2O of the last sample collection with and without C_2H_2 application in the headspace (varieties $-C_2H_2$ and $+C_2H_2$) of each soil (*Soil 1* to *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}]	$\delta^{18}O_{N2O}$ [‰]	$\delta^{15}N^{bulk}_{N2O}$ [‰]	SP_{N2O} [‰]
Soil 1 (Loamy sand, winter 2012)					
A / $-C_2H_2$	272.0 (38.4)a	1233.8 (170.5)a	13.1 (0.2)a	-21.9 (1.7)a	1.6 (0.8)a
B / $-C_2H_2$	180.9 (16.8)b	1284.8 (168.0)a	13.0 (<0.1)*	-24.2 (0.7)*	-1.3 (0.2)*
C / $-C_2H_2$	203.1 (14.4)b	1124.8 (54.8)a	14.6 (0.4)a	-20.0 (0.8)a	-1.6 (0.5)a
D / $-C_2H_2$	207.8 (32.6)b	1371.7 (35.3)a	15.2 (0.5)*	-20.2 (1.8)*	-0.3 (0.5)*
A / $+C_2H_2$	554.9 (46.5)a	1700.9 (98.1)a	8.5 (0.1)a	-22.1 (0.3)a	-0.4 (0.3)a
B / $+C_2H_2$	353.5 (14.0)b	1610.7 (47.2)a	7.5 (0.1)a	-26.1 (0.2)a	-1.2 (1.0)b
C / $+C_2H_2$	441.8 (18.5)c	1604.1 (60.3)a	9.3 (0.2)a	-22.4 (0.4)a	-0.9 (0.4)b
D / $+C_2H_2$	331.0 (20.5)b	1438.0 (141.9)a	7.8 (0.3)*	-24.2 (0.1)*	-2.3 (0.7)*
Soil 2 (Sand, winter 2012)					
A / $-C_2H_2$	315.0 (35.0)a	1316.7 (97.7)a	15.5 (1.8)a	-18.9 (2.6)a	-0.9 (2.5)a
B / $-C_2H_2$	241.7 (3.0)b	1209.2 (24.6)a	15.0 (1.3)a	-23.4 (2.5)a	-0.8 (<0.1)a
C / $-C_2H_2$	247.6 (22.8)b	1201.9 (48.2)a	14.3 (0.1)a	-21.8 (0.2)a	-1.8 (0.2)a
D / $-C_2H_2$	198.4 (26.8)b	1102.4 (101.7)a	13.4 (0.3)a	-24.5 (0.1)a	-1.2 (0.3)a
A / $+C_2H_2$	270.9 (36.3)a	1271.6 (203.5)a	12.6 (0.3)a	-18.9 (4.6)a	-1.4 (0.3)a
B / $+C_2H_2$	263.1 (19.1)a	1338.7 (71.9)a	12.3 (0.1)a	-24.6 (0.2)b	-2.0 (0.2)a
C / $+C_2H_2$	247.3 (15.9)a	1220.2 (50.0)a	12.7 (0.1)*	-23.3 (0.2)*	-1.7 (0.4)*
D / $+C_2H_2$	187.3 (21.8)b	1173.1 (55.1)a	12.2 (0.3)a	-26.0 (0.1)b	-1.5 (0.9)a
Soil 3 (Silt loam, winter 2013)					
A / $-C_2H_2$	355.0 (18.4)a	1227.6 (95.2)a	26.0 (0.5)a	-20.8 (0.5)a	-0.5 (0.4)a
B / $-C_2H_2$	325.4 (36.3)a	1159.3 (178.2)a	24.1 (0.2)b	-22.0 (0.2)b	-0.1 (0.4)a
C / $-C_2H_2$	278.9 (9.8)a	1056.0 (59.6)a	27.3 (0.1)b	-20.6 (0.3)a	0.6 (0.2)a
D / $-C_2H_2$	291.1 (38.5)a	1118.5 (70.3)a	26.3 (0.3)a	-21.0 (0.1)a	0.0 (0.2)a
A / $+C_2H_2$	612.8 (25.2)a	1332.5 (116.9)a	15.2 (0.1)a	-25.6 (0.8)a	-2.8 (0.2)a
B / $+C_2H_2$	546.9 (27.5)b	1235.7 (83.4)a	14.9 (0.2)a	-26.3 (<0.1)a	-3.5 (0.4)a
C / $+C_2H_2$	519.8 (19.2)b	1173.5 (25.7)a	16.2 (<0.1)*	-25.2 (0.1)*	-4.0 (0.4)*
D / $+C_2H_2$	511.7 (3.5)b	1295.6 (63.3)a	16.0 (0.1)b	-25.1 (0.1)a	-4.3 (0.5)a
Soil 4 (Loamy sand, summer 2011)					
A / $-C_2H_2$	175.3 (6.6)a	2448.5 (135.8)a	25.7 (0.3)a	-30.6 (0.2)a	12.1 (1.6)a
B / $-C_2H_2$	121.3 (74.0)a	2091.3 (19.5)b	28.0 (5.0)a	-32.3 (0.7)a	7.7 (1.4)b
C / $-C_2H_2$	104.5 (5.3)a	1844.7 (192.1)b	29.3 (0.1)a	-30.0 (0.5)a	4.3 (1.0)c
D / $-C_2H_2$	73.8 (63.0)a	1632.2 (115.3)b	28.9 (1.2)a	-31.8 (2.2)a	3.4 (2.0)c
A / $+C_2H_2$	263.5 (31.7)a	2076.6 (305.3)a	13.5 (0.5)*	-34.7 (0.1)*	-1.0**
B / $+C_2H_2$	233.0 (15.6)a	1794.9 (238.9)a	14.3 (1.7)a	-33.8 (0.9)a	-4.9 (0.9)a
C / $+C_2H_2$	119.5 (102.7)a	1736.8 (424.7)a	19.0 (7.0)a	-33.1 (2.8)a	-1.7 (2.7)b
D / $+C_2H_2$	161.6 (7.6)a	1497.0 (138.7)a	14.8 (0.5)a	-35.7 (0.2)a	-4.9 (0.7)c

Letters denote significant differences (P < 0.1) among treatments and varieties within a soil.
Asterisks indicate that only two samples (*) or one sample (**) of triplicates were analysable due to logistical difficulties.

3.2 Isotopologues of N_2O produced in different varieties and treatments

3.2.1 Variety $-C_2H_2$

SP_{N2O} values of all *Soils* and inhibitor treatments of variety $-C_2H_2$ were within a range of -1.8 to 12.1 ‰ (Table 2) and differed among inhibitor treatments ($P = 0.037$). SP_{N2O} values in variety $-C_2H_2$ of *Soil 4* was particularly large (3.4 to 12.1 ‰) compared to the other *Soils* (1.6 to -1.6 ‰). SP_{N2O} values of variety $-C_2H_2$ were significantly larger than SP_{N2O} values of variety $+C_2H_2$ (up to 2.4, 1.5, 4.6 and 4.1 ‰ in *Soil 1, 2, 3* and *4*, respectively). Generally, most SP_{prod} values of variety $-C_2H_2$ (Eq. 7) were smaller than SP_{N2O} values of variety $-C_2H_2$ but still larger than SP_{N2O} values of variety $+C_2H_2$ and are presented in Table S2 (Supplementary Material).

3.2.2 Variety $+C_2H_2$

SP_{N2O} values of all *Soils* and all treatments of variety $+C_2H_2$ were within a narrow range between -4.9 and -0.4 ‰ (Table 2), and differed only significantly among treatments of *Soil 4* ($P = 0.002$). In general, there were only small differences among treatments: SP_{N2O} values of treatments A in variety $+C_2H_2$ differed significantly among soils ($P < 0.001$), with largest SP_{N2O} values in *Soil 1* (-0.4 ‰) and smallest SP_{N2O} values in *Soil 3* (-2.8 ‰). SP_{N2O} values of treatment D in variety $+C_2H_2$ of all soils varied between -1.5 and -4.9 ‰, but only SP_{N2O} values of *Soil 2* differed significantly from SP_{N2O} values of the other *Soils* ($P = 0.006$). For treatments B of variety $+C_2H_2$, SP_{N2O} values differed only significantly between *Soil 1* and *4, 2* and *4*, and *1* and *3* (each $P = 0.002$). SP_{N2O} values from treatment C in variety $+C_2H_2$ did not differ significantly ($P = 0.600$). For every soil, we found significantly larger $\delta^{18}O_{N2O}$, $\delta^{15}N^{bulk}{}_{N2O}$ and SP_{N2O} values in variety $-C_2H_2$ than in variety $+C_2H_2$ ($P < 0.001$), except for *Soil 2*, where $\delta^{15}N^{bulk}{}_{N2O}$ values of variety $-C_2H_2$ were indistinguishable from those of variety $+C_2H_2$ ($P = 0.400$). However, only in a few varieties there were significant differences in $\delta^{18}O_{N2O}$, $\delta^{15}N^{bulk}{}_{N2O}$ or SP_{N2O} values between treatments with fungal and bacterial inhibition (B and C, respectively) (Table 2). As explained in section 3.3, N_2O reduction blockage in varieties $+C_2H_2$ was successful in most cases (*Soil 2, 3* and *4*). SP_{N2O} values of this variety are thus assumed to be valid estimates of $\delta 0$, i.e. SP_{prod} values of N_2O production, and can thus be used for applying the IEM.

3.2.3 Variety *traced*

The ^{15}N-labeling of N_2O ($^{15}N_{N2O}$) or N_2 produced ($^{15}N_{N2}$) gave information about the incorporated N from ^{15}N-labeled NO_3^- into N_2O or N_2 as well as about the N_2O reduction to N_2. Microorganisms in each treatment used the ^{15}N-labeled NO_3^- in variety *traced* (Table 3) and expected $^{15}N_{N2O}$ depended on the initial N abundance in NO_3^- of unfertilized soil (Eq. 7). *Soil 4* is the only one showing a large discrepancy between measured (about 30 at%) and calculated $^{15}N_{N2O\_exp}$ (49 at%) in N_2O, whereas the other *Soils* showed close agreement (Table 3).

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

r_{C2H2} as well as r_{15N} determined with *Soil 2* were significantly larger than with the other *Soils* ($P \leq 0.001$) (Table 3). r_{15N} of treatment B was significantly larger thanof treatment C and D of *Soil 4* ($P = 0.032$), while all other treatments of other *Soils* did not differ. r_{C2H2} did not differ significantly among treatments ($P = 0.400$). In order to test the efficiency of blockage of N_2O reduction by C_2H_2 application, r_{C2H2} (Eq. 5) was compared with r_{15N} (Eq. 6). In *Soil 1*, r_{C2H2} was by far smaller than r_{15N}, while both calculated product ratios were in similar ranges in the other three *Soils* and thus a successful blockage of N_2O reduction was assumed for those *Soils*.

485 **Table 3: Average CO_2 and N_2O production rates of the last sample collection after 10 or 8 hours of variety *traced*, respectively, with ^{15}N labeling in N_2O ($^{15}N_{N2O}$) and the calculated r_{15N} of variety *traced* and r_{C2H2} calculated from N_2O production rates of variety $-C_2H_2$ and $+C_2H_2$ of each soil (Soil *1* to *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}]	$^{15}N_{N2O}$ [at%]	$^{15}N_{N2O\_exp}$ [at%][a]	Calc. total r_{15N}[b]	Calc. total r_{C2H2}[c]
Soil 1 (Loamy Sand, 2012)						
A	255.6 (43.5)	1310.0 (167.3)	36.8 (0.1)	39	0.80 (0.02)	0.48 (0.07)
B	154.5 (29.6)	1153.5 (238.4)	36.4 (0.2)		0.76 (0.02)	0.48 (0.05)
C	191.6 (30.7)	1219.6 (109.1)	36.9 (<0.1)		0.72 (0.05)	0.45 (0.04)
D	148.1 (1.9)	1253.8 (54.5)	36.8 (0.1)		0.69 (0.02)	0.54 (0.05)
Soil 2 (Sand, 2012)						
A	240.7 (0.95)	1286.2 (5.6)	43.2 (<0.1)	44	0.94 (0.01)	1.04 (0.10)
B	185.1 (3.9)	1157.4 (17.3)	43.0 (0.1)		0.94 (0.01)	0.81 (0.04)
C	241.1 (13.4)	1282.1 (63.4)	43.2 (0.1)		0.95 (0.01)	0.99 (0.09)
D	167.3 (34.9)	1199.0 (34.6)	42.7 (0.1)		0.93 (0.01)	0.98 (0.04)
Soil 3 (Silt loam, 2013)						
A	285.9 (20.4)	1044.0 (46.6)	35.8 (<0.1)	34	0.62 (<0.01)	0.52 (0.04)
B	320.5 (14.7)	1204.2 (86.5)	35.5 (<0.1)		0.62 (0.01)	0.59 (0.02)
C	216.4 (34.9)	980.5 (202.5)	35.5 (<0.1)		0.59 (0.02)	0.48 (0.04)
D	231.4 (11.4)	988.5 (74.4)	35.3 (<0.1)		0.62 (0.01)	0.51 (0.04)
Soil 4 (Loamy Sand, 2011)						
A	156.9 (62.7)	3111.4 (1252.5)	31.1**	49	0.54 (0.05)	0.63 (0.10)
B	169.2 (6.1)	2314.6 (307.1)	26.5**		0.59 (0.03)	0.63 (0.17)
C	117.2 (3.1)	1785.6 (79.3)	30.1 (1.1)*		0.50 (0.01)	0.62 (0.02)
D	115.2 (3.1)	1706.7 (38.1)	33.5 (0.5)*		0.50 (0.01)	0.53 (0.12)

Asterisks indicate that only two samples (*) or one sample (**) were analysed due to logistical difficulties.

490 **[a] $^{15}N_{N2Oexp}$ [at%] was calculated from Eq. 8.**

[b] r_{15N} = [N_2O/(N_2+N_2O)] with N_2O or N_2 production rates from variety *traced*; see Eq. 5

[c] r_{C2H2} = [N_2O_{-C2H2}/N_2O_{+C2H2}] with N_2O production rate from varieties -C_2H_2 and -C_2H_2; see Eq. 6, cf. Table 2

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

495 When calculating f_{FDmi}, N_2O production rates of treatment D must be significantly smaller compared to the other three treatments and the flux balance according to Eq. 1 and 2 must be consistent. Taking the large ranges of N_2O production rates of each treatment (minimum and maximum values) into account, for each *Soil* (A-D) was indistinguishable from ((B-D)+(C-D)) (Eq. 2), showing good agreement between Eqs. 1 and 2. However, N_2O production in treatment D was large within all varieties. Only with *Soil 2* of the variety $+C_2H_2$, the N_2O 500 production rates of treatment D were significantly smaller than those of the other three treatments. Thus, for *Soil 2,* f_{FDmi} could be calculated (Eq. 3) and amounted to 28 ± 9 % (Table 5) with a corresponding fungal N_2O production rate of 23.7 ± 1.8 µg N kg^{-1} h^{-1}. Although the N_2O production rate of Treatment D was smaller than that of treatment A (*Soil 2*), it must be pointed out that due to the large amount of non-inhibitable production (treatment D), even the result for *Soil 2* is actually very unsure. For all other *Soils*, calculation of f_{FDmi} was not 505 possible, i.e., SIRIN was not successful.

3.5 Fungal contribution to N_2O production from denitrification by the SP endmember mixing approach (IEM) and SP/$\delta^{18}O$ isotope mapping approach (SP/$\delta^{18}O$ Map)

The IEM revealed that f_{FD_SP} was small in all *Soils* (≤15 %, ≤14 %, ≤ 9%, and ≤ 11 % with *Soil 1* to *4*, respectively) (Table 5). When applying SP/$\delta^{18}O$ Map, we can assess the plausibility of the determined f_{FD} values based on the $\delta^{18}O_{H2O}$ values obtained from the fitting ($\delta^{18}O_{H2O}$ value in Table 4) and the fitting outcome, i.e. the difference between r_{15N} and r_{MAP} (*Diff*, see Table 4). The most probable $\delta^{18}O_{H2O}$ value for our *Soils* can be assumed based on the fact that Braunschweig tap water was used and the original soil water also represents the isotope characteristics typical for this region, which is about -7.4 ‰ (long-term mean Braunschweig precipitation water (Stumpp et al., 2014)). Thus, in the presented application of SP/$\delta^{18}O$ Map, $\delta^{18}O_{H2O}$ values were fitted and it has to be pointed out that the precision of such calculations can be improved by measuring $\delta^{18}O_{H2O}$ instead. Depending on the season and evaporative losses, $\delta^{18}O_{H2O}$ may slightly vary and the most possible range of soil water in our *Soils* may vary from about -11 to -4 ‰ as observed in other experiments used in our laboratory experiments with similar conditions (Lewicka-Szczebak et al., 2014; Rohe et al., 2014a; Lewicka-Szczebak et al., 2017; Rohe et al., 2017). Taking this into account, we can say that for *Soil 1*, the fungal contribution must be below 2 %, because to obtain any larger f_{FD} values, unrealistically small $\delta^{18}O_{H2O}$ values (of -14.9 ‰) must be fitted (see Table 4). For *Soil 2*, both the smaller f_{FD_MAP} values of 1 % and the larger ones up to 15 % are possible, since they are associated with very realistic $\delta^{18}O_{H2O}$ values (of -6.3 and -10.1, respectively) and identical *Diff* of 0.04 (Table 4). For *Soil 3*, the only plausible fitting can be obtained for the smallest SP_{BD} values, which are associated with a $\delta^{18}O_{H2O}$ value of -5.6 ‰ (Table 4). Although the *Diff* for this fitting is slightly higher, the other fittings must be rejected due to unrealistic $\delta^{18}O_{H2O}$ values (of -1.7 and +3.7 ‰), hence f_{FD_MAP} values must be between 4 and 9 %. Similarly, for *Soil 4*, the only plausible fitting can be obtained for the smallest SP_{BD} values, which are associated with a $\delta^{18}O_{H2O}$ value of -6.8 ‰ (Table 4) and indicate f_{FD_MAP} values from 11 to 20 %. Here this fitting also shows clearly the smallest *Diff* of only 0.01 (Table 4). However, except for *Soil 4*, where the *Diff* is smallest for the last fitting, the *Diff* values for other *Soils* are very similar for different fittings with the largest values in *Soil 3*. A better fit (showing smaller *Diff* values) was not possible with any other SP_{BD} and $\delta^{18}O_{H2O}$ values. Since the precision of r_{15N} (expressed in standard deviation in Table 3) was always ≤ 0.05, this uncertainty of r_{15N} did not reduce the precision of the fitting (compare large ranges of $\delta^{18}O_{H2O}$ and r_{MAP} values, respectively, in Table 4). The f_{FD_SP} ranged between 0 and approximately 15 % (Table 5). The results obtained from SP/$\delta^{18}O$ Map show f_{FD_MAP} reaching up to 14, 15, 9 and 20 % for *Soils 1, 2, 3*, and *4*, respectively (Figure 3, Table 4, Table 5).

Table 4: Summary of the results provided by SP/$\delta^{18}O$ Map for fraction of fungal denitrification (f_{FD_MAP}) and N_2O product ratio (r_{MAP}) in the acetylated (+C_2H_2) and non-acetylated (-C_2H_2) varieties for three possible SP_{N2O} values from bacterial denitrification (SP_{BD}): mean (-1.9 ‰), maximal (3.7 ‰), and minimal (-7.5 ‰). The $\delta^{18}O$ values of soil water ($\delta^{18}O_{H2O}$) were fitted to get the lowest difference (*Diff*) between product ratio determined with ^{15}N treatment (r_{15N}) and SP/$\delta^{18}O$ Map (r_{MAP}). The most plausible fittings are shown in bold (see discussion for reasons of this choice).

Soil	Variety	r_{15N}	SP_{BD} [‰]	$\delta^{18}O_{H2O}$ [‰]	r_{MAP}	*Diff*	f_{FD_MAP} [%]*
1	**-C_2H_2**	**0.66**	**-1.9**	**-11.2**	**0.66**	**0.00**	**-1**
	+C_2H_2	**1**	**-1.9**	**-11.2**	**1.00**	**0.00**	**2**
	-C_2H_2	0.66	3.7	-6.1	0.65	0.01	-14
	+C_2H_2	1	3.7	-6.1	1.00	0.00	-16
	-C_2H_2	0.66	-7.5	-14.9	0.66	0.00	8
	+C_2H_2	1	-7.5	-14.9	1.00	0.00	14
2	**-C_2H_2**	**0.94**	**-1.9**	**-6.3**	**0.90**	**0.04**	**1**
	+C_2H_2	**1**	**-1.9**	**-6.3**	**1.04**	**0.04**	**1**
	-C_2H_2	0.94	3.7	-1.2	0.90	0.04	-16
	+C_2H_2	1	3.7	-1.2	1.04	0.04	-18
	-C_2H_2	**0.94**	**-7.5**	**-10.1**	**0.90**	**0.04**	**13**
	+C_2H_2	**1**	**-7.5**	**-10.1**	**1.04**	**0.04**	**15**
3	-C_2H_2	0.61	-1.9	-1.7	0.54	0.07	-3
	+C_2H_2	1	-1.9	-1.7	1.04	0.04	-5
	-C_2H_2	0.61	3.7	3.7	0.54	0.07	-14
	+C_2H_2	1	3.7	3.7	1.03	0.03	-24
	-C_2H_2	**0.61**	**-7.5**	**-5.6**	**0.53**	**0.08**	**4**
	+C_2H_2	**1**	**-7.5**	**-5.6**	**1.04**	**0.04**	**9**
4	-C_2H_2	0.60	-1.9	-3.3	0.66	0.06	15
	+C_2H_2	1	-1.9	-3.3	0.96	0.04	-30
	-C_2H_2	0.60	3.7	1.5	0.72	0.12	8
	+C_2H_2	1	3.7	1.5	0.91	0.09	-21
	-C_2H_2	**0.60**	**-7.5**	**-6.8**	**0.61**	**0.01**	**20**
	+C_2H_2	**1**	**-7.5**	**-6.8**	**0.99**	**0.01**	**11**

***Negative values for f_{FD_MAP} are non-realistic and therefore discarded for further interpretation.**

Table 5: Ranges of the fraction of N_2O produced by fungi (f_{FD}) from four *Soils* using four different approaches: Fungal fraction was calculated using a) the microbial inhibitor approach (modified SIRIN) (f_{FDmi}), b) the isotopomer endmember mixing approach (IEM) by SP isotope mixing balance (f_{FD_SP}), c) the IEM for results from variety -C_2H_2 with reduction correction to calculate the SP_{N2O} values (f_{FD_SPcalc}), and d) the $\delta^{18}O$/SP Map (f_{FD_MAP}) with $\delta^{18}O_{N2O}$ and SP_{N2O} values from variety -C_2H_2 and variety +C_2H_2.

Soil	f_{FDmi} [%][a]	f_{FD_SP} [%][b*]	f_{FD_SPcalc} [%][c*]	f_{FD_MAP} [%][d*]
1	n.d.	-14 to 15	-6 to 19	<2
2	19 to 37	-18 to 14	-12 to 15	1 to 15
3	n.d.	-25 to 9	-9 to 18	4 to 9
4	n.d.	-23 to 11	1 to 21	11 to 20

[a]Fungal fraction of N_2O production calculated by Eq. 3 taking variations of three replicates into account

[b]Fungal fraction of N_2O production calculated by Eq. 4 for variety +C_2H_2 with assuming SP_{N2O} values of N_2O produced by bacteria were 3.7 ‰ or -7.5 ‰. Using the minimum and maximum SP_{N2O} values known for bacteria resulted in a f_{FD_SP} range.

[c]Eq. 4 to solve for fungal fraction in variety -C_2H_2 with assuming SP_{N2O} values of N_2O produced by bacteria was 3.7 (resulting in negative fraction and therefore set to zero) or -7.5 ‰ and using reduction correction with η_r=-6 ‰ to calculate SP_{prod} values (Senbayram et al., 2018; Yu et al., 2020). Using the minimum and maximum SP_{N2O} values known for bacteria resulted in a f_{FD_SPcalc} range.

[d]Fungal fraction of N_2O production calculated by SP/$\delta^{18}O$ Map with assuming most probable SP_{N2O} values from bacterial denitrification (according to Table 4). Using the minimum and maximum SP_{N2O} values known for bacteria and ranges of fitted $\delta^{18}O$ values resulted in a f_{FD_MAP} range.

***Negative values for f_{FD_SP}, f_{FD_SPcalc}, f_{FD_MAP} are non-realistic and therefore discarded for further interpretation.**

n.d.-not determined because of insufficient inhibition.

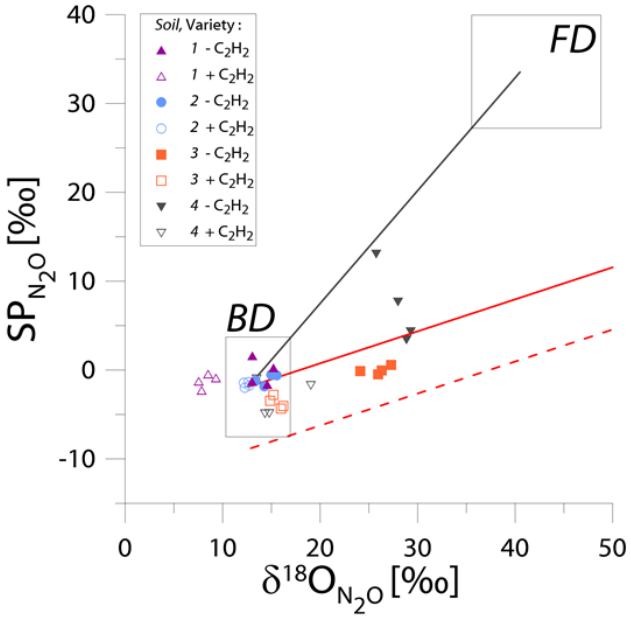


Figure 3: SP/$\delta^{18}O$ isotope mapping approach (SP/$\delta^{18}O$ Map) to estimate the contribution of bacteria or fungi to N_2O produced according to Lewicka-Szczebak et al. (2017) and Lewicka-Szczebak et al. (2020). The isotopic values for natural abundance treatments with acetylene addition (+C_2H_2, empty symbols) and without acetylene addition (-C_2H_2, corresponding filled symbols) are shown for four *Soils* (*1 to 4*). The grey rectangles indicate expected ranges of isotopic signatures for heterotrophic bacterial denitrification (*BD*) and fungal denitrification (*FD*) (Yu et al., 2020). The black solid line is the mixing line connecting the average expected values for *BD* and *FD*, while the red solid line is the mean reduction (for the mean *SP* values for *BD*) line and the red dashed line is the minimum reduction line (for the minimal SP_{N2O} values for *BD*).

4. Discussion

To our knowledge, this was the first attempt to determine SP_{N2O} values by fungi or bacteria from soil communities using microbial growth inhibitors with a modification of SIRIN and comparing microbial inhibitor and isotopic approaches (IEM and SP/δ^{18}O Map) to estimate fungal contribution to N_2O production from denitrification in anoxic incubation. Using isotopic approaches revealed that the fungal contribution to N_2O production was small ($f_{FD\_SP} \leq 15$ % or $f_{FD\_MAP} \leq 20$ %) in the soils tested (Table 5). The modified SIRIN approach was not successful, because large amounts of non-inhibitable N_2O production were observed with all four *Soils* (Table 2, Table 3). The fungal fraction producing N_2O during denitrification (f_{FDmi}) was only estimated for *Soil 2*, where significantly smaller N_2O production in treatment D was observed compared to that of treatment A and resulted in a range of 19 to 37 %, which was probably overestimated due to uncertainties resulting from the large N_2O production of non-inhibitable sources. While the three approaches coincided in showing dominance of bacterial denitrification, the isotopic approaches yielded small estimates for f_{FD} (≤ 20 %) and thus did not confirm largest f_{FDmi} of *Soil 2*. 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). All SIRIN results obtained with respect to N_2O production by the fungal or bacterial fraction were unsatisfactory and led to unsolved questions, which are discussed in the following sections.

4.1 Experimental setup and inhibitor effects

In accordance with other studies, N_2O production was analysed after the addition of glucose as substrate (Laughlin and Stevens, 2002; McLain and Martens, 2006; Blagodatskaya et al., 2010; Long et al., 2013). Glucose initiates the growth of active heterotrophic organisms. Since pure cultures were shown to synthesize enzymes capable of denitrification within two to three hours (USEPA, 1993), pre-incubation of soil under anaerobic conditions is not needed. Thus, when gas sample collection started organisms should have produced denitrifying enzymes and microbial growth of initially active organisms should have started too. However, in accordance to Anderson and Domsch (1975) experimental duration should be as short as possible to ensure the CO_2 production by initially active organisms only. Thus, short-time incubation is recommended when conducting a modified SIRIN approach, as the incubation period should cause changes in conditions for microorganisms and initiate growth on the one hand, while it should avoid the use of inhibitors as C sources by organisms on the other.

With incubation time, production rates of CO_2 decreased, probably because experimental incubation conditions provoked unfavourable conditions and physiological changes, e. g. due to anaerobic conditions or local substrate depletion (e. g. C supplied as glucose). Decreasing CO_2 fluxes might also be explained by CO_2 accumulation in pore space as this effect is shown by modelled diffusive fluxes from soil in closed systems (Well et al., 2019).

Previous studies found much larger inhibitor effects (Laughlin and Stevens, 2002; Blagodatskaya et al., 2010; Long et al., 2013; Chen et al., 2014). It is therefore important to discuss considerable differences among the experimental design of the present study compared to that of other studies (e. g., Laughlin and Stevens, 2002; Blagodatskaya et al., 2010).

The conventional practice of SIRIN implies determination of c_{opt}(glucose), c_{opt}(streptomycin) or c_{opt}(cycloheximide) with an "Ultragas 3" CO_2 analyser (WösthoffCo., Bochum) (Anderson and Domsch, 1973) with continuous gas flow. We used this method to determine optimal concentrations for SIRIN in the pre-

experiment and used these concentrations for the modified SIRIN approach as well. This optimization procedure was not used in other studies (Laughlin and Stevens, 2002; Blagodatskaya et al., 2010; Long et al., 2013). We supposed that optimal concentrations for CO_2 respiration should work as well for denitrification, if both inhibitors inhibit the denitrification process as well. However, although SIRIN has so far been tested with isolated cultures and soils for microbial growth for CO_2 production only (Anderson and Domsch, 1973, 1975), information on N_2O producing processes, especially denitrification, is still lacking and should be investigated in further studies. Additionally, as presented by Ladan and Jacinthe (2016) the bactericide bronopol and the fungicide captan were more effective inhibitors than streptomycin or cycloheximide and should be included when evaluating inhibition approaches and isotopic endmember approaches.

Previous studies that found much larger inhibitor effects were conducted with pre-incubating the soil with selective inhibitors (Laughlin and Stevens, 2002; Blagodatskaya et al., 2010; Long et al., 2013; Chen et al., 2014). In contrast to that, the experimental design of our incubation setup was without soil pre-incubation with selective inhibitors to minimize disturbance of the soil microbial community and our approach was thus in agreement with the original SIRIN method for respiration (Anderson and Domsch, 1973, 1975, 1978). Another study performing similar experiments without pre-incubation with inhibitors did not find effectiveness of application of both antibiotics during long-term application (up to 48 h) (Ladan and Jacinthe, 2016), although streptomycin and cycloheximide are commonly used to inhibit denitrification of selective groups. Nevertheless, as we expected that pre-incubation with selective inhibitors would induce changes in the F:B ratio of soil, we decided to conduct the modified SIRIN approach without a pre-incubation step. This assumption was supported by findings of Blagodatskaya et al. (2010), where pre-incubation of about one to twenty hours with cycloheximide resulted in increasing inhibitor efficiency with time, while this was not the case when pre-incubating with streptomycin. This suggests that microbial communities might change after exposition to cycloheximide.

In the present study, even with both growth inhibitors (treatment D), N_2O production was large in all experiments, i.e., in most cases not significantly smaller than in treatments A, B or C. Thus, we suppose similar contributions of non-inhibitable organisms and processes in all treatments. Non-inhibitable organisms could be, for example, bacteria or fungi that are not in growth stage or may be not affected by inhibitors. Recently, Pan et al. (2019) summarized findings of other studies and pointed out that some microorganisms can use inhibitors as growth substrates, that dead organisms may serve as energy sources for others, and that interactions of microbial species may change due to non-inhibitable organisms occurring in soil communities. Non-inhibitable organisms could be archaea as well, which are also known to be capable of denitrification (Philippot et al., 2007; Hayatsu et al., 2008). It is known that archaea are not affected by streptomycin or cycloheximide (Seo and DeLaune, 2010). However, effects of archaeal occurrence in soil or secondary effects on fungi or bacteria were not tested in this study. Additionally, abiotic N_2O production cannot be quantified with the experimental setup, but might be contributing to each inhibitor treatment.

In summary, the present experimental setup without pre-incubating soil samples with selective inhibitors was not successful in complete inhibition of bacterial or fungal denitrifiers. Although pre-incubation with selective inhibitors may lead to more successful inhibition, we do not recommend this due to induced changes in soil communities. For further studies focusing on application of modified SIRIN to determine the fraction of bacterial or fungal N_2O derived from denitrification a method validation using also different inhibitors is recommended.

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

In order to determine SP_{N2O} values without alteration by partial reduction of N_2O to N_2, C_2H_2 was used to quantitatively block N_2O reduction during denitrification. We found the expected effect of C_2H_2 application, i.e. larger N_2O production rates in variety $+C_2H_2$ compared to variety $-C_2H_2$. Calculated product ratios varied between 0.5 and 0.95 (r_{15N}) in all *Soils*, showing that N_2O reduction can have significant effects on measured N_2O production and isotopic values.

The calculated r_{C2H2} was within the same range as r_{15N} in *Soil 2, 3* and *4* (maximal 9 % difference), indicating effective blockage of N_2O reductase in variety $+C_2H_2$ in these *Soils*. Only in *Soil 1,* r_{15N} and r_{C2H2} differed by about 34 % with larger calculated reduction in the *traced* variety, which might point to incomplete inhibition by the C_2H_2 method. Artifacts with C_2H_2 were found in previous studies, resulting in smaller N_2O production rates due to NO oxidation accelerated by C_2H_2 application in the presence of very small O amounts (Bollmann and Conrad, 1997a, b; Nadeem et al., 2013). Moreover, incomplete C_2H_2 diffusion into denitrifying aggregates might also lead to incomplete N_2O reductase blockage (Groffman et al., 2006). Both potential methodological errors cannot be excluded for *Soil 1*.

For the other three *Soils* (*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 $-C_2H_2$ and $+C_2H_2$, isotopologue values of all these *Soils* (except $\delta^{15}N^{bulk}{}_{N2O}$ values of *Soil 2*) of variety $-C_2H_2$ were significantly larger than those of variety $+C_2H_2$. The enrichment of residual N_2O in heavy isotopes results from the isotope effect associated with N_2O reduction (Jinuntuya-Nortman et al., 2008; Well and Flessa, 2009; Lewicka-Szczebak et al., 2014). This explains why C_2H_2 application is essential for analysing N_2O produced by different microbial organism groups from soil using solely the modified SIRIN approach without additional isotopic approaches.

Moreover, when applying SIRIN without quantifying N_2O reduction, fungal denitrification is potentially overestimated due to the impact of SIRIN inhibitors on N_2O reduction. It is evident that N_2O fluxes represent net N_2O production, i.e. the difference between gross N_2O production by the microbial community and N_2O reduction, mainly by heterotrophic bacterial denitrifiers (Müller and Clough, 2014). It has been shown that N_2O released by microorganisms to air-filled pore space can be partially consumed by denitrifiers before being emitted (Clough et al., 1998). This means that fungal N_2O can also be subject to reduction by bacterial denitrifiers. Consequently, inhibiting bacterial denitrification by SIRIN would enhance the measured flux of fungal N_2O. Until now, this effect has not been considered in SIRIN papers on fungal N_2O (e. g. Laughlin and Stevens, 2002; Ladan and Jacinthe, 2016; Chen et al., 2014). This effect can only be evaluated by measuring N_2O reduction in all inhibitor treatments as in our study. If true, the N_2O reduction with bacterial inhibition should be smaller than that of the treatments without inhibition or with fungal inhibition. However, with fungal inhibition, N_2O reduction is also assumed to be smaller that without inhibition, because N_2O produced by fungi is missed for bacterial reduction.

The product ratio is a measure for the N_2O reduction to N_2. However, regarding the r_{15N}, there was no evidence of different N_2O reduction effects between the SIRIN treatments. The r_{C2H2} also revealed indistinguishable values between SIRIN treatments in *Soil 1* and *4*, but it was slightly larger in *Soil 3* with bacterial inhibition compared to the other treatments. However, this effect was very small, which would only cause small overestimation of fungal contribution. The smallest N_2O reduction was found in *Soil 2* (r_{C2H2} values near 1), with

smallest r_{C2H2} with bacterial inhibition (0.81). This could result in an overestimation of bacterial contribution, since with blockage of N_2O reduction, gross N_2O production of bacteria is measured.

The r_{15N} and r_{C2H2} were between 0.5 and 1 and N_2O reduction was thus never consuming most of the produced N_2O. Hence, both the C_2H_2 and streptomycin effects on SIRIN results were probably low. However, as the product ratio in soil denitrification exhibited the full range from 0 to 1, this effect can be quite relevant and must thus be considered in future studies. Therefore, we recommend to estimate the effectiveness of C_2H_2 in blocking the N_2O reductase by performing parallel ^{15}N approaches with and without C_2H_2 in studies using the modified SIRIN to determine the fraction of bacterial or fungal N_2O production.

4.3 SP_{N2O} values of N_2O produced by microbial communities

As discussed above, all N_2O fluxes of modified SIRIN treatments of *Soil 1, 3* and *4* were dominated by N_2O from non-inhibitable organisms or processes. This made it impossible to calculate SP_{N2O} values for active bacteria or fungi (modified SIRIN B and C), also with *Soil 2,* where a relatively large N_2O production was observed with treatment D (Sutka et al., 2008; Rohe et al., 2014a; Maeda et al., 2015) (see section 4.4).

Despite this, the SP_{N2O} values from $+C_2H_2$ variety as well as SP_{prod} values (i.e., reduction corrected SP_{N2O} values of $-C_2H_2$ variety) of each *Soil*, represented by treatment A of modified SIRIN, indicated predominantly bacteria to be responsible for N_2O production during denitrification, assuming that results of SP_{N2O} values of denitrification by pure bacterial cultures is transferable to bacteria of soil communities contributing to denitrification. Also in many soil incubation studies SP_{N2O} values (without reduction effects) within the range of bacterial pure cultures have been found (Lewicka-Szczebak et al., 2015; Lewicka-Szczebak et al., 2017; Senbayram et al., 2018). Therefore, there was no unequivocal evidence of fungi contributing to N_2O production during denitrification, although here, the isotopic approaches revealed a fungal contribution to N_2O production during denitrification of up to 20 % on N_2O production during denitrification.

The SP_{N2O} values of variety $+C_2H_2$ within treatment A are not affected by reduction effects and therefore might give evidence of the microbial community contributing to N_2O production (Sutka et al., 2006; Sutka et al., 2008; Frame and Casciotti, 2010; Rohe et al., 2014a). However, variations in SP_{N2O} values of treatments A of variety $+C_2H_2$ were very small and do not give a clear evidence of any differences in microbial soil community producing N_2O. Lewicka-Szczebak et al. (2014) analysed SP_{N2O} values of denitrification with blockage of N_2O reduction by C_2H_2 for the same soils as used in the present study (*Soil 1* and *4* as well as *Soil 3*) and revealed SP_{N2O} values between -3.6 and -2.1 ‰, which is similar to the respective SP_{N2O} values of the present study from -4.9 to -0.4 ‰. This reinforces the conclusion that bacteria dominated gross N_2O production under anoxic conditions in both studies.

SP_{prod} values (variety $-C_2H_2$) differed from SP_{N2O} values (variety $+C_2H_2$), which may result from deviations between the actual fractionation factor that was not estimated in the present study and the used fractionation factor of -6 ‰ adapted from the literature (Yu et al., 2020). If so, we could assume smaller fractionation effects in the present study as decreasing this average fractionation factor would lead to increasing SP_{prod} values, which in turn would result in values more similar to SP_{N2O} values of variety $-C_2H_2$.

4.4 Potential influence of co-denitrification

The influence of co-denitrification, which is predominantly associated with fungi (Spott et al., 2011), may have a large impact on N_2O production. E.g. Laughlin and Stevens (2002) found 92 % of N_2O production in their

experiment to be derived from co-denitrification and only 8 % from denitrification. So far, there is no study on SP_{N2O} values of N_2O produced by co-denitrification. Co-denitrification could have been a contributing process in *Soil 4*. When N in N_2O originates only from ^{15}N-labeled soil NO_3^-, measured $\delta^{15}N^{bulk}_{N2O}$ values as well as the ^{15}N enrichment of the labelled N pool producing N_2O (a_p) should show identical ^{15}N enrichment to the labeled soil NO_3^-. During co-denitrification, when one N atom in N_2O originates from labeled NO_3^- and the other one from an unlabeled N source, this results in a_p values and ^{15}N enrichment of produced N_2O smaller than the respective enrichment of the NO_3^- pool. The ^{15}N enrichment NO_3^- in *Soil 4* was about 60 % larger than the analyzed ^{15}N enrichment in N_2O, leading to the assumption that N_2O was produced not only by denitrification. We also calculated a_p values of the other three *Soils* (data not shown) which coincided with the ^{15}N enrichment of N_2O (Table 3), showing no indication of co-denitrification. Since a_p would not be affected by contributions of unlabeled N_2O we can thus exclude the possibility that this smaller enrichment could be caused by dilution of enriched N_2O from denitrification by N_2O production from an unknown N source and thus verified that this was due to formation of hybrid N_2O, probably via co-denitrification (Spott et al., 2011). The question arises, why hybrid N_2O formation was only found when the loamy sand was sampled in summer (June, *Soil 4*) but not when it was sampled during winter (December, *Soil 1*). Since environmental conditions may vary within one year in arable soils, soil pH, F:B ratio, or biomass as presented in Table 1 might have been different for samples collected in summer 2011. However, as the soil was amended with C and N, the current state of the soil was changed before incubation in any case. Although soil properties, microbial community or biomass may have changed over time, we assumed pre-incubating the soil for seven days, applying C and N, and changing the environmental conditions during denitrification induced a rapid growth of specific organisms. It has to be presumed that the denitrifying community and the abundance of these organisms in incubation experiments may differ from the community in the field. Since these possible factors were not assessed in our study and their impact on co-denitrification is still poorly understood, it is currently not possible to give an answer here. Thus, only the SP_{N2O} values in *Soil 4* might be influenced by co-denitrification. But since SP_{N2O} values of the acetylated treatments of *Soil 4* coincided with the SP_{N2O} value range of bacterial denitrification and also with SP_{N2O} values of the other *Soils*, our data give no indication that co-denitrification produces N_2O with SP_{N2O} values differing from bacterial denitrification.

4.5 Calculating the fungal fraction contributing to N_2O production

Due to the inefficiency of the inhibition of microbial N_2O production in most cases, calculation of f_{FDmi} contributing to N_2O production was possible for *Soil 2* only, although even this calculated value included inaccuracies. The isotopic approaches, however, which are independent of modified SIRIN results, yielded similar estimates of f_{FD} for all *Soils*. As recently published (Wu et al., 2019), uncertainty analysis is a complex issue and large uncertainties of the results from the SP/$\delta^{18}O$ Map approach can be assumed when all the possible sources of errors are taken into account. Regarding the presented application of SP/$\delta^{18}O$ Map, calculation would be more precise when measuring $\delta^{18}O(H_2O)$ rather than using the fitted $\delta^{18}O(H_2O)$ values. Still, the analysis of $\delta^{18}O_{N2O}$ values can give information about O exchange between water and denitrification intermediates by various microorganisms (Aerssens et al., 1986; Kool et al., 2007; Rohe et al., 2014b; Rohe et al., 2017). The range of $\delta^{18}O_{N2O}$ values in our study for variety $+C_2H_2$ (7.5 to 19.0 ‰) was 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 the O exchange was probably also very high. There were also no

remarkable differences in $\delta^{18}O_{N2O}$ values among treatments within one variety and soil and therefore we assume no differences in O exchange among the treatments. The information on $\delta^{18}O_{N2O}$ values combined with known $\delta^{18}O_{H2O}$ values is also precious information for differentiation between N_2O mixing and reduction processes (Lewicka-Szczebak et al., 2017). Due to parallel *traced* variety experiments, possible $\delta^{18}O_{H2O}$ values for the particular SP_{N2O} values of bacterial denitrification mixing endmembers could be determined (Table 4). Since the $\delta^{18}O_{H2O}$ value for the particular geographic region can be assessed based on the known isotopic signatures of meteoric waters (Lewicka-Szczebak et al., 2014; Stumpp et al., 2014; Lewicka-Szczebak et al., 2017; Buchen et al., 2018), the most plausible ranges of $\delta^{18}O_{H2O}$ values can be used to indicate the plausible ranges of f_{FD_MAP} values. Here we showed that in case of missing $\delta^{18}O_{H2O}$ values but known product ratio, the SP/δ^{18}O Map can also provide information on N_2O production pathway contributions. Comparing the modified SIRIN with the isotopic approaches revealed that the fungal contribution to N_2O production was consistently estimated to be smaller (about 28 % in modified SIRIN, ≤15 % with IEM, ≤20 % with SP/δ^{18}O Map) than the bacterial fraction. In some soil studies using helium incubations, the SP_{Prod} values obtained by correction for the reduction effect on SP_{N2O} values showed significantly larger values than SP_{N2O} of bacterial denitrification (Köster et al., 2013a; Lewicka-Szczebak et al., 2017; Lewicka-Szczebak et al., 2014; Senbayram et al., 2018; Senbayram et al., 2020). However, those results were obtained in an experimental setup with ambient oxygen concentration. Short incubations under static conditions as presented here may, however, promote bacterial over fungal growth, which may also be transferable to denitrification activity by both organism groups (Lewicka-Szczebak et al., 2014; Lewicka-Szczebak et al., 2017). Obviously, based on the estimations from isotopic approaches, soils may largely differ in the microbial community that contributes to N_2O from denitrification.

However, all our tested soils seemed to contain a microbial community where fungi have minor contributions to N_2O emissions from denitrification compared to bacteria. This may also have been due to the applied experimental setup favoring bacterial denitrification by static and strictly anoxic conditions. Additionally, the use of glucose as substrate in the selected concentration may further promote bacteria compared to fungi (Koranda et al., 2014; Reischke et al., 2014). Senbayram et al. (2018) could show in an incubation experiment with sufficient NO_3^- supply, that fungal contribution to denitrification was larger with straw compared to a control without straw addition. Thus, experimental conditions need to be carefully set and more information is needed here in order to get a good representation of soil conditions in incubation experiments.

The isotopic approaches should be further investigated with soils where fungi are presumed to contribute largely to N_2O production (e. g. acid forest soils, or litter-amended arable soils) (Senbayram et al., 2018) and using SIRIN with more suitable inhibitors (Ladan and Jacinthe, 2016). The critical question whether the isotopic signatures of fungal N_2O determined in pure culture studies are transferable to natural soil conditions could not be answered with this study due to large uncertainties associated with the results of the SIRIN method. The latter precluded determination of SP_{N2O} values of N_2O from fungal denitrification. Further experiments would be needed with improved selective inhibition to assure that SP_{N2O} values known from a few pure cultures or soil isolates (Sutka et al., 2008; Rohe et al., 2014a; Maeda et al., 2015) are true for fungal soil communities as well. This could be accompanied by studies mixing various fungal species known to occur in soil or by isolating fungal communities from soil and conduct similar experiments under anoxic conditions with supply of electron acceptors and C sources to investigate denitrification. In such incubations, parallel ^{15}N tracing experiments should be conducted to assure denitrification as the dominating process for N_2O production and quantify the possible contribution of co-denitrification.

5. Conclusions

Based on the presented results we conclude that the modified SIRIN approach presented here is not appropriate to estimate the contribution of selected communities (bacteria or fungi) on denitrification from soil. Here, the quantification of the fungal fraction with modified SIRIN could be done with one soil only and was possibly overestimated when compared the results of isotopic approaches. Both isotope approaches (IEM and SP/$\delta^{18}O$ Map) revealed similar results of the fungal fraction contributing to denitrification and thus could be recommended as equally suitable for future studies. The present study could show that consideration of N_2O reduction is indispensable. It has to be pointed out, however, that the fungal fraction estimated applies only for the soil under presented experimental conditions, i.e. anaerobic conditions, but not for the investigated soil in general.

Further studies are needed to cross-validate methods, e. g. with improved inhibitor approaches or molecular-based methods. Due to the mentioned difficulties, the SP_{N2O} values of fungal N_2O could not be calculated from the modified SIRIN approach.Several potential artefacts in the modified SIRIN approach should be further investigated, e.g. the effectiveness of inhibitors, changes in microbial community during pre-incubation with inhibitors and effects of bacterial consumption of N_2O produced by fungi.

Data availability. Gas emission and isotopic data are available from the authors on request.

Author contribution. HF, NWM, RW and THA designed the experiment. LR carried out the experiment at Thünen Institute for Climate-Smart Agriculture in Braunschweig. AG, DLS and RW helped with isotopic analysis and DLS performed the $\delta^{18}O$/SP Map. LR, RW and DLS prepared the manuscript with contributions from all co-authors.

Competing interests. The authors declare that they have no conflict of interest.

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