# Comparing modified substrate induced respiration with selective inhibition (SIRIN) and N$_2$O isotope approaches to estimate fungal contribution to denitrification in three arable soils under anoxic conditions

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## Abstract

The coexistence of many N$_2$O production pathways in soil hampers differentiation of microbial pathways. The question whether fungi are significant contributors to soil emissions of the greenhouse gas nitrous oxide (N$_2$O) from denitrification has not yet been resolved. Here, as far as we know, three approaches to independently investigate the fungal fraction contributing to N$_2$O from denitrification were used simultaneously for the first time (modified substrate induced respiration with selective inhibition (SIRIN) approach, and two isotopic approaches, i.e. endmember mixing approach (IEM) using the $^{15}$N site preference of N$_2$O produced ($SP_{N2O}$), and the SP/$\delta^{18}$O mapping approach (SP/$\delta^{18}$O Map)). This enabled a comparison of methods and a quantification of the importance of fungal denitrification in soil.

Three soils were incubated in four treatments of the SIRIN approach under anaerobic conditions to promote denitrification. 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$_2$O reduction on N$_2$O produced, while two other ~~varieties~~ were performed under natural isotopic conditions with and without acetylene.

All three approaches revealed a small ~~fungal~~ contribution to N$_2$O fluxes ($f_{FD}$) under anaerobic conditions in the soils tested. Quantifying the fungal fraction with modified SIRIN was not successful due to large amounts of uninhibited N$_2$O production. In only one soil, $f_{FD}$ could be estimated using modified SIRIN 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.

40    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
45    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.

50    Denitrification describes 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). 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 that although only some fungal species (e.g. *Fusarium* strains) are performing respiratory denitrification, these may produce
55    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, since~~, 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, due to a lacking $N_2O$ reductase (Nos) (Shoun et al., 1992; Shoun et al., 2012; Higgins et al.,
60    2018), $N_2O$ is the major end product of fungal denitrification. However, although there are methodological approaches to ~~disentangle~~ sources of $N_2O$, it is still challenging to clearly attribute $N_2O$ emitted from soil to bacterial or fungal denitrification.

One approach to differentiate between $N_2O$ produced by fungi and bacteria during denitrification comprises the addition of two antibiotics to soil incubation experiments, i.e. streptomycin and cycloheximide to inhibit bacterial
65    or fungal protein biosynthesis, i.e. growth, respectively. This method is known as substrate induced respiration with selective inhibition (SIRIN) and was originally developed to determine the bacterial or fungal contribution to $CO_2$ respiration (Anderson and Domsch, 1975). A few studies used a modification of this method for $N_2O$ analysis (Laughlin and Stevens, 2002; Crenshaw et al., 2008; Blagodatskaya et al., 2010; Long et al., 2013) and found a greater decrease of $N_2O$ production with fungal than with bacterial growth inhibition (i.e. 89 vs. 23 % decrease,
70    respectively (Laughlin and Stevens, 2002)). This indicated 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). However, difficulties of this method may be to achieve complete inhibition of selective groups (Ladan and Jacinthe, 2016) and to avoid shifts in the structure of microbial communities as response of pre-incubation or duration of experiments. Another opportunity to distinguish between
75    $N_2O$ from bacterial and fungal denitrification and other pathways is the analysis of the isotopic composition of $N_2O$. Especially the isotopomer ratios of $N_2O$ (i.e. $N_2O$ molecules with the same bulk $^{15}N$ isotopic enrichment but 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). Isotopomer ratios of $N_2O$ can be expressed as $^{15}N$ site

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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 bacterial cultures 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). While it is generally assumed that $SP_{N2O}$ values of $N_2O$ produced by fungal pure cultures during denitrification are 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 large ranges of fungal $SP_{N2O}$ values (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.

The $SP_{N2O}$ value of $N_2O$ produced by pure bacterial cultures during nitrification is approximately 33 ‰ and thus interferes with that of fungal denitrification (Sutka et al., 2006; Sutka et al., 2008; Rohe et al., 2014a). This demonstrates the difficulty to use solely $SP_{N2O}$ values as an indicator for different organism groups contributing to $N_2O$ production from soil, where different pathways may co-occur. Based on the above cited ranges for the isotopomer ~~endmembers~~ of fungal and bacterial denitrification, and assuming that only fungal and bacterial denitrification 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 does not occur (Ostrom et al., 2010; Ostrom and Ostrom, 2011). If there is a $N_2O$ reduction, $SP_{N2O}$ and also $\delta^{15}N$ and $\delta^{18}O$ values of produced $N_2O$ ($\delta^{15}N^{bulk}_{N2O}$ and $\delta^{18}O_{N2O}$, respectively) are affected by isotopic fractionation (Ostrom et al., 2007; Ostrom and Ostrom, 2011). This means that the $^{14}N^{16}O$ bond of $N_2O$ is preferentially broken compared to $^{14}N^{18}O$ or $^{15}N^{16}O$, resulting in $N_2O$ that is isotopically enriched in $^{15}N$ and $^{18}O$ and shows larger $SP_{N2O}$ values compared to $N_2O$ from denitrification without the reduction step (Popp et al., 2002; Ostrom et al., 2007).

In controlled laboratory experiments, the $N_2O$ reduction to $N_2$ can be inhibited 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. However, problems due to incomplete inhibition of $N_2O$ reduction and unwanted inhibition of other pathways may occur (Wrage et al., 2004b; Wrage et al., 2004a). Another possibility to quantify $N_2O$ reduction to $N_2$ during denitrification is also possible with $^{15}N$ tracing experiments using $^{15}N$ enriched substrates and analysing $^{15}N_2$ fluxes (Well et al., 2006; Lewicka-Szczebak et al., 2014). The $^{15}N$ tracer approach also enables to distinguish between $N_2O$ from fungal denitrification and co-denitrification, i.e. 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; Laughlin and Stevens, 2002; Rohe et al., 2017).

$N_2O$ reduction can be quantified using $N_2O$ natural abundance isotopic signatures, which also enables simultaneous differentiation of selected pathways ~~producing $N_2O$~~. Here, the isotope mapping approach uses 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 (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., 2017) and different slopes of $N_2O$ reduction and mixing lines in the $\delta^{18}O$ – SP isotope plot. While $SP_{N2O}$ values are independent of isotopic signatures of the precursors, $\delta^{15}N^{bulk}_{N2O}$ and $\delta^{18}O_{N2O}$ result from the isotopic signature of the precursor and isotopic

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fractionation during N₂O 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 (Kool et al., 2009; Snider et al., 2009; Lewicka-Szczebak et al., 2016). However, interpretation of $\delta^{18}O_{N2O}$ values from different microbial groups may be more complex due to incomplete O exchange, because 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). Importantly, fungal and bacterial N₂O showed different ranges for $\delta^{18}O_{N2O}$ values, hence this isotopic signature may also be helpful in differentiation of these pathways (Lewicka-Szczebak et al., 2016). This SP/$\delta^{18}O$ Map approach thus allows for an estimation of the contributions of N₂O reduction and admixture of fungal N₂O.

So far, the described methods for distinguishing between fungal and bacterial N₂O emission have not been compared in the same soil and their accuracy and possible bias ~~remains~~ unknown. A better knowledge of the comparability of the methods would enable comparison of results of studies using different methods and thus further improve our understanding of processes of N₂O production. It would also reveal weaknesses of approaches and might lead to the development of better methods.

Therefore, this study aims at (i) determining the fungal contribution to N₂O production by denitrification under anoxic conditions and glucose addition using three arable soils and three approaches (modified SIRIN, IEM and the *SP/δ¹⁸O* 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. We hypothesized that the fungal fraction contributing to N₂O from denitrification in different soils using a modified SIRIN approach and isotopic methods will be correlated but not exactly matched 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 N₂O from denitrification will yield fungal $SP_{N2O}$ ~~endmember~~ values within the range of values previously reported in the literature.

## 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 N₂O 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 1.1*", "*Soil 1.2*", "*Soil 2*", and "*Soil 3*": *Soil 1.1 and Soil 1.2* with loamy sand (*Soil 1*) sampled in December 2012 and in June 2011, respectively, *Soil 2* with sand sampled in January 2013, and *Soil 3* with silt loam sampled in December 2012 (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 1.1, 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) (Table 1).

The three soils were also sampled in summer 2010 for pre-experiments to gain information on the respiratory biomass by analyzing the 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) by a computer-generated selectivity analysis: "SIR-SBA 4.00" (Heinemeyer, copyright MasCo Analytik, Hildesheim, Germany) (Anderson and Domsch, 1975) (Table 1). 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.
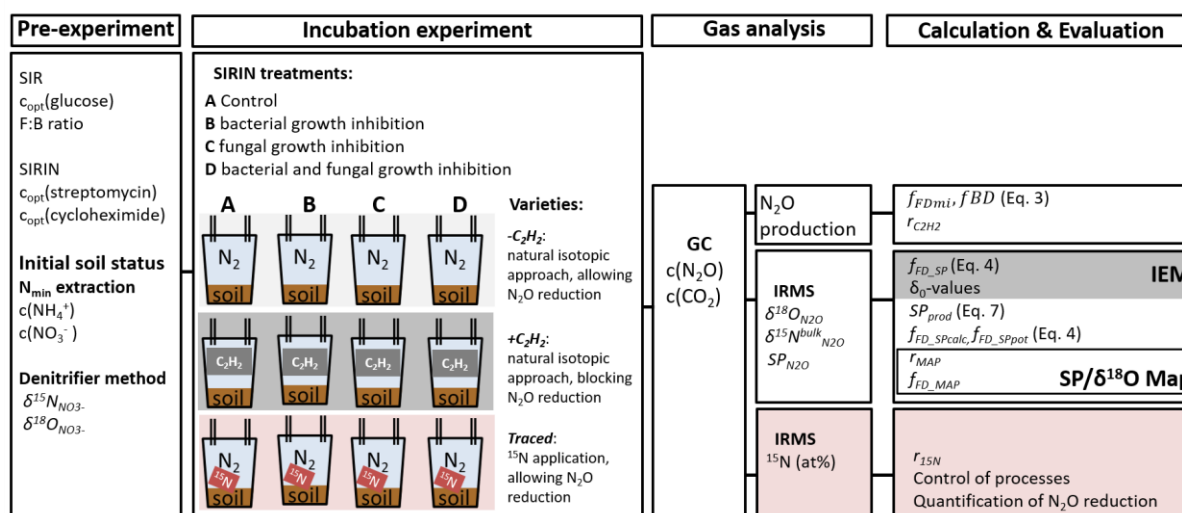


**Figure 1: The methodical approach comprised a pre-experiment with substrate induced respiration (SIR) to estimate the optimal glucose concentration ($c_{opt}$(glucose)) and the fungal-to-bacterial ~~ration~~ in the soil (F:B ratio), and the substrate induced respiration with selective inhibition approach (SIRIN) to determine the optimal inhibitor concentration ($c_{opt}$(streptomycin and $c_{opt}$(cycloheximide)). The initial soil status, i.e. ammonium and nitrate concentration of the soil ($c(NH_4^+)$ and $c(NO_3^-)$, respectively), was measured in $N_{min}$ extracts and the isotopic signature of soil $NO_3^-$ was analysed by the denitrifier method. The incubation experiment comprised the SIRIN approach with three experimental varieties: without acetylene ($-C_2H_2$), with $C_2H_2$ ($+C_2H_2$), and without $C_2H_2$ but with $^{15}N$ labelled $NO_3^-$ (*traced*), while $NO_3^-$ with natural isotopic composition was added to the other two varieties. 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) for its isotopic composition. 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.

195 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

200 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

205 (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, on the

210 assumption that concentrations optimal for $CO_2$ respiration also allow denitrification. Examples of respiration curves derived from SIR and SIRIN pre-experiments are represented in Figure S1 and S2, respectively.

**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 (C, N, pH, $\delta^{15}N_{NOx}$ and $\delta^{18}O_{NOx}$) 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 (CaCl₂) | $\delta^{15}N_{NOx}$ [‰][e] | $\delta^{18}O_{NOx}$ [‰][e] | F:B[f] | Biomass[g] [µg C gdw⁻¹ soil] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.1 (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 |
| 1.2 (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) in a pre-experiment in 2010.
[g]Respiratory biomass analysed by $CO_2$ production from SIR method (Anderson and Domsch, 1978) in a pre-experiment in 2010..

**2.2.2 Soil incubation with selective inhibition to determine $N_2O$ forming processes**

225 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

230 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)

235 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

240 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, +$C_2H_2$*

245 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 1.2* 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 1.1, 2* and *3*. In variety *traced,* $NO_3^-$ with a

250 $^{15}N$ 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

255 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 1.1, 1.2,* and *2* 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

260 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 1.1, 2* and *3*) or two, four and eight hours (*Soil 1.2*) 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 1.2*).

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 1.1, 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 1.2* 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 1.2* (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):

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

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:

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

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The fungal contribution to N$_2$O production during denitrification with microbial inhibition ($f_{FDmi}$) can be calculated, when N$_2$O production of treatment D is significantly smaller than N$_2$O production of treatments A, B and C by:

$$f_{FDmi} = \frac{(A-C)}{(A-D)}$$ (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$_2$O 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$_2$O production. Assuming that bacteria ($BD$) and fungi ($FD$) are the only sources of N$_2$O in soil, the $^{15}$N site preference values of produced N$_2$O ($SP_{prod}$) results from the $SP_{N2O}$ mixing balance:

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

where $f_{FD}$ and $f_{BD}$ represent the fraction of N$_2$O produced by fungi and other N$_2$O 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$_2$O 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$_2$O production ($f_{FD\_SP}$), as microorganisms of this variety produce N$_2$O that is not affected by reduction to N$_2$. The $f_{FD\_SP}$ contributing to N$_2$O 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.

Based on $SP_{N2O}$ values from $-C_2H_2$ variety, it was possible to solve Eq. 4 also to estimate the maximum potential fungal contribution to denitrification ($f_{FD\_SPpot}$), assuming that we did not have any estimations for N$_2$O reduction. While bacterial denitrification and nitrifier denitrification would result in low $SP_{N2O}$ values ($SP_{BD/ND}$=-10.7 to +3.7 ‰ (Frame and Casciotti, 2010; Yu et al., 2020)), large $SP_{N2O}$ values would be expected from fungal denitrification and nitrification ($SP_{FB/N}$=16 to 37 ‰ (Sutka et al., 2008; Decock and Six, 2013; Rohe et al., 2014a; Maeda et al., 2015; Rohe et al., 2017)). N$_2$O reduction could have further increased the $SP_{prod}$ values. If the contribution of this process on $SP_{prod}$ values cannot be precisely estimated, by neglecting these effects we can determine the maximal potential fungal contribution. $f_{FD}$ calculated from Eq. 4 (variety $-C_2H_2$) would thus be lower if N$_2$O reduction had occurred. However, assuming the impact of N$_2$O reduction on $SP_{N2O}$ was negligible, this IEM enabled to calculate the maximum potential $f_{FD}$ as $f_{FD\_SPpot}$ = 1-(($SP_{N2O}$-$SP_{FD/N}$)/($SP_{BD/ND}$-$SP_{FD/N}$)).

**2.5.2 Product ratio [N₂O/(N₂+N₂O)] 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:

$$r_{15N} = \frac{{}^{15}N_{N2O}}{{}^{15}N_{N2} + {}^{15}N_{N2O}}$$ (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 $C_2H_2$ in blocking 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 +$C_2H_2$:

$$r_{C2H2} = \frac{N_2O_{-C2H2}}{N_2O_{+C2H2}}$$ (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 as follows. 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 $N_2O$ ($SP_{N2O-r}$), were corrected with the net isotope effect of $N_2O$ reduction ($\eta r$) and the $r_{15N}$ as follows:

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

According to Yu et al. (2020) the $\eta r$ 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/$\delta^{18}$O isotope mapping approach (SP/$\delta^{18}$O Map)**

The $f_{FD}$ contributing to $N_2O$ production from denitrification in soil samples was also estimated with the SP/$\delta^{18}$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/$\delta^{18}$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

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

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 $^{15}N_{fert}$ the $^{15}N$ 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 0.05 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/$\delta^{18}O$ 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, Figure 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 (Figure 2). 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 1.1, 2* and *3* and eight hours for *Soil 1.2*.

$N_2O$ 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 1.1* and *3*, respectively, while in *Soil 2* and *1.2* $N_2O$ production rates

were smaller (271 and 264 µg N kg$^{-1}$h$^{-1}$, respectively). N$_2$O and CO$_2$ production rates were significantly larger in variety₁ +C$_2$H$_2$ than in variety₁ -C$_2$H$_2$ of *Soil 1.1, 1.2* and *3* ($P < 0.001$, $P = 0.002$, and $P < 0.001$ for N$_2$O production rate and $P < 0.001$, $P = 0.027$, and $P = 0.008$ for CO$_2$ production rate, respectively) (Table 2), while -C$_2$H$_2$ and +C$_2$H$_2$ varieties₁ of *Soil 2* did not differ in N$_2$O and CO$_2$ production rates *($P = 0.640$ and $P = 0.342$, respectively).*

**Figure 2: Time series of average N$_2$O and CO$_2$ production rates during incubation of variety +C$_2$H$_2$ at the three sample collection times of each soil (*Soil 1* to *3*) 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 α ≤ 0.05). For all significant regressions, *R²*-values were ≥ 0.46 and in the case of non-significance, *R²*-values were ≤ 0.40.**
**n.d.: There was no detectable CO$_2$ production in *Soil 1.2* at the first sampling time after 2 hours. (Figure is continued on next page)**
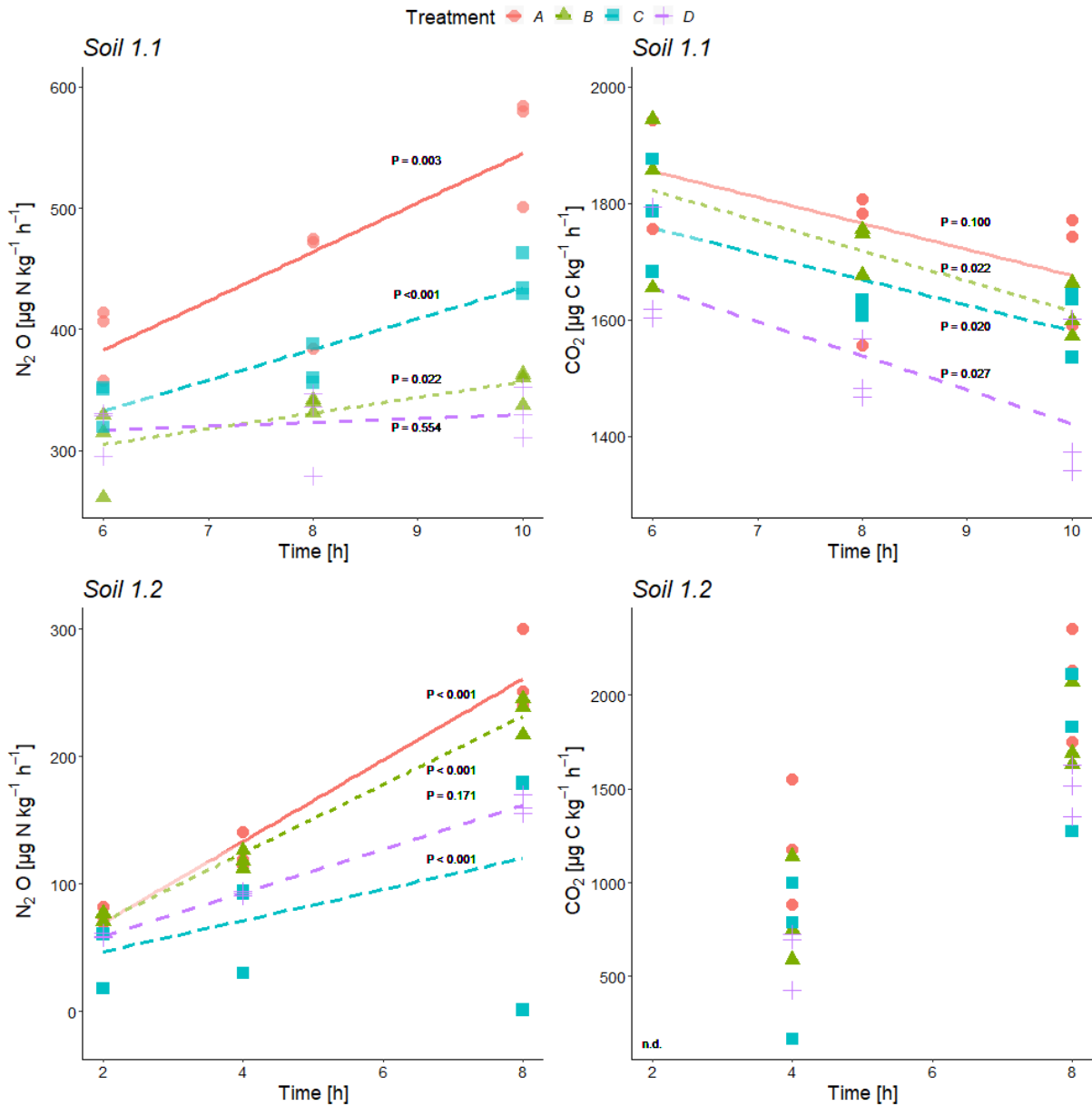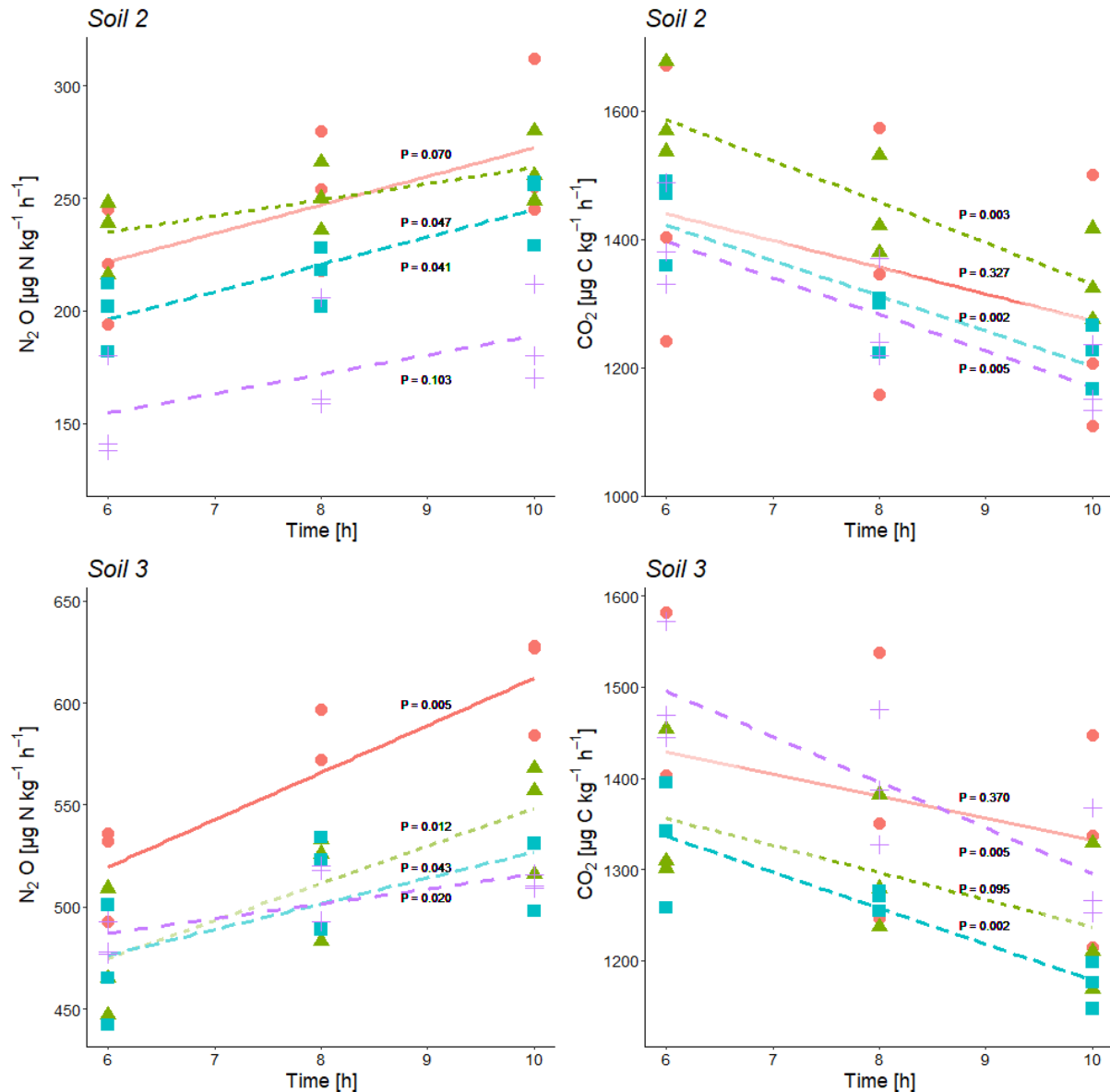
**Figure 2 continued.**

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 ($P < 0.001$) (Table 2). In *Soil 1.1*, $N_2O$ production rate was significantly larger (272 µg N kg⁻¹ h⁻¹) than in *Soil 1.2* (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 1.2, 2*, and *3*) or treatment C (fungal growth inhibitor; more $N_2O$ compared to treatment B in *Soil 1.1*). 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 1.1*, varieties -$C_2H_2$ and traced of *Soil 3* and variety *traced* of *Soil 1.2*). Microbial inhibitor treatments differed significantly in $N_2O$ fluxes of variety +$C_2H_2$ of each *Soil* (always $P \leqslant 0.042$), while this was not the case for inhibitor treatments of varieties -$C_2H_2$ and *traced* of *Soil 1.2* ($P = 0.154$ 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 1.1* (+$C_2H_2$ and -$C_2H_2$), *Soil 2* (-$C_2H_2$) and *Soil 3* (+$C_2H_2$); treatment D was

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significantly smaller compared to the other three treatments in *Soil 2* ($+C_2H_2$) only and compared to treatments A and C in *Soil 1.1* ($+C_2H_2$). 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.

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**Table 2: Average CO₂ and N₂O production rates and N₂O isotopic values of N₂O of the last sample collection with and without C₂H₂ application in the headspace (~~varieties~~ -C₂H₂ and +C₂H₂) of each soil (*Soil 1 to 3*) 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 1.1* (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)ab | 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)ab | 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)a |
| 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)a |
| 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 1.2* (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)ab | 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)b | 1736.8 (424.7)a | 19.0 (7.0)a | -33.1 (2.8)a | -1.7 (2.7)a |
| D / +$C_2H_2$ | 161.6 (7.6)ab | 1497.0 (138.7)a | 14.8 (0.5)a | -35.7 (0.2)a | -4.9 (0.7)a |
| *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)ab | -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)ab | -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)b | -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)ab | 1159.3 (178.2)a | 24.1 (0.2)a | -22.0 (0.2)a | -0.1 (0.4)a |
| C / -$C_2H_2$ | 278.9 (9.8)b | 1056.0 (59.6)a | 27.3 (0.1)a | -20.6 (0.3)a | 0.6 (0.2)a |
| D / -$C_2H_2$ | 291.1 (38.5)ab | 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)a | -25.1 (0.1)a | -4.3 (0.5)a |

Letters denote significant differences (P < 0.05) 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 N₂O 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 1.2* 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~~ +$C_2H_2$ ($P < 0.001$) (up to 2.4, 1.5, 4.6 and 4.1 ‰ in *Soil 1.1, 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). 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 1.1* (-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 1.1* and *1.2, 2* and *1.2*, and *1.1* 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, N₂O 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 $\delta0$, i.e. $SP_{prod}$ values of N₂O production, and can thus be used for applying the IEM.

#### 3.2.3 ~~Variety~~ *traced*

The ¹⁵N-labeling of N₂O ($^{15}N_{N2O}$) or N₂ produced ($^{15}N_{N2}$) gave information about the incorporated N from ¹⁵N-labeled NO₃⁻ into N₂O or N₂ as well as about the N₂O reduction to N₂. Microorganisms in each treatment used the ¹⁵N-labeled NO₃⁻ in ~~variety~~ *traced* (Table 3) and expected $^{15}N_{N2O}$ depended on the initial N abundance in NO₃⁻ of unfertilized soil (Eq. 7). *Soil 1.2* is the only one showing a large discrepancy between measured (about 30 at%) and calculated $^{15}N_{N2O\_exp}$ (49 at%) in N₂O, whereas the other *Soils* showed close agreement (Table 3).

### 3.3 Product ratios of denitrification and efficiency of N₂O 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 than of treatment C and D of *Soil 1.2* ($P = 0.032$), 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 N₂O reduction by $C_2H_2$ application, $r_{C2H2}$ (Eq. 5) was compared with $r_{15N}$ (Eq. 6). In *Soil 1.1*, $r_{C2H2}$ 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 N₂O reduction was assumed for those *Soils*.

**Table 3: Average CO$_2$ and N$_2$O production rates of the last sample collection after 10 or 8 hours of variety *traced*, respectively, with $^{15}$N labeling in N$_2$O ($^{15}$N$_{N2O}$) and the calculated $r_{15N}$ of variety *traced* and $r_{C2H2}$ calculated from N$_2$O production rates of variety -$C_2H_2$ and +$C_2H_2$ of each soil (*Soil 1* to *3*) 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$_2$O [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 1.1* (Loamy Sand, 2012) | | | | | | |
| A | 255.6 (43.5) | 1310.0 (167.3) | 36.8 (0.1) | | 0.80 (0.02) | 0.48 (0.07) |
| B | 154.5 (29.6) | 1153.5 (238.4) | 36.4 (0.2) | 39 | 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 1.2* (Loamy Sand, 2011) | | | | | | |
| A | 156.9 (62.7) | 3111.4 (1252.5) | 31.1** | | 0.54 (0.05) | 0.63 (0.10) |
| B | 169.2 (6.1) | 2314.6 (307.1) | 26.5** | 49 | 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) |
| *Soil 2* (Sand, 2012) | | | | | | |
| A | 240.7 (0.95) | 1286.2 (5.6) | 43.2 (<0.1) | | 0.94 (0.01) | 1.04 (0.10) |
| B | 185.1 (3.9) | 1157.4 (17.3) | 43.0 (0.1) | 44 | 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) | | 0.62 (<0.01) | 0.52 (0.04) |
| B | 320.5 (14.7) | 1204.2 (86.5) | 35.5 (<0.1) | 34 | 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) |

**Asterisks indicate that only two samples (*) or one sample (**) were analysed due to logistical difficulties.**
[a]$^{15}$N$_{N2Oexp}$ **[at%] was calculated from Eq. 8.**
[b]$r_{15N}$ **= [N$_2$O/(N$_2$+N$_2$O)] with N$_2$O or N$_2$ production rates from variety *traced*; see Eq. 5**
[c]$r_{C2H2}$ **= [N$_2$O$_{-C2H2}$/N$_2$O$_{+C2H2}$] with N$_2$O production rate from varieties -$C_2H_2$ and +$C_2H_2$; see Eq. 6, cf. Table 2**

### 3.4 Fungal contribution to N$_2$O production from denitrification by microbial inhibitor approach (modified SIRIN)

When calculating $f_{FDmi}$, N$_2$O 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$_2$O 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$_2$O production in treatment D was large within all varieties. Only with *Soil 2* of the variety +$C_2H_2$, the N$_2$O 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$_2$O production rate of 23.7 ± 1.8 µg N kg$^{-1}$ h$^{-1}$. Although the N$_2$O 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 possible, i.e. SIRIN was not successful.

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**3.5 Fungal contribution to N₂O production from denitrification by the SP endmember mixing approach (IEM) and SP/δ¹⁸O isotope mapping approach (SP/δ¹⁸O Map)**

515    The IEM revealed that $f_{FD\_SP}$ was small in all *Soils* (≤15 %, ≤14 %, ≤ 9%, and ≤ 11 % with *Soil 1* to *3*, respectively) (Table 5). Regardless of influence of N₂O reduction on $SP_{N2O}$ values, only in *Soil 1.2* $f_{FD\_SPpot}$ could have reached 66%, while fungal denitrification could not have dominated with the other three soils (Table 5).

When applying SP/δ¹⁸O 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 $r_{15N}$

520    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/δ¹⁸O 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

525    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 1.1*, 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

530    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 1.2*, the

535    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 1.2*, 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 combination of $SP_{BD}$ and $\delta^{18}O_{H2O}$ values. Since the precision of $r_{15N}$

540    (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/δ¹⁸O Map show $f_{FD\_MAP}$ reaching up to 14, 20, 15, and 9 % for *Soils 1.1, 1.2, 2,* and *3*, respectively (Figure 3, Table 4, Table 5). Importantly, due to the fitting procedure applied the estimations of $f_{FD\_MAP}$ values are based not only on $SP_{N2O}$ and $\delta^{18}O_{N2O}$ values but also on the

545    results obtained in the ¹⁵N treatment ($r_{15N}$ values).

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**Table 4: Summary of the results provided by SP/$\delta^{18}$O Map for fraction of fungal denitrification ($f_{FD\_MAP}$) and N$_2$O 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 | $f_{FD\_MAP}$ [%]* |
|------|---------|-----------|---------------|--------------------------|-----------|------|--------------------|
| *1.1* | **-$C_2H_2$** | **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 |
| *1.2* | -$C_2H_2$ | 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** |
| *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** |

**\*Negative values for $f_{FD\_MAP}$ are non-realistic and therefore discarded for further interpretation.**

**Table 5: Ranges of the fraction of N₂O produced by fungi ($f_{FD}$) from four *Soils* using different approaches: Fungal fraction was calculated using a) the microbial inhibitor approach (modified SIRIN) ($f_{FDmi}$), the isotopomer endmember mixing approach (IEM) by SP isotope mixing balance using b) ~~variety~~ +$C_2H_2$ ($f_{FD\_SP}$), c) $f_{FD\_SPpot}$ for results from ~~variety~~ -$C_2H_2$ assuming the SP effect of N₂O reduction was negligible, d) for results from variety -$C_2H_2$ with reduction correction to calculate the $SP_{N2O}$ values ($f_{FD\_SPcalc}$), and e) the δ¹⁸O/SP Map ($f_{FD\_MAP}$) with δ¹⁸O$_{N2O}$ and $SP_{N2O}$ values from ~~variety~~ -$C_2H_2$ and variety +$C_2H_2$.**

| Soil | $f_{FDmi}$ [%][a] | $f_{FD\_SP}$ [%][b*] | $f_{FD\_SPpot}$ [%][c*] | $f_{FD\_SPcalc}$ [%][d*] | $f_{FD\_MAP}$ [%][e*] |
|---|---|---|---|---|---|
| 1.1 | n.d. | -14 to 15 | -12 to 39 | -6 to 19 | <2 |
| 1.2 | n.d. | -23 to 11 | 10 to 66 | 1 to 21 | 11 to 20 |
| 2 | 19 to 37 | -18 to 14 | -14 to 36 | -12 to 15 | 1 to 15 |
| 3 | n.d. | -25 to 9 | -11 to 40 | -9 to 18 | 4 to 9 |

[a]**Fungal fraction of N₂O production calculated by Eq. 3 taking variations of three replicates into account**

[b]**Fungal fraction of N₂O production calculated by Eq. 4 for variety +$C_2H_2$ with assuming $SP_{N2O}$ values of N₂O produced by bacteria were 3.7 ‰ or -7.5 ‰ (Yu et al., 2020) and by fungi on average 33.6 ‰ (Sutka et al., 2008; Rohe et al., 2014a; Maeda et al., 2015; Rohe et al., 2017). Using the minimum and maximum $SP_{N2O}$ values known for bacteria resulted in a $f_{FD\_SP}$ range.**

[c]**Maximum potential fungal fraction of N₂O production calculated by Eq. 4 as an average range for all treatments of variety -$C_2H_2$ assuming $SP_{N2O}$ values of N₂O produced by bacterial denitrification or nitrifier denitrification were between 3.7 and -10.7 ‰ (Frame and Casciotti, 2010; Yu et al., 2020) or produced by fungal denitrification or nitrification were between 16 and 37 ‰ (Sutka et al., 2008; Decock and Six, 2013; Rohe et al., 2014a; Maeda et al., 2015; Rohe et al., 2017). Using the minimum and maximum $SP_{N2O}$ values known from pure cultures resulted in the given $f_{FD\_SPpot}$ range. Here, the effect of partial reduction of N₂O on $SP_{N2O}$ values was assumed to be negligible.**

[d]**Eq. 4 to solve for fungal fraction in variety -$C_2H_2$ with assuming $SP_{N2O}$ values of N₂O produced by bacteria was 3.7 (resulting in negative fraction and therefore set to zero) or -7.5 ‰ and using reduction correction with $\eta_r$=-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.**

[e]**Fungal fraction of N₂O production calculated by SP/δ¹⁸O 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 δ¹⁸O$_{H2O}$ values (the fitting is based also on results obtained in ¹⁵N treatment) resulted in a $f_{FD\_MAP}$ range.**

*****Negative values for $f_{FD\_SP}$, $f_{FD\_SPpot}$, $f_{FD\_SPcalc}$, $f_{FD\_MAP}$ are non-realistic and therefore discarded for further interpretation.**

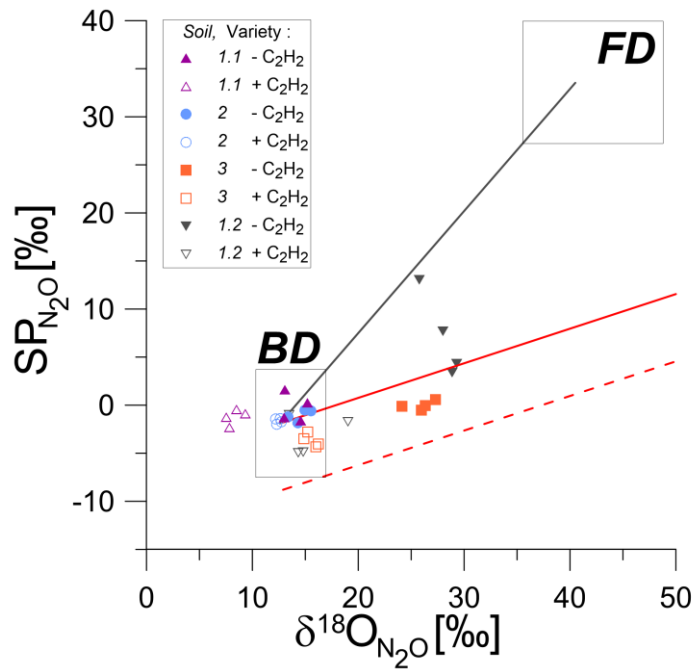**n.d.-not determined because of insufficient inhibition.**

**Figure 3: SP/δ¹⁸O isotope mapping approach (SP/δ¹⁸O Map) to estimate the contribution of bacteria or fungi to N₂O produced according to Lewicka-Szczebak et al. (2017) and Lewicka-Szczebak et al. (2020). The isotopic values for natural abundance treatments with acetylene addition (+C₂H₂, empty symbols) and without acetylene addition (-C₂H₂, corresponding filled symbols) are shown for four *Soils* (*1 to 3*). 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/δ¹⁸O Map) to estimate fungal contribution to N₂O production from denitrification in anoxic incubation. The isotopic approaches revealed that the fungal contribution to N₂O production was small ($f_{FD\_SP} \leq 15$ % or $f_{FD\_MAP} \leq 20$ %) in the soils tested (Table 5). A dominant contribution of fungi over bacteria was also excluded by the potential maximum fungal denitrification for *Soil 1.1, 2,* and *3* ($f_{FD\_SPpot}$ between 37 and 40 %, Table 5), even though effects of N₂O reduction are not included. The modified SIRIN approach was not successful, because large amounts of non-inhibitable N₂O production were observed with all four *Soils* (Table 2, Table 3). The fungal fraction producing N₂O during denitrification $(f_{FDmi})$ was only estimated for *Soil 2*, where significantly smaller N₂O 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 N₂O production of non-inhibitable sources. While the three approaches coincided in showing dominance of bacterial denitrification, the isotopic approaches yielded small estimates for $f_{FD}$ ($\leq$ 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 N₂O production by the fungal or bacterial fraction were unsatisfactory, thus fungal $SP_{N2O}$ values could not be assessed, and the overall results led to unsolved questions, which are discussed in the following sections.

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### 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 consumption of inhibitors as C sources 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 after 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 $C_2H_2$ application a suitable and necessary treatment for examining 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 1.2, 2,* and *3* (maximal 9 % difference), indicating effective blockage of $N_2O$ reductase in variety $+C_2H_2$ in these *Soils*. Only in *Soil 1.1*, $r_{15N}$ 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 1.1*.

For the other three *Soils* (*1.2, 2,* and *3*), 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 ($SP_{prod}$). 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 groups from soil. This has particular relevance for experiments with modified SIRIN approaches. Although the modified SIRIN approach presented here was not successful, it should be noted that comparable soil incubation experiments without quantifying $N_2O$ reduction potentially overestimate fungal denitrification due to the impact of SIRIN inhibitors on $N_2O$ reduction.

Of course, $N_2O$ 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, successful inhibition of 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. 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.

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 1.1, 1.2,* and *3* 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 3.4).

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 are 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 so far no unequivocal evidence of fungi contributing to $N_2O$ production during denitrification in soils, although here, the isotopic approaches were consistent with a fungal contribution of up to 20 % of $N_2O$ 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 1.1* and *1.2* 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 hybrid $N_2O$

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 of $N_2O$ in *Soil 1.2* was about 60 % smaller than the ^{15}N enrichment in soil $NO_3^-$, 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 hybrid $N_2O$. 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 $N_2O$, potentially via co-denitrification (Spott et al., 2011). So far, there is no study on $SP_{N2O}$ values of $N_2O$ produced by co-denitrification. But since $SP_{N2O}$ values of the acetylated treatments of *Soil 1.2* 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 the $SP_{N2O}$ values of hybrid $N_2O$, potentially produced during co-denitrification, differed from that of bacterial denitrification. It was however, remarkable that the maximum potential contribution of fungal denitrification to $N_2O$ ($f_{FD\_SPpot}$) was higher for *Soil 1.2* compared to that of *Soil 1.1* from the winter period. *Soil 1.2* was the only soil where $f_{FD\_SPpot}$ exceeded 50%, thus fungi may potentially dominate $N_2O$ emissions only in this *Soil*.

### 4.5 Steps towards quantifying 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 $\delta^{18}O_{H2O}$ rather than using the fitted $\delta^{18}O_{H2O}$ 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/$\delta^{18}$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/$\delta^{18}$O Map) than the bacterial fraction. This was supported by estimates for maximum potential contribution of fungal denitrification to $N_2O$ in variety $-C_2H_2$ ($f_{FD\_SPpot}$) for *Soil 1.1, 2* and *3*. 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 in the form presented here is not appropriate to estimate the contribution of selected communities (bacteria or fungi) on denitrification from soil. The quantification of the fungal fraction with modified SIRIN could be done with one soil only and was possibly overestimated when compared with 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 and with glucose amendment, 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.

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