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

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Abstract

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

This study aimed to identify the fungal contribution to N_2O emissions under anaerobic conditions in incubated repacked soil samples by using different approaches to disentangle sources of N_2O. Three soils were incubated under anaerobic conditions to promote denitrification with four treatments of a modified substrate induced respiration with selective inhibition (SIRIN) approach. While one treatment without microbial inhibition served as a control the other three treatments were amended with inhibitors to selectively inhibit bacterial, fungal or bacterial and fungal growth. These treatments were performed in three varieties. In one variety the ^{15}N tracer technique was used to estimate the effect of N_2O reduction on N_2O produced, while two other varieties were performed under natural isotopic conditions but with and without acetylene. Three approaches were established to estimate the N_2O production by a fungal community in soil: i) A modification of the SIRIN approach was used to

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calculate N_2O evolved from selected organism groups, and ii) SP_{N2O} values from the acetylated treatment were used in the isotope endmember mixing approach (IEM), and iii) the SP/$\delta^{18}O$ mapping approach (SP/$\delta^{18}O$ Map) was used to estimate the fungal contribution to N_2O production and N_2O reduction under anaerobic conditions from the non-acetylated treatment.

The three approaches tested revealed a small fungal contribution to N_2O fluxes under anaerobic conditions in the soils tested. Quantifying the fungal fraction with modified SIRIN was only possible in one soil and totaled 0.28±0.09. This was higher than the results obtained by IEM and SP/$\delta^{18}O$ Map, which accounted zero to 0.20 of N_2O produced to the fungal community.

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

1. Introduction

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

For a long time, it was believed that solely bacteria are involved in N_2O formation during denitrification (Firestone and Davidson, 1989); however, also several fungi are capable of denitrification (Bollag and Tung, 1972; Shoun et al., 1992). Denitrification describes the reduction of nitrate (NO_3^-) to dinitrogen (N_2), with the intermediates nitrite (NO_2^-), nitric oxide (NO) and N_2O (Knowles, 1982). While this entire reaction chain including the ability to reduce N_2O to N_2 is found among bacterial denitrifiers, most fungi lack N_2O reductase (Nos) (Shoun et al., 1992; Shoun et al., 2012; Higgins et al., 2018). Recently, pure culture studies showed that N_2O from fungal denitrification was often accompanied with N_2O from abiotic production (Phillips et al., 2016a; Phillips et al., 2016b), which may lead to overestimate the importance of fungal N_2O production. Other studies indicated that only some fungal species (e.g. *Fusarium* strains) performing respiratory denitrification with substantial amounts of N_2O production (Higgins et al., 2018; Keuschnig et al., 2020). Even though only a few fungal species were identified to be capable of respiratory denitrification, N_2O produced by fungi may contribute largely to N_2O from denitrification in soil. Firstly, fungi dominate the biomass in soil (up to 96%) compared to bacteria in general and thus fungi could potentially play a dominant role in N_2O production (Ruzicka et al., 2000; Braker and Conrad, 2011). Thus, a respiratory

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fungal-to-bacterial (F:B) ratio of 4 is typical for arable soils (Anderson and Domsch, 1975; Blagodatskaya and Anderson, 1998). Secondly, the fact that N_2O is the major end product of fungal denitrification led to the assumption that the potential activity of fungal N_2O production in soil may exceed that of bacteria, provided that both microbial groups have the same specific denitrification activity (Shoun et al., 1992; Sutka et al., 2008). Thirdly, co-denitrification was found to often co-occur with fungal denitrification (Shoun and Tanimoto, 1991; Tanimoto et al., 1992). During this fungal pathway, a hybrid N_2O is formed using one N atom from NO_2^- and one N atom from compounds like azide or ammonium (NH_4^+) for N_2O production (Tanimoto et al., 1992; Shoun et al., 1992; Rohe et al., 2017; Spott et al., 2011). A ^{15}N tracing approach was used to identify and quantify co-denitrification, which contributed about 92% to N_2O produced in an incubation experiment with a grassland soil under anaerobic conditions (Laughlin and Stevens, 2002). This again stresses the large potential N_2O production by fungi. However, in pure culture studies not only co-denitrification, but also abiotic N_2O formation may co-occur with fungal denitrification (Phillips et al., 2016a; Phillips et al., 2016b; Rohe et al., 2017) and pathway differentiation is still challenging.

Soil incubation experiments could serve to differentiate between N_2O produced by fungi and bacteria during denitrification by the application of two antibiotics: streptomycin and cycloheximide, which inhibit bacterial or fungal growth, respectively, by inhibition of the protein biosynthesis. This method is known as substrate induced respiration with selective inhibition (SIRIN) (Anderson and Domsch, 1975; Laughlin and Stevens, 2002; Crenshaw et al., 2008; Blagodatskaya et al., 2010; Long et al., 2013). A few studies used a modification of this method for N_2O analysis and found a greater decrease of N_2O production with fungal than with bacterial growth inhibition (e.g. 89 vs. 23% decrease (Laughlin and Stevens, 2002)), indicating that fungi might dominate N_2O production (Laughlin and Stevens, 2002; McLain and Martens, 2006; Crenshaw et al., 2008; Blagodatskaya et al., 2010; Long et al., 2013; Chen et al., 2014; Chen et al., 2015).

Analysing the isotopic composition of N_2O might be a promising tool to distinguish between N_2O from bacterial and fungal denitrification and other pathways. Especially, the isotopomer ratios of N_2O (i.e. N_2O molecules with the same bulk ^{15}N isotopic enrichment but showing different positions of ^{15}N in the linear N_2O molecule (Ostrom and Ostrom, 2017)) in pure culture studies showed differences in N_2O of bacterial and fungal denitrification (Sutka et al., 2006; Sutka et al., 2008; Frame and Casciotti, 2010; Rohe et al., 2014a; Rohe et al., 2017) and might be suitable for distinguishing between N_2O produced by bacteria or fungi under denitrifying conditions. Isotopomer ratios of N_2O can be expressed as ^{15}N site preference (SP_{N2O}), i.e. the difference between $\delta^{15}N$ of the central and terminal N-position of the asymmetric N_2O molecule (Toyoda and Yoshida, 1999). The SP_{N2O} values of N_2O of six pure fungal cultures was between 16 and 37 ‰ (Sutka et al., 2008; Rohe et al., 2014a; Maeda et al., 2015; Rohe et al., 2017), whereas several bacteria produced N_2O with SP_{N2O} values between -7.5 and +3.5 ‰ during denitrification (Toyoda et al., 2005; Sutka et al., 2006; Rohe et al., 2017). However, the SP_{N2O} value of N_2O produced by pure bacterial cultures during nitrification is approximately 33 ‰ and

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interferes with SP_{N2O} values of fungal denitrification (Sutka et al., 2006; Sutka et al., 2008; Rohe et al., 2014a). This demonstrates the difficulty to use SP_{N2O} values as an indicator for different organism groups contributing to N_2O production from soil, where different pathways may co-occur. Although SP_{N2O} values are independent of isotopic signatures of the precursors, $\delta^{15}N$ and $\delta^{18}O$ values of produced N_2O ($\delta^{15}N^{bulk}_{N2O}$ and $\delta^{18}O_{N2O}$, respectively) result from the isotopic signature of the precursor and isotopic fractionation during N_2O production (Toyoda et al., 2005; Frame and Casciotti, 2010). Interpretation of $\delta^{18}O_{N2O}$ values is even more complex, because O exchange during denitrification between water and denitrification intermediates alters 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). However, recently fungal and bacterial N_2O showed different ranges for $\delta^{18}O_{N2O}$ values and this isotopic signature may also be helpful in differentiation of these pathways (Lewicka-Szczebak et al., 2016). Moreover, $\delta^{15}N^{bulk}_{N2O}$, $\delta^{18}O_{N2O}$ and SP_{N2O} values are in the course of denitrification affected by isotopic fractionation due to N_2O reduction. During N_2O reduction, the $^{14}N^{16}O$ bond is preferentially broken compared to $^{14}N^{18}O$ or $^{15}N^{16}O$, resulting in residual N_2O, that is relatively isotopically enriched in ^{15}N and ^{18}O and shows larger SP_{N2O} values compared to SP_{N2O} values of N_2O from denitrification without the reduction step (Popp et al., 2002; Ostrom et al., 2007). Quantification of N_2O reduction to N_2 during denitrification is possible by analyzing $^{15}N_2$ fluxes in ^{15}N tracing experiments using ^{15}N enriched substrates (Well et al., 2006; Lewicka-Szczebak et al., 2014). To quantify N_2O reduction and the pathways producing N_2O based on N_2O isotopocules (i.e. N_2O with differing number or positions of N or O isotopes (Ostrom and Ostrom, 2017)), the isotope mapping approach was developed using isotope fractionation factors together with $\delta^{15}N^{bulk}$ values of N_2O 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 of N_2O and $\delta^{18}O$ values of precursors (Lewicka-Szczebak et al., 2014; Lewicka-Szczebak et al., 2017). This approach uses different slopes of N_2O reduction and mixing lines in the $\delta^{18}O$ – SP isotope plot and allows for differentiation of isotope effects due to N_2O reduction and admixture of fungal N_2O.

Based on the above cited ranges for the isotopomer endmembers of fungal and bacterial denitrification, and assuming that only fungi and bacteria are responsible for N_2O production the fraction of fungal N_2O can be calculated using the isotope endmember mixing approach (IEM) with SP_{N2O} values of N_2O produced in soil (SP_{prod}), provided N_2O reduction, which is altering SP_{N2O} values of emitted N_2O, does not occur (Ostrom et al., 2010; Ostrom and Ostrom, 2011). This can be ensured in laboratory experiments by inhibiting N_2O reduction to N_2 using acetylene (C_2H_2) during anaerobic incubation experiments (Yoshinari and Knowles, 1976; Groffman et al., 2006; Well and Flessa, 2009; Nadeem et al., 2013). Hence, C_2H_2 inhibition might be suitable to quantify SP_{prod} values in soils exhibiting significant N_2O reduction and would thus allow quantification of fungal N_2O fluxes based on SP_{prod} values. For the SP/$\delta^{18}O$ Map, the inhibition of N_2O reduction is not needed. Hence, N_2O reduction can be estimated together with the N_2O mixing due to application of two isotopic signatures

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

So far, the described methods for distinguishing between fungal and bacterial N_2O emission have not been evaluated and compared in the same soil and their accuracy and possible bias remains unknown. Therefore, this study aims at (i) determining the fungal contribution on N_2O production by denitrification under anoxic conditions and glucose addition using three arable soils and approaches: modified SIRIN, IEM and the SP/$\delta^{18}O$ Map, (ii) to compare the fungal contribution on N_2O production determined by these approaches and thus assess factors of potential bias of the methods, and (iii) to estimate the SP_{N2O} values from a fungal soil community and thus to evaluate the transferability of the pure culture range of the fungal SP_{N2O} endmember values.

2. Materials and Methods

2.1 Soil samples

All experiments were conducted with three arable soils differing in texture to provide different conditions for denitrification. As one soil was sampled at two different time points, we conducted four experiments: Experiment 1 with loamy sand sampled in December 2012, Experiment 2 with sand sampled in January 2013, Experiment 3 with silt loam sampled in December 2012, and Experiment 4 with loamy sand sampled in June 2011.

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, total contents of C and N in soil samples were analyzed by dry combustion of grinded samples (LECO TruSpec, Germany). The soil pH was measured in 0.01 M $CaCl_2$. The mineral nitrogen content (Nmin) of soil samples was determined before and after fertilization by extracting NO_3^- and NH_4^+ with 0.01 M calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$) according to ISO 14255 and analyzing NO_3^- and NH_4^+ concentrations in the extracts with a Continuous-Flow-Analyzer (SKALAR, Germany). The $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^- and NO_2^- ($\delta^{15}N_{NOx}$ and $\delta^{18}O_{NOx}$, respectively) in soil extracts (with 0.01 M calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$)) were analyzed by the bacterial denitrifier method (Casciotti et al., 2002). Respiratory biomass of the three soils was analyzed with substrate induced respiration (SIR) according to Anderson and Domsch (1978) and the respiratory F:B ratio was analyzed with substrate induced respiration with selective inhibition (SIRIN) in summer 2010 by a computer-generated selectivity analysis: “SIR-SBA 4.00” (Heinemeyer, copyright MasCo Analytik, Hildesheim, Germany)

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(Anderson and Domsch, 1975). The scheme of glucose and growth inhibitor combinations is listed below in section "Methodological approach". For further characteristics of the soils, see Table 1.

2.2 Methodological approach

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

We conducted SIRIN for determining the respiratory F:B ratio according to Anderson and Domsch (1975). Selectivity of the inhibitor combinations of streptomycin (bacterial respiratory inhibitor) and cycloheximide (fungal respiratory inhibitor) were tested with the following concentrations, 0.75, 1.0, 1.5 mg 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)).

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

Exp. (Year)	Soil texture	Soil type (WRB)	Location	C content [%]	N content [%]	NH_4^+ [mg N L^{-1}]	NO_3^- [mg N L^{-1}]	pH ($CaCl_2$)	$\delta^{15}N_{NOx}$ [‰][e]	$\delta^{18}O_{NOx}$ [‰][e]	F:B[f]	Biomass[g] [µg C gdw^{-1} soil]
1 (2012) 4 (2011)	Loamy sand	Haplic Luvisol	Braun-schweig[a]	1.43 (<0.01)	0.10 (<0.01)	0.04	1.25	5.67	3.98	-4.82	2.6	234
2 (2013)	Sand	Gleyic Podzol	Wenne-bostel[b]	2.31 (0.04)	0.14 (<0.01)	0.02	0.56	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]	2.05	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 0.005 mg L^{-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 analyzed by SIRIN method (Anderson and Domsch, 1973, 1975)
[g]Respiratory biomass analyzed by CO_2 production from SIR method.

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215 2.2.2 Soil incubation with selective inhibition to determine N_2O forming processes

216 The experimental design included two factors, (i.) microbial inhibition by fungal and/or bacterial
217 inhibitors and (ii.) activity of N_2O reductase analyzed either by inhibition with C_2H_2 or quantification
218 by ^{15}N tracing. To address factor (i.), the SIRIN method for determination of the respiratory F:B ratio
219 based on CO_2 emission was modified to determine N_2O production by microbial groups. However, in
220 contrast to previous studies by Laughlin and Stevens (2002), McLain and Martens (2006),
221 Blagodatskaya et al. (2010) and Long et al. (2013), we did not pre-incubate the soil with the growth
222 inhibitors, as this could result in changes of the microbial community (e.g. preferential growth of
223 selected organisms). We intended to disturb microbial communities as little as possible.
224 The soil was sieved (2 mm) and pre-incubated at 22 °C for five to seven days in the dark with cotton
225 wool stoppers to allow respiration and aerobic conditions in soil bags. Four microbial inhibitor
226 treatments (each in triplicate) with c_{opt}(glucose) for each soil were established:
227 A Control, without growth inhibitors
228 B With streptomycin sulfate ($C_{42}H_{84}N_{14}O_{36}S_3$) to inhibit bacterial growth
229 C With cycloheximide ($C_{15}H_{23}NO_4$) to inhibit fungal growth
230 D With streptomycin and cycloheximide, to inhibit bacterial and fungal growth
231 To address factor (ii.), all microbial inhibitor treatments were conducted in three N_2O reductase
232 varieties, i.e.: with ^{15}N-NO_3 fertilizer (variety "*traced*") to quantify N_2O reduction to N_2, with natural
233 abundance NO_3^- and 10 kPa C_2H_2 in the headspace (variety "+C_2H_2") to block N_2O reductase, and
234 with natural abundance NO_3^- but without blocking N_2O reductase, i.e. no C_2H_2 added (variety "-
235 C_2H_2"). In total, there were 48 experimental treatments and 144 vessels (four Experiments with four
236 inhibitor treatments (A, B, C, D) and three varieties (*traced,* +C_2H_2 and -C_2H_2,) each in triplicates).
237 The soil was adjusted to 80% water filled pore space (WFPS) with distilled water and simultaneously
238 fertilized with NO_3^- (varieties -C_2H_2 and +C_2H_2 with 50 mg N kg^{-1} KNO_3 in Experiment 1, 2 and 3 and
239 with 60 mg N kg^{-1} $NaNO_3$ in Experiment 4 and *traced* variety with 50 mg N kg^{-1} ^{15}N-KNO_3 in
240 Experiment 1, 2 and 3 and 60 mg N kg^{-1} ^{15}N-KNO_3 in Experiment 4 with a ^{15}N-labeling of 50 atom%
241 (at%)). For each treatment, we incubated 100 g dw soil in 850 mL preserving jars (J. WECK GmbH u.
242 Co KG, Wehr, Germany) with gas inlet and outlet equipped with three port luer lock plastic stopcocks
243 (Braun, Melsungen, Germany). According to the original SIRIN method (Anderson and Domsch,
244 1973, 1978) and a mixture of c_{opt}(glucose) and carrier material talcum (5 mg talcum g dw^{-1}) was added
245 to soil of treatment A and together with the growth inhibitors to the soil of treatments B, C and D. The
246 soil and additives of each treatment were mixed for 90 seconds with a handheld electric mixer. During
247 packing, the soil density was adjusted to a target soil density of 1.6 g cm^{-3} in Experiment 1, 2 and 4
248 and of 1.3 g cm^{-3} in Experiment 3. To ultimately achieve denitrifying conditions in all treatments and
249 to avoid catalytic NO decomposition in the +C_2H_2 variety (Nadeem et al., 2013), the headspace of the
250 closed jars was flushed with N_2 to exchange the headspace 10 times. Directly following, 85 mL of the

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gas in the headspace in variety $+C_2H_2$ were exchanged by pure C_2H_2 resulting in 10 kPa C_2H_2 in the headspace. The manual sample collection of 14 mL gas in duplicates with a plastic syringe was performed after six, eight and ten hours (Experiment 1, 2 and 3) or two, four and eight (Experiment 4) of incubation time, respectively. The removed gas was replaced by the same amount of N_2.

2.3 Gas analysis

Gas samples were analyzed for N_2O and CO_2 concentrations ($c(N_2O)$ and $c(CO_2)$) with gas chromatography (GC, Agilent 7890A, Agilent, Böblingen, Germany). The detection limit of N_2O was 0.04 ng N h^{-1} with a measurement precision of 1% and for CO_2 the detection limit was 4 ng C h^{-1} with a measurement precision of 0.5%. As a control, N_2 and O_2 concentrations in the samples were analyzed with GC to ensure anaerobic conditions during the incubation for N_2O production from denitrification. The N_2O isotopic analysis of the gas samples of varieties $-C_2H_2$ and $+C_2H_2$ were performed on a pre-concentrator (PreCon, Thermo–Finnigan, Bremen, Germany) interfaced with a GC (Trace Gas Ultra, Thermo Scientific, Bremen, Germany) and analyzed by isotope ratio mass spectrometry (IRMS, Delta V, Thermo Fisher Scientific, Bremen, Germany) (Brand, 1995; Toyoda and Yoshida, 1999; Köster et al., 2013b). 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.

The gas samples of variety *traced* from Experiment 1, 2, and 3 were analyzed for the 29/28 and 30/28 ratios of N_2 according to Lewicka-Szczebak et al. (2013) using a modified GasBench II preparation system coupled to IRMS (MAT 253, Thermo Scientific, Bremen, Germany). The gas samples of variety *traced* from Experiment 4 were analyzed at the Centre for Stable Isotope Research and Analysis (University of Göttingen, Germany). The N_2 produced was analyzed using an elemental analyzer (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 Experiment 4 (variety *traced*) were analyzed in the same lab using a pre-concentration unit coupled to IRMS (Precon-DeltaXP, Thermo Scientific, Bremen, Germany) (Well et al., 2006). Isotope ratios were used applying the non-random distribution approach to calculate the fraction of N_2 and N_2O originating from the ^{15}N-labelled N pool as well as the ^{15}N enrichment of that N pool (a_p) (Bergsma et al., 2001; Spott et al., 2006).

2.4 Inhibitor effects

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

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

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

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production was equal to treatment D, N_2O produced by bacteria or fungi should show the following relation between the four treatments:

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

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

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

2.5 Isotope methods

2.5.1 Isotope endmember mixing approach (IEM)

The fungal fraction (F_{FD}) contributing to N_2O production from denitrification in soil samples was calculated according to the isotope mixing model (IEM) proposed by Ostrom et al. (2010), which was established for calculating the bacterial fraction (F_{BD}) of N_2O production. Assuming that bacteria (*BD*) and fungi (*FD*) are the only microorganisms responsible for denitrification in soil, the ^{15}N site preference values of produced N_2O (SP_{prod}) results from the SP_{N2O} mixing balance:

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

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

2.5.2 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 estimation of both: the F_{FD} and N_2O product ratio [$N_2O/(N_2+N_2O)$] (*product ratio*$_{Map}$). 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

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the N_2O product ratio from the *traced* variety, 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 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 *product ratio*$_{Map}$ and measured in *traced* variety, *i.e.*, the minimal value of (*product ratio*$_{15N}$ - *product ratio*$_{Map}$)^2 for -C_2H_2 and +C_2H_2 variety (for explanation of the product ratio see next section). This further allows obtaining the possible ranges for F_{FD} for particular fitted values (Table 4). 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 yield almost identical results (maximal difference of 0.02 in N_2O product ratio and F_{FD} was found), due to F_{BD} near 1. Hence, we only provide the results assuming the reduction of bacterial N_2O followed by mixing with fungal N_2O.

2.5.3 Product ratio [$N_2O/(N_2+N_2O)$] of denitrification

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

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

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

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

with N_2O_{-C2H2} and N_2O_{+C2H2} representing the N_2O produced in varieties -C_2H_2 and +C_2H_2, respectively. If *product ratio*$_{15N}$ and *product ratio*$_{C2H2}$ were in agreement, a complete blockage of N_2O reduction could be assumed. This enabled to estimate reduction effects on the isotopic signatures of N_2O by comparing the isotopic values of N_2O produced without N_2O reduction effects of variety +C_2H_2 ($\delta 0$ values) 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. First, the Rayleigh-type model presented by Lewicka-Szczebak et al. (2017) and Senbayram et al. (2018) for similar closed-system incubations, the ^{15}N site preference values of produced N_2O, i.e. without its reduction to N_2O (SP_{prod}), of variety -C_2H_2 was calculated by correcting SP values of emitted N_2O, i.e. after partial reduction of produced N_2O ($SP_{N2O\text{-}r}$) from variety -C_2H_2 with the net isotope effect of N_2O reduction (ηr) and the *product ratio*$_{15N}$ as follows:

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

According to (Yu et al., 2020) the ηr was assumed to be -6‰. Secondly, 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

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352 2.6 Sources of N_2O produced

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

355 $$^{15}N_{N2O\_exp}\ [at\%] = \frac{(N_{soil}\ x\ ^{15}N_{nat}) + (N_{fert}\ x\ ^{15}N_{fert})}{N^{bulk}} \qquad \text{(Eq. 8)}$$

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

361 2.7 Statistical Analysis

362 We conducted several three-way analyses of variance (ANOVA) to test significant effects of soil,
363 experimental variety and treatment on N_2O production, CO_2 production, and SP_{N2O}, $\delta^{15}N^{bulk}{}_{N2O}$ and
364 $\delta^{18}O_{N2O}$ values. The pairwise comparison with Tukey's HSD test was made to find differences between
365 soils, varieties and treatments influencing N_2O production, CO_2 production, and isotopic values.
366 Significant effects of soils and treatments on *product ratio*$_{C2H2}$ and *product ratio*$_{15N}$ were tested by
367 two-way ANOVA, while differences between soils and treatments influencing the product ratios were
368 tested with pairwise comparison with Tukey's HSD test. Effects of varieties -C_2H_2 and *traced* on N_2O
369 and CO_2 production were tested by ANOVA. For this ANOVA, the N_2O production rate had to be
370 $\log_{10}$-transformed to achieve homogeneity of variance and normality. The significance level α was 0.1
371 for every ANOVA. For some ANOVAs treatments were excluded, when replicates were n < 3. The
372 N_2O or CO_2 production rates of variety +C_2H_2 were followed over three sampling times by regression.
373 For statistical analysis, we used the program R (R Core Team, 2013). Excel Solver tool was used to
374 determine the $\delta^{18}O_{H2O}$ values in the application of SP/$\delta^{18}O$ Map calculations.

375 **3. Results**

376 3.1 N_2O production rates

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

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385 N_2O and CO_2 production rates of all $+C_2H_2$ varieties differed significantly among soils ($P < 0.001$) and
386 N_2O production rates differed also significantly among treatments ($P < 0.001$). Largest N_2O
387 production rate about 5.5 to 6.1 µg N $kg^{-1}h^{-1}$ was obtained in Experiment 1 and 3, while in Experiment
388 2 and 4 N_2O production rates were lower (2.6 and 2.7 µg N $kg^{-1}h^{-1}$, respectively). N_2O and CO_2
389 production rates were significantly larger in variety $+C_2H_2$ than in variety $-C_2H_2$ of Experiment 1, 3
390 and 4 ($P = 0.002$, $P < 0.010$ and $P < 0.010$ for N_2O production rate and $P = 0.027$, $P < 0.010$ and
391 $P = 0.008$ for CO_2 production rate, respectively) (Table 2), while $-C_2H_2$ and $+C_2H_2$ varieties of
392 Experiment 2 did not differ in N_2O and CO_2 production rates ($P = 0.402$ and $P = 0.288$, respectively).
393

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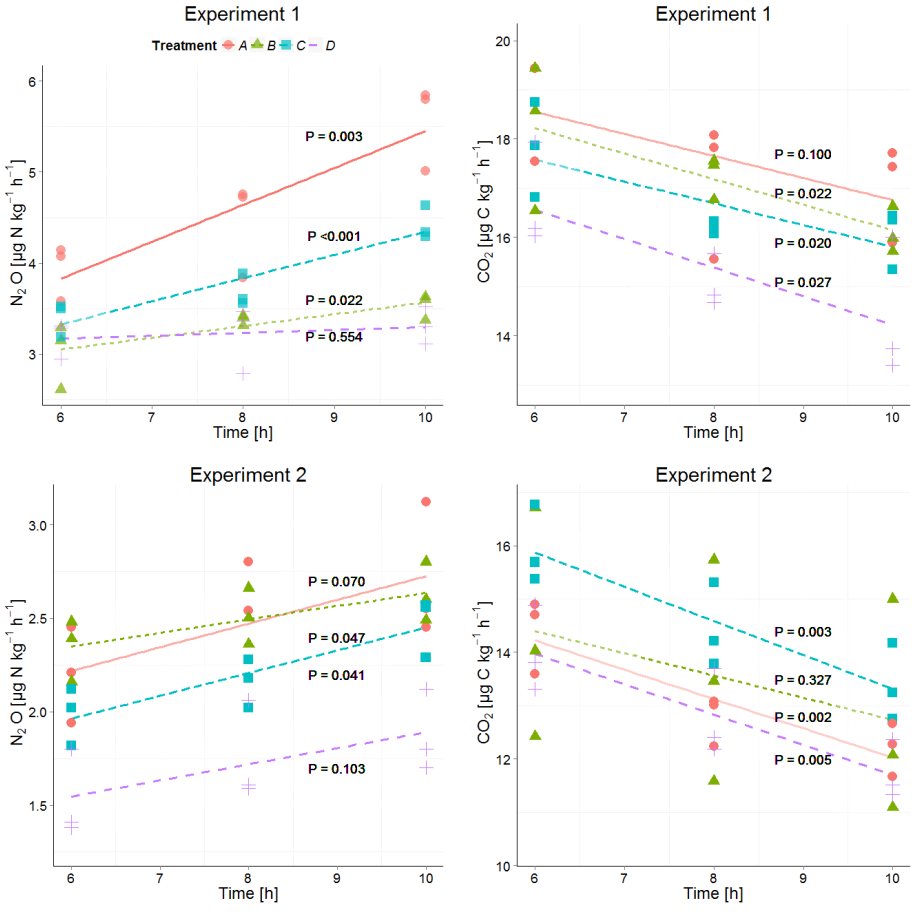
Figure 1: Time series of average N_2O and CO_2 production rates during incubation of variety $+C_2H_2$ at the three sample collection times of each soil (Experiment 1 - 4) for treatment A without growth inhibitors, B with bacterial growth inhibition, C with fungal growth inhibition, and D with bacterial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



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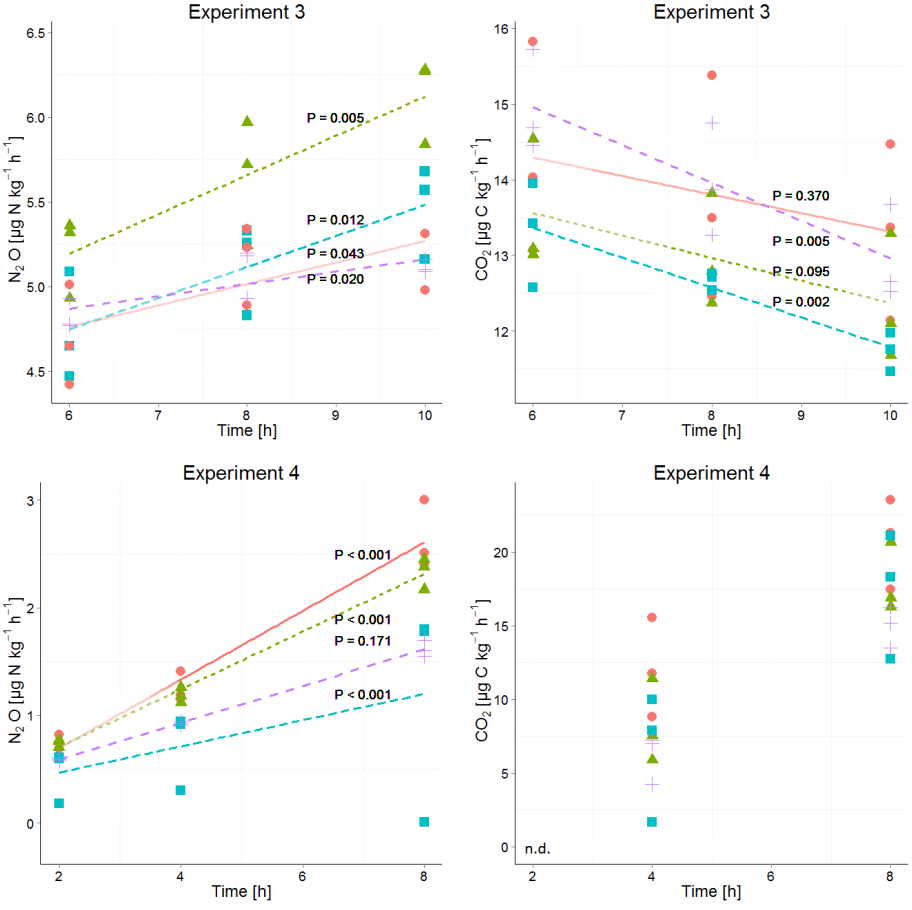

Figure 1 continued.

Without blockage of N_2O reductase (variety -C_2H_2), N_2O production rates of treatment A varied significantly among experiments with mean values between 1.6 and 3.6 µg N kg^{-1} h^{-1} ($P \leq 0.001$) (Table 2). In Experiment 1, N_2O production rate was significantly larger (2.7 µg N kg^{-1} h^{-1}) than in Experiment 4 (1.6 µg N kg^{-1} h^{-1}) ($P = 0.028$) in variety -C_2H_2. The inhibitor application of each variety revealed in most cases that treatment A (without growth inhibitors) produced most N_2O, followed by either treatment B (bacterial growth inhibitor; more N_2O compared to treatment C in Experiments 2, 3 and 4) or treatments C (fungal growth inhibitor; more N_2O compared to treatment B in Experiment 1). In varieties -C_2H_2, +C_2H_2 and *traced* varieties, non-inhibitable organisms (treatment D) showed smallest N_2O production rates in most cases (i. e. except of variety -C_2H_2 of Experiment 1, varieties -C_2H_2 and traced of Experiment 3 and variety *traced* of Experiment 4). Microbial inhibitor treatments differed significantly in N_2O fluxes of variety +C_2H_2 of each experiment (always $P \leq 0.040$), while this was not the case for inhibitor treatments of varieties -C_2H_2 and *traced* of Experiment 4 ($P = 0.154$ and $P = 0.154$, respectively). Significant deviations of treatments without (A) or with full inhibition

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418 (D) were found in the following cases (Table 2): N_2O production rate of treatment A was significantly
419 larger compared to the other three treatments of Experiment 1 (+C_2H_2 and -C_2H_2), Experiment 2
420 (+C_2H_2) and Experiment 3 (+C_2H_2); treatment D was significantly smaller compared to the other three
421 treatments in Experiment 2 (-C_2H_2) only and compared to treatments A and C of Experiment 1
422 (+C_2H_2). Comparing varieties -C_2H_2 and *traced*, N_2O and CO_2 rates did not differ ($P = 0.991$ for N_2O
423 production rate and $P = 0.490$ for CO_2 production rate, respectively), confirming that ^{15}N-labeling did
424 not affect N_2O and CO_2 processes.
425

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

Treatment/ variety	mean N_2O [µg N kg^{-1} h^{-1}]	mean CO_2 [µg C kg^{-1} h^{-1}]	$\delta^{18}O_{N2O}$ [‰]	$\delta^{15}N^{bulk}{}_{N2O}$ [‰]	SP_{N2O} [‰]
Experiment 1 (Loamy sand, winter 2012)					
A / -C_2H_2	2.7 (0.4)a	12.3 (1.7)a	13.1 (0.2)a	-21.9 (1.7)a	1.6 (0.8)a
B / -C_2H_2	1.8 (0.2)b	12.8 (1.6)a	13.0 (<0.1)*	-24.2 (0.7)*	-1.3 (0.2)*
C / -C_2H_2	2.0 (0.1)b	11.2 (0.5)a	14.6 (0.4)a	-20.0 (0.8)a	-1.6 (0.5)a
D / -C_2H_2	2.1 (0.3)b	13.7 (0.4)a	15.2 (0.5)*	-20.2 (1.8)*	-0.3 (0.5)*
A / +C_2H_2	5.5 (0.5)a	17.0 (1.0)a	8.5 (0.1)a	-22.1 (0.3)a	-0.4 (0.3)a
B / +C_2H_2	3.5 (0.1)b	16.1 (0.5)a	7.5 (0.1)a	-26.1 (0.2)a	-1.2 (1.0)b
C / +C_2H_2	4.4 (0.2)c	14.0 (0.6)a	9.3 (0.2)a	-22.4 (0.4)a	-0.9 (0.4)b
D / +C_2H_2	3.3 (0.2)b	14.4 (1.4)a	7.8 (0.3)*	-24.2 (0.1)*	-2.3 (0.7)*
Experiment 2 (Sand, winter 2012)					
A / -C_2H_2	3.2 (0.4)a	13.1 (1.0)a	15.5 (1.8)a	-18.9 (2.6)a	-0.9 (2.5)a
B / -C_2H_2	2.4 (<0.1)b	12.1 (0.2)a	15.0 (1.3)a	-23.4 (2.5)a	-0.8 (<0.1)a
C / -C_2H_2	2.5 (0.2)b	12.0 (0.5)a	14.3 (0.1)a	-21.8 (0.2)a	-1.8 (0.2)a
D / -C_2H_2	2.0 (0.3)b	11.0 (0.2)a	13.4 (0.3)a	-24.5 (0.1)a	-1.2 (0.3)a
A / +C_2H_2	2.7 (0.4)a	12.7 (2.0)a	12.6 (0.3)a	-18.9 (4.6)a	-1.4 (0.3)a
B / +C_2H_2	2.6 (0.2)a	13.4 (0.7)a	12.3 (0.1)a	-24.6 (0.2)b	-2.0 (0.2)a
C / +C_2H_2	2.5 (0.2)a	12.2 (0.5)a	12.7 (0.1)*	-23.3 (0.2)*	-1.7 (0.4)*
D / +C_2H_2	1.9 (0.2)b	11.7 (0.6)a	12.2 (0.3)a	-26.0 (0.1)b	-1.5 (0.9)a
Experiment 3 (Silt loam, winter 2013)					
A / -C_2H_2	3.6 (0.2)a	12.3 (1.0)a	26.0 (0.5)a	-20.8 (0.5)a	-0.5 (0.4)a
B / -C_2H_2	3.3 (0.4)a	11.6 (1.8)a	24.1 (0.2)b	-22.0 (0.2)b	-0.1 (0.4)a
C / -C_2H_2	2.8 (0.1)a	10.6 (0.6)a	27.3 (0.1)b	-20.6 (0.3)a	0.6 (0.2)a
D / -C_2H_2	2.9 (0.4)a	11.2 (0.7)a	26.3 (0.3)a	-21.0 (0.1)a	-0.04 (0.18)a
A / +C_2H_2	6.1 (0.3)a	13.3 (1.2)a	15.2 (0.1)a	-25.6 (0.8)a	-2.8 (0.2)a
B / +C_2H_2	5.5 (0.3)b	12.4 (0.8)a	14.9 (0.2)a	-26.3 (<0.1)a	-3.5 (0.4)a
C / +C_2H_2	5.2 (0.2)b	11.7 (0.3)a	16.2 (<0.1)*	-25.2 (0.1)*	-4.0 (0.4)*
D / +C_2H_2	5.1 (<0.1)b	13.0 (0.6)a	16.0 (0.1)b	-25.1 (0.1)a	-4.3 (0.5)a
Experiment 4 (Loamy sand, summer 2011)					
A / -C_2H_2	1.8 (0.1)a	24.5 (1.4)a	25.7 (0.3)a	-30.6 (0.2)a	12.1 (1.6)a
B / -C_2H_2	1.2 (0.7)a	20.9 (0.2)b	28.0 (5.0)a	-32.3 (0.7)a	7.7 (1.4)b
C / -C_2H_2	1.0 (0.05)a	18.4 (1.9)b	29.3 (0.1)a	-30.0 (0.5)a	4.3 (1.0)c
D / -C_2H_2	0.7 (0.6)a	16.3 (1.2)b	28.9 (1.2)a	-31.8 (2.2)a	3.4 (2.0)c
A / +C_2H_2	2.6 (0.3)a	20.8 (3.1)a	13.5 (0.5)*	-34.7 (0.1)*	-1.0**
B / +C_2H_2	2.3 (0.2)a	17.9 (2.4)a	14.3 (1.7)a	-33.8 (0.9)a	-4.9 (0.9)a
C / +C_2H_2	1.2 (1.0)a	17.4 (4.2)a	19.0 (7.0)a	-33.1 (2.8)a	-1.7 (2.7)b
D / +C_2H_2	1.6 (0.1)a	15.0 (1.3)a	14.8 (0.5)a	-35.7 (0.2)a	-4.9 (0.7)c

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

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433 3.2 Isotopologues of N_2O produced in different varieties and treatments

434 3.2.1 Variety $+C_2H_2$

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

452 3.2.2 Variety $-C_2H_2$

453 SP_{N2O} values of all experiments and inhibitor treatments of variety $-C_2H_2$ were within a range of -1.8
454 to 12.1 ‰ (Table 2) and did not differ among inhibitor treatments ($P = 0.037$). SP_{N2O} values in variety
455 $-C_2H_2$ of Experiment 4 was particularly large (3.4 - 12.1 ‰) compared to the other experiments (1.6 to
456 -1.6 ‰). As already stated above, SP_{N2O} values of variety $-C_2H_2$ were significantly larger than SP_{N2O}
457 values of variety $+C_2H_2$ (up to 2.4, 1.5, 4.6 and 4.1‰ in Experiment 1, 2, 3 and 4, respectively).
458 Generally, most SP_{prod} values of variety $-C_2H_2$ (Eq. 7) were smaller than SP_{N2O} values of variety $-C_2H_2$
459 but still larger than SP_{N2O} values of variety $+C_2H_2$ and are presented in Table S1 (supplementary
460 Material).

461 3.2.3 Variety *traced*

462 The ^{15}N-labeling of N_2O ($^{15}N_{N2O}$) or N_2 produced ($^{15}N_{N2}$) gave information about the incorporated N
463 from ^{15}N-labeled NO_3^- into N_2O or N_2 as well as about the N_2O reduction to N_2. Microorganisms in
464 each treatment used the ^{15}N-labeled NO_3^- in variety *traced* (Table 3) and expected $^{15}N_{N2O}$ depended on
465 the initial N abundance in NO_3^- of unfertilized soil (Eq. 7). Experiment 4 is the only one showing a

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large discrepancy between measured (about 30 at%) and calculated $^{15}N_{N2O\_exp}$ (49 at%) in N_2O, whereas the other experiments showed close agreement (Table 3).

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

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

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

Treatment	mean N_2O [µg N kg^{-1} h^{-1}]	mean CO_2 [µg N kg^{-1} h^{-1}]*	$^{15}N_{N2O}$ [at%]	$^{15}N_{N2O\_exp}$ [at%][a]	Calc. total *product ratio*$_{15N}$[b*]	Calc. total *product ratio*$_{C2H2}$[c*]
Experiment 1 (Loamy Sand, 2012)						
A	2.6 (0.4)	13.1 (1.7)	36.8 (0.1)	39	0.80 (0.02)	0.48 (0.07)
B	1.5 (0.3)	11.5 (2.4)	36.4 (0.2)		0.76 (0.02)	0.48 (0.05)
C	1.9 (1.5)	12.2 (1.1)	36.9 (<0.1)		0.72 (0.05)	0.45 (0.04)
D	1.5 (<0.1)	12.5 (0.5)	36.8 (0.1)		0.69 (0.02)	0.54 (0.05)
Experiment 2 (Sand, 2012)						
A	2.4 (<0.1)	12.9 (0.1)	43.2 (<0.1)	44	0.94 (0.01)	1.04 (0.10)
B	1.9 (<0.1)	11.6 (0.2)	43.0 (0.1)		0.94 (0.01)	0.81 (0.04)
C	2.4 (0.1)	12.8 (0.6)	43.2 (0.1)		0.95 (0.01)	0.99 (0.09)
D	1.7 (0.1)	12.0 (0.3)	42.7 (0.1)		0.93 (0.01)	0.98 (0.04)
Experiment 3 (Silt loam, 2013)						
A	2.9 (0.2)	10.4 (0.5)	35.8 (<0.1)	34	0.62 (<0.01)	0.52 (0.04)
B	3.2 (0.2)	12.0 (0.9)	35.5 (<0.1)		0.62 (0.01)	0.59 (0.02)
C	2.2 (0.3)	9.8 (2.0)	35.5 (<0.1)		0.59 (0.02)	0.48 (0.04)
D	2.3 (0.1)	9.9 (0.7)	35.3 (<0.1)		0.62 (0.01)	0.51 (0.04)
Experiment 4 (Loamy Sand, 2011)						
A	1.6 (0.6)	31.1 (12.5)	31.1**	49	0.54 (0.05)	0.63 (0.10)
B	1.7 (<0.1)	23.2 (3.0)	26.5**		0.59 (0.03)	0.63 (0.17)
C	1.2 (<0.1)	17.9 (0.8)	30.1*		0.50 (0.01)	0.62 (0.02)
D	1.2 (<0.1)	17.1 (0.4)	33.5*		0.50 (0.01)	0.53 (0.12)

Asterisks indicate that only two samples (*) or one sample (**) were analyzed.
[a] $^{15}N_{N2Oexp}$ [at%] was calculated from Eq. 7.
[b] *product ratio*$_{15N}$ = [N_2O/(N_2+N_2O)] with N_2O or N_2 production rates from variety *traced*; see Eq. 5
[c] *product ratio*$_{C2H2}$ = [N_2O_{-C2H2}/N_2O_{+C2H2}] with N_2O production rate from varieties $-C_2H_2$ and $-C_2H_2$; see Eq. 6, cf. Table 2

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

When calculating F_{FDmi}, N_2O production rates of treatment D must be significantly smaller compared to the other treatments and the flux balance according to Eq. 1 and 2 must be consistent. This was only the case in Experiment 2 of variety $+C_2H_2$. The calculated F_{FDmi} (Eq. 3) was 0.28 ± 0.90 (Table 5). The respective flux of fungal N_2O was 0.24 ± 0.08 µg N kg^{-1} h^{-1}. For all other experiments calculation of F_{FDmi} was not possible.

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

When applying SP/$\delta^{18}O$ Map, we can assess the plausibility of the determined F_{FD} values based on the $\delta^{18}O_{H2O}$ values obtained from the fitting ($\delta^{18}O_{H2O}$ value in Table 4) and the fitting outcome, i.e. the difference between *product ratio*$_{15N}$ and *product ratio*$_{MAP}$ (*Diff* in Table 4). The most probable $\delta^{18}O_{H2O}$ value for our experiments can be assumed based on the fact that Braunschweig tap water was added to soil and the original soil water also represent the isotope characteristics typical for this region which is about -7.4‰ (long-term mean Braunschweig precipitation water (Stumpp et al., 2014)). Depending on the season and evaporative losses, this value may slightly vary and the most possible range of soil water in our experiments may vary from about -11 to -4‰ as observed in other experiments conducted in our laboratory in 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 Experiment 1, the fungal contribution must be below 0.02, 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 Experiment 2 both the smaller F_{FD_MAP} values of 0.01 and the larger ones up to 0.15 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 Experiment 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 0.04-0.09. Similarly, for Experiment 4, the only plausible fitting can be obtained for the smallest SP_{BD} values, which are associated with a $\delta^{18}O_{H2O}$ value of -6.8‰ (Table 4) and indicate F_{FD_MAP} values from 0.11 to 0.20. Here this fitting also shows clearly the smallest *Diff* of only 0.01 (Table 4). However, except for Experiment 4, where the *Diff* is smallest for the last fitting, the *Diff* values for other experiments are very similar for different fittings with the largest values in Experiment 3. A better fit (showing smaller *Diff* values) was not possible with any other SP_{BD} and $\delta^{18}O_{H2O}$ values. The F_{FD_SP} ranged between 0 and approximately 0.15 (Table 5). The results obtained from SP/$\delta^{18}O$ Map show F_{FD_MAP} reaching up to 0.14, 0.15, 0.09 and 0.20 for Experiments 1, 2, 3, and 4 respectively (Table 4, Table 5).

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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_2O product ratio (*product ratio*$_{MAP}$) in the acetylated (+C_2H_2) and non-acetylated (-C_2H_2) treatments for 3 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 and SP/$\delta^{18}O$ Map (*product ratio*$_{15N}$ and *product ratio*$_{MAP}$). The most plausible fittings are bolded (see discussion for reasons of this choice).

Experiment	Variety	*product ratio*$_{15N}$	SP_{BD} [‰]	$\delta^{18}O_{H2O}$ [‰]	*product ratio*$_{MAP}$	*Diff*	F_{FD_MAP}
1	**-C_2H_2**	**0.66**	**-1.9**	**-11.2**	**0.66**	**0.00**	**-0.01**
	+C_2H_2	**1**	**-1.9**	**-11.2**	**1.00**	**0.00**	**0.02**
	-C_2H_2	0.66	3.7	-6.1	0.65	0.01	-0.14
	+C_2H_2	1	3.7	-6.1	1.00	0.00	-0.16
	-C_2H_2	0.66	-7.5	-14.9	0.66	0.00	0.08
	+C_2H_2	1	-7.5	-14.9	1.00	0.00	0.14
2	**-C_2H_2**	**0.94**	**-1.9**	**-6.3**	**0.90**	**0.04**	**0.01**
	+C_2H_2	**1**	**-1.9**	**-6.3**	**1.04**	**0.04**	**0.01**
	-C_2H_2	0.94	3.7	-1.2	0.90	0.04	-0.16
	+C_2H_2	1	3.7	-1.2	1.04	0.04	-0.18
	-C_2H_2	**0.94**	**-7.5**	**-10.1**	**0.90**	**0.04**	**0.13**
	+C_2H_2	**1**	**-7.5**	**-10.1**	**1.04**	**0.04**	**0.15**
3	-C_2H_2	0.61	-1.9	-1.7	0.54	0.07	-0.03
	+C_2H_2	1	-1.9	-1.7	1.04	0.04	-0.05
	-C_2H_2	0.61	3.7	3.7	0.54	0.07	-0.14
	+C_2H_2	1	3.7	3.7	1.03	0.03	-0.24
	-C_2H_2	**0.61**	**-7.5**	**-5.6**	**0.53**	**0.08**	**0.04**
	+C_2H_2	**1**	**-7.5**	**-5.6**	**1.04**	**0.04**	**0.09**
4	-C_2H_2	0.60	-1.9	-3.3	0.66	0.06	0.15
	+C_2H_2	1	-1.9	-3.3	0.96	0.04	-0.03
	-C_2H_2	0.60	3.7	1.5	0.72	0.12	0.08
	+C_2H_2	1	3.7	1.5	0.91	0.09	-0.21
	-C_2H_2	**0.60**	**-7.5**	**-6.8**	**0.61**	**0.01**	**0.20**
	+C_2H_2	**1**	**-7.5**	**-6.8**	**0.99**	**0.01**	**0.11**

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

Experiment	F_{FDmi} [a]	F_{FD_SP} [b]	F_{FD_SPcalc} [c]	F_{FD_MAP} [d]
1	n.d.	0-0.15	0-0.19	0-0.02
2	0.19-0.37	0-0.14	0-0.15	0.01-0.15
3	n.d.	0-0.09	0-0.18	0.04-0.09
4	n.d.	0-0.11	0-0.21	0.11-0.20

[a]Fungal fraction on N_2O production calculated Eq. 3.

[b]Fungal fraction on N_2O production calculated by Eq. 4 for variety +C_2H_2 with assuming SP_{N2O} values of N_2O produced by bacteria were 3.7 ‰ (resulting in negative fraction and therefore set to zero) or -7.5 ‰.

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

[d]Fungal fraction on N_2O production calculated by SP/$\delta^{18}O$ Map with assuming most probable SP_{N2O} values from bacterial denitrification (according to Table 4)

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

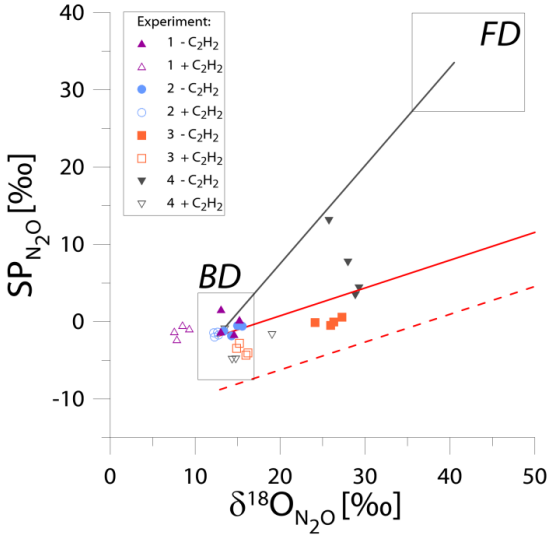

Figure 2: SP/$\delta^{18}O$ isotope mapping approach (SP/$\delta^{18}O$ Map) to estimate the contribution of bacteria or fungi to N_2O produced according to Lewicka-Szczebak et al. (2017) and Lewicka-Szczebak et al. (2020). The isotopic values for natural abundance treatments with acetylene addition (+C_2H_2, empty symbols) and without acetylene addition (-C_2H_2, corresponding filled symbols) are shown for four experiments (1-4). The grey rectangles indicate expected ranges of isotopic signatures for heterotrophic bacterial denitrification (*BD*) and fungal denitrification (*FD*) (Yu et al. 2020). The black solid line is the mixing line connecting the average expected values for *BD* and *FD*, while the red solid line is the mean reduction (for the mean *SP* values for *BD*) line and the red dashed line is the minimum reduction line (for the minimal SP_{N2O} values for *BD*).

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3.6 SP_{N2O} values of N_2O produced by the fungal soil community

Solving Eq. 4 for SP_{FD} enables to calculate SP_{N2O} values from the fungal soil community for Experiment 2 (Table 6). Estimates for the ranges of F_{FD} and F_{BD} from the results ($+C_2H_2$) of the modified SIRIN were obtained (F_{FDmi}=0.19-0.37 and F_{BD}=*1-* F_{FDmi} resulted in a range between 0.63 and 0.81, respectively, see section "*3.4 Fungal contribution to N_2O production from denitrification by microbial inhibitor approach (modified SIRIN)*"). The SP_{prod} values of N_2O (SP_{prod} = -1.4 ‰) of the respective treatment A (Table 2, variety $+C_2H_2$) served to calculate SP_{N2O} values for fungal denitrification for Experiment 2. Assuming -7.5 or 3.7 ‰ for the bacterial SP_{N2O} endmember values of N_2O (Toyoda et al., 2005; Sutka et al., 2006; Yu et al., 2020) resulted in SP_{FD} values between -10 ‰ (SP_{BD} = 3.7 ‰) and 25 ‰ (SP_{BD} = -7.5 ‰) (Table 6). The respective SP_{FD} value for variety $-C_2H_2$ was in a very similar range between -17 ‰ and 27 ‰ (Table 6) using SP_{prod} values (SP_{prod} = -1.0 ‰) of the respective treatment A (Table S1).

Table 6: SP_{FD} values (i.e. SP_{N2O} values of N_2O produced by fungi) by solving Eq. 4 using F_{FDmi} and F_{BD} from results of modified SIRIN approach and using SP_{prod} values of varieties $+C_2H_2$ and $-C_2H_2$ of Experiment 2.

Treatment	SP_{prod} [‰]	SP_{BD} [‰][a]	F_{FDmi}[b]	F_{BD}[b]	SP_{FD} [‰]
$+C_2H_2$	-1.4	-7.5	0.19	0.81	25
		3.7	0.19	0.81	-23
		-7.5	0.37	0.63	9
		3.7	0.37	0.63	-10
$-C_2H_2$	-1.0	-7.5	0.19	0.81	27
		3.7	0.19	0.81	-17
		-7.5	0.37	0.63	10
		3.7	0.37	0.63	-9

SP_{N2O} endmember values of bacterial denitrification were taken for calculation (Eq. 4) according to studies with pure cultures (Toyoda et al., 2005; Sutka et al., 2006; Yu et al., 2020).
[b]Ranges of F_{FDmi} and F_{BD} were calculated using the modified SIRIN approach.

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/$\delta^{18}O$ Map) to estimate fungal contribution to N_2O production from denitrification in anoxic incubation. Using IEM revealed that the fungal contribution to N_2O production was small ($F_{FD\_SP} \leq 0.15$ or $F_{FD\_MAP} \leq 0.20$) in the three soils tested (Table 5). Only one experiment with modified SIRIN allowed the calculation of the fungal fraction producing N_2O during denitrification (F_{FDmi} between 0.19 and 0.37 in Experiment 2), which was larger than the F_{FD} by two isotope approaches (≤0.20). While the three approaches coincided in showing dominance of bacterial denitrification, the isotopic approaches yielded similar estimates of F_{FD} and thus did not confirm largest F_{FD} of Experiment 2. The strict application of the SIRIN method prescribes proof of

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selectivity of the inhibitors (i.e., streptomycin should not inhibit fungi and cycloheximide should not inhibit bacteria). The SIRIN results obtained with respect to N_2O production by the fungal or bacterial fraction were rather unsatisfactory and led to unsolved questions, which are discussed in the following sections.

4.1 Experimental setup

Inhibitor effects, expressed by smaller N_2O production with selective inhibitors (treatments B, C and D) compared to treatments without inhibitors (A), were only minor in the present study. Previous studies found much larger inhibitor effects by pre-incubating the soil with selective inhibitors (Laughlin and Stevens, 2002; Blagodatskaya et al., 2010; Long et al., 2013; Chen et al., 2014). The experimental design of our incubation setup was, however, in agreement with the original SIRIN method for respiration (Anderson and Domsch, 1975, 1978) without soil pre-incubation with selective inhibitors to minimize disturbance of the soil microbial community. 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). Inhibitor application without pre-incubating with inhibitors was contrary to previous studies focusing on N_2O production (Laughlin and Stevens, 2002; Blagodatskaya et al., 2010; Long et al., 2013) and we suppose that pre-incubation with selective inhibitors changes the F:B ratio compared to the undisturbed soil considerably more than soil incubation without this pre-incubation step. Additionally, although Blagodatskaya et al. (2010) did not find more inhibitor efficiency after a period of 1 to 20 hours of pre-incubation with streptomycin, they found greater inhibitor effects of cycloheximide with pre-incubation phases. This could indicate that microbial distribution changed after exposition to this inhibitor. Anderson and Domsch (1975) stated already that CO_2 production of initially active organisms can only be ensured up to six or eight hours of experimental duration and biomass activity is changed by both inhibitors.

It has to be noticed that pre-incubation in previous studies was without glucose, while N_2O production was analyzed after the addition of glucose as substrate in the present as well as previous studies (Laughlin and Stevens, 2002; McLain and Martens, 2006; Blagodatskaya et al., 2010; Long et al., 2013). Glucose initiates the growth of active heterotrophic organisms. Pre-incubation under denitrifying conditions is not needed for microorganisms to produce denitrifying enzymes as pure cultures synthesized enzymes capable of denitrification within two to three hours (USEPA, 1993). We started gas sample collection after two or four hours, when organisms should have produced denitrifying enzymes and microbial growth of initially active organisms should have started. With incubation time production rates of CO_2 decreased, probably because experimental incubation conditions provoked unfavorable conditions and physiological changes, e.g. increasing partial pressure within the closed jars.

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The conventional practice of SIRIN implies determination of c_{opt}(glucose), c_{opt}(streptomycin) or c_{opt}(cycloheximide) with an "Ultragas 3" CO_2 analyzer (WösthoffCo., Bochum) (Anderson and Domsch, 1973) with continuous gas flow and we used this method to determine optimal concentrations for SIRIN and used these concentrations for the modified SIRIN approach as well. This 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 could work as well for denitrification, if both inhibitors are apt to inhibit the denitrification process as well. SIRIN has so far been tested with isolated cultures and soils for microbial growth on agar and CO2 production (Anderson and Domsch, 1975, 1973), but information on N_2O producing processes, especially denitrification, is still lacking and should be investigated in further studies.

4.2 Inhibitor effects

Even with both growth inhibitors (treatment D) N_2O production was large in all experiments, i.e., often not significantly smaller than in the other three treatments. Thus, we suppose similar contributions of non-inhibitable organisms 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. These 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. As stated before, Ladan and Jacinthe (2016) did not find effective inhibition of denitrification by either inhibitor for denitrification although streptomycin and cycloheximide are commonly used to inhibit denitrification of selective groups. Thus, similar experiments with different inhibitors, such as the bactericide bronopol and the fungicide captan presented by Ladan and Jacinthe (2016), should be conducted to evaluate inhibition approaches and isotopic endmember approaches.

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

In order to determine SP_{N2O} values without alteration by partial reduction of N_2O to N_2, C_2H_2 was used to quantitatively block N_2O reduction during denitrification. We found the expected effect of C_2H_2 application, i.e. larger N_2O production rates in variety $+C_2H_2$ compared to variety $-C_2H_2$. Calculated product ratios varied between 0.5 and 0.95 ($product\ ratio_{15N}$) in all soils, showing that N_2O reduction can have significant effects on measured N_2O production and isotopic values. The product ratio is controlled by the reaction rate or by the activity of enzymes capable of N_2O reduction (Nos) in the system. The calculated $product\ ratio_{C2H2}$ was within the same range as $product\ ratio_{15N}$ in Experiment 2, 3 and 4 (maximal 9% difference), providing the effective blockage of N_2O reductase in variety $+C_2H_2$. Only in Experiment 1 $product\ ratio_{15N}$ and $product\ ratio_{C2H2}$ differed by about 34% with larger calculated reduction in the *tracer* variety, which might be explained by potential incomplete inhibition

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682 by the C_2H_2 method. Nadeem et al. (2013) found some artifacts with C_2H_2, which resulted in smaller
683 N_2O production rates due to NO oxidation accelerated by C_2H_2 application in the presence of very
684 small oxygen (O) amounts (≥ 0.19 mL L^{-1}). Moreover incomplete C_2H_2 diffusion into denitrifying
685 aggregates might also lead to incomplete N_2O reductase blockage (Groffman et al., 2006). Both
686 potential methodological errors cannot be excluded for Experiment 1. For the other three experiments
687 (2, 3 and 4) it can be supposed that the isotopic signature of N_2O of variety $+C_2H_2$ showed isotopic
688 signatures of produced N_2O without influences of N_2O reduction. By comparing varieties $-C_2H_2$ and
689 $+C_2H_2$, isotopologue values of all soils (except $\delta^{15}N^{bulk}_{N2O}$ values of Experiment 2) of variety $-C_2H_2$
690 were significantly larger than those that of variety $+C_2H_2$. The enrichment of residual N_2O in heavy
691 isotopes results from the isotope effect associated with N_2O reduction (Jinuntuya-Nortman et al., 2008;
692 Well and Flessa, 2009; Lewicka-Szczebak et al., 2014). This explains why C_2H_2 application is
693 essential for analyzing N_2O produced by different microbial organism groups from soil using solely
694 the modified SIRIN approach without additional isotopic approaches.
695 Moreover, when applying SIRIN without quantifying N_2O reduction, fungal denitrification is
696 potentially overestimated due to the impact of SIRIN inhibitors on N_2O reduction. It is evident that
697 N_2O fluxes represent net N_2O production, i. e. the difference between gross N_2O production by the
698 microbial community and N_2O reduction, mainly by heterotrophic bacterial denitrifiers (Müller and
699 Clough, 2014). The goal of SIRIN application has been to determine the contribution of fungi and
700 bacteria, respectively, to net N_2O production. It has been shown that N_2O released by microorganisms
701 to air filled pore space can be partially consumed by denitrifiers before being emitted (Clough et al.,
702 1998). This means that fungal N_2O can also be subject to reduction by bacterial denitrifiers.
703 Consequently, inhibiting bacterial denitrification by SIRIN would lead to an overestimation of fungal
704 contribution to N_2O production. Until now, this effect has not been considered in previous SIRIN
705 papers on fungal N_2O. This effect can only be evaluated by measuring N_2O reduction in all inhibitor
706 treatments as in our study. If true, the N_2O reduction with bacterial inhibition should be smaller than
707 that of the treatments without inhibition or with fungal inhibition. Though, with fungal inhibition, N_2O
708 reduction is also assumed to be smaller than that without inhibition, because N_2O produced by fungi is
709 missed for bacterial reduction. The product ratio is a measure for the N_2O reduction to N_2. However,
710 regarding the $product\ ratio_{15N}$, there was no evidence of different N_2O reduction effects between the
711 SIRIN treatments. The $product\ ratio_{C2H2}$ also revealed indistinguishable values between SIRIN
712 treatments in Experiment 1 and 4, but it was slightly larger in Experiment 3 with bacterial inhibition
713 compared to the other treatments. However, this effect was very small, which would only cause small
714 overestimation of fungal contribution. The smallest N_2O reduction was found in Experiment 2
715 ($product\ ratio_{C2H2}$ values near 1), with smallest $product\ ratio_{C2H2}$ with bacterial inhibition (0.81). This
716 could result in an overestimation of bacterial contribution, since with blockage of N_2O reduction, gross
717 N_2O production of bacteria is measured. The $product\ ratio_{15N}$ and $product\ ratio_{C2H2}$ were between 0.5
718 and 1 and N_2O reduction was thus never consuming most of the produced N_2O. Hence both the C_2H_2

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and Streptomycin effects on SIRIN results were probably low. But the product ratio in soil denitrification exhibits the full range from 0 to 1, meaning that this effect can be quite relevant and must thus be considered in future studies.

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

The SP_{N2O} values of each soil indicated predominantly bacteria to be responsible for N_2O production during denitrification, assuming that results of SP_{N2O} values of denitrification by pure bacterial cultures is transferable to bacteria of soil communities contributing to denitrification. The latter assumption has been confirmed repeatedly in soil incubation studies, where in absence of N_2O reduction smallest SP_{N2O} values have been found that were within the range of bacterial pure cultures (Lewicka-Szczebak et al., 2015; Lewicka-Szczebak et al., 2017; Senbayram et al., 2018). Therefore, there was no unequivocal evidence of fungi contributing to N_2O production during denitrification, although the isotopic approaches revealed a fungal contribution up to 0.20 on N_2O production during denitrification. The SP_{N2O} values of treatment A within variety $+C_2H_2$ showed that the signature of produced N_2O was not affected by reduction effects and might give evidence of the microbial community contributing to N_2O production regarding differences in SP_{N2O} values of pure bacterial or fungal culture studies (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 $+C_2H_2$ are very small and do not give a clear evidence of any differences in microbial soil community producing N_2O. Lewicka-Szczebak et al. (2014) analyzed SP_{N2O} values of denitrification with blockage of N_2O reduction by C_2H_2 for the same soils as used in the present study for Experiment 1 and 4 as well as Experiment 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 dominate gross N_2O production under anoxic conditions in both these soils. However, other studies found larger SP_{N2O} values of produced N_2O unaffected by the reduction effect of up to +6 ‰ (Köster et al., 2013a) most probably as a result of larger contributions of fungi to N_2O production. However, those results were obtained in an experimental setup with ambient oxygen concentration, without glucose amendment and without C_2H_2 inhibition of N_2O reduction since N_2 gas fluxes were directly measured. It was also discussed before that short-time incubations under static conditions as presented here, may promote bacterial over fungal growth, which may also be transferable to denitrification activity by both organism groups (Lewicka-Szczebak et al., 2017; Lewicka-Szczebak et al., 2014). Additionally to this, the selection of glucose as substrate in the selected concentration may promote bacteria compared to fungi even more (Koranda et al., 2014; Reischke et al., 2014).

4.5 $\delta^{18}O_{N2O}$ values

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

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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. However, there were 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). However, for this study, $\delta^{18}O_{H2O}$ values were not analyzed. However, due to parallel *traced* variety experiments, we could determine possible $\delta^{18}O_{H2O}$ values for the particular SP_{N2O} values of bacterial denitrification mixing endmember (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. In case of precisely determined $\delta^{18}O_{H2O}$ values, the calculated F_{FD_MAP} values could be more precise, however, here we show 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.

4.6 Co-denitrification

The influence of co-denitrification, which is predominantly associated to fungi (Spott et al., 2011), may have a large impact on N_2O production, since Laughlin and Stevens (2002) found N_2O production in their experiment derived to 92% from co-denitrification and only 8% from denitrification. So far, there is no study on SP_{N2O} values of N_2O produced by co-denitrification. Co-denitrification could have been a contributing process in Experiment 4. When N in N_2O originates only from ^{15}N-labeled soil NO_3^-, measured $\delta^{15}N^{bulk}_{N2O}$ values as well as the ^{15}N enrichment of the labelled N pool producing N_2O (a_p) should show identical ^{15}N enrichment to the labeled soil NO_3^-. During co-denitrification, when one N atom in N_2O originates from labeled NO_3^- and the other one from another unlabeled and unknown 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 soil NO_3^- was about 60% larger than the analyzed ^{15}N enrichment in N_2O, leading to the assumption that N_2O was produced not only by denitrification. We also calculated a_p values of the other three experiments (data not shown) which coincided with the ^{15}N enrichment of N_2O (Table 3). Since a_p would not be affected by contributions of unlabelled N_2O we can thus exclude the possibility that this smaller enrichment could be caused by dilution of enriched N_2O from denitrification by N_2O production from an unknown N source and thus verified that this was due to formation of hybrid N_2O, probably via co-denitrification (Spott et al., 2011). In the other experiments there was no indication of co-denitrification being relevant for N_2O production since ^{15}N enrichments of NO_3^- and N_2O coincided. The question arises, why hybrid N_2O

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790 formation was only found when the loamy sand was sampled in summer (June, Experiment 4) but not
791 when it was sampled during winter (December, Experiment 1). Information on substrates for co-
792 denitrification, i.e. NO_2^- and NH_4^+ or certain organic N compounds could have been different due to
793 seasonal effects. Moreover, seasonal impacts on microbial community could have been relevant. Since
794 these possible factors were not assessed in our study and their impact on co-denitrification is still
795 poorly understood, it is currently not possible to give an answer here. Thus, only the SP_{N2O} values in
796 Experiment 4 might be influenced by co-denitrification. But since SP_{N2O} values of the acetylated
797 treatments of Experiment 4 coincided with the SP_{N2O} value range of bacterial denitrification and also
798 with SP_{N2O} values of the other experiments, our data give no indication that co-denitrification produces
799 N_2O with SP_{N2O} values differing from bacterial denitrification.

800 4.7 Calculating the fungal fraction contributing to N_2O production and SP_{FD} values

801 Due to the inefficiency of microbial inhibition regarding N_2O production in most cases, calculation of
802 F_{FDmi} contributing to N_2O production was only possible for Experiment 2. Comparing the modified
803 SIRIN with the isotopic approaches revealed that the fungal fraction contribution to N_2O production
804 was smaller (about 0.28 in modified SIRIN, ≤0.15 with IEM, ≤0.20 with SP/$\delta^{18}O$ Map) than the
805 bacterial fraction. Although we did not obtain a very clear picture of various microorganisms
806 contributing to N_2O production due to the large uncertainties of the calculated fractions, all approaches
807 coincided by showing dominance of bacterial N_2O. In contrast to SIRIN, the isotopic approaches
808 yielded similar estimates of F_{FD} for all experiments.
809 In some soil studies using helium incubations the SP_{Prod} values obtained by correction for the
810 reduction effect on SP_{N2O} values showed significantly larger values than SP_{N2O} of bacterial
811 denitrification (Köster et al., 2013a; Lewicka-Szczebak et al., 2017; Lewicka-Szczebak et al., 2014;
812 Senbayram et al., 2018; Senbayram et al., 2020). Therefore, it can be supposed that based on the
813 isotopic approaches various soils may largely differ in the microbial community that contributes to
814 N_2O from denitrification. The three tested soils seemed to contain a microbial community where fungi
815 have minor contributions to N_2O emissions from denitrification compared to bacteria. However, this
816 may also be due to the applied experimental setup favoring bacterial denitrification by static and
817 strictly anoxic conditions and due to the choice of glucose as substrate. Senbayram et al. (2018) could
818 show in an incubation experiment with sufficient NO_3^- supply, that fungal contribution to
819 denitrification was larger with straw compared to a control without straw addition.
820 The fungal SP_{FD} values (section 3.6 "*SP of N_2O produced by the fungal soil community*") by SIRIN
821 were highly variable with values between -23 and +25 ‰, which is smaller than the SP_{N2O} range of
822 N_2O known from pure cultures (16 - 37 ‰) (Sutka et al., 2008; Rohe et al., 2014a). Unfortunately,
823 both ranges exhibit a large overlap but also some discrepancy, which precludes a clear conclusion
824 whether or not Experiment 2 yielded valid estimates of fungal SP_{N2O} values. There may be different
825 reasons why estimating the SP_{N2O} values using SIRIN of the fungal community was imprecise: the

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826 fungal fraction contributing to denitrification of the tested soils was only small compared to that of
827 bacteria, SP_{N2O} values were estimated using a large endmember range known from pure culture studies
828 only, and possible SIRIN artefacts may have occurred as discussed above. The isotopic approaches
829 should thus be further investigated with soils, where presumable fungi contribute largely to N_2O
830 production during (e. g. acid forest soils, or litter-amended arable soils) (Senbayram et al., 2018) and
831 using SIRIN with suitable inhibitors (Ladan and Jacinthe, 2016). The critical question whether the
832 isotopic signatures of fungal N_2O determined in pure culture studies are transferable to natural soil
833 conditions cannot be fully answered with this study due to large uncertainties associated with the
834 results of the SIRIN method.

835 **5. Conclusions**

836 Selective inhibitor and isotopic approaches coincided in showing dominance of bacterial
837 denitrification. Neither the modified SIRIN approach, nor IEM or SP/$\delta^{18}O$ Map approaches yielded
838 larger contributions of the fungal N_2O fraction in any experiment. Both selective growth inhibitors of
839 modified SIRIN confirmed the expected effect on N_2O production only in one out of four experiments,
840 and SP_{N2O} values of fungal N_2O calculated from this treatment did not appear to be a valid estimate of
841 this value and need further evaluation. There might be several artefacts in the modified SIRIN, where
842 further studies should focus on, e.g. including the effectiveness of inhibitors, changes in microbial
843 community during pre-incubation with inhibitors and effects of bacterial consumption of N_2O
844 produced by fungi in the presence of bacterial growth inhibitors. The present study could show that
845 consideration of N_2O reduction in further studies is inevitably necessary. Further studies should also
846 determine the range of SP_{N2O} values known from fungal denitrification as well as the effect of specific
847 inhibitors on microbial groups producing N_2O and reducing N_2O during denitrification.

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

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

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

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