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Long term farming systems affect soils potential for N_2O production and reduction processes under denitrifying conditions

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ABSTRACT

N_2O is a potent greenhouse gas with an atmospheric lifetime of 114 years which also contributes to ozone layer destruction. Mitigating N_2O emissions is especially challenging to the agricultural sector that is responsible for the majority of anthropogenic N_2O release. In order to develop effective mitigation strategies, a detailed understanding of drivers for N_2O production and reduction in agriculturally managed soils is needed. Denitrification is recognized as one of the most important source processes for N_2O emissions from soils. However, the last step in denitrification, the reduction of N_2O to N_2 is the only known sink for N_2O in soil. Although the impact of single parameters on denitrification is quite well documented, there is still a knowledge gap when it comes to the impact of complex farming systems on N_2O production and reduction. In this experiment, we incubated soil samples from the DOK long term field trial in Therwil/Switzerland comparing organic (BIOORG) and conventional (CONMIN) farming systems with an a non-fertilized control (NOFERT). Soil samples were incubated under 90% WFPS after fertilization with $NH_4^{15}NO_3$ equivalent to a moderate fertilization event in the field with 40 kg N ha^{-1}. In order to assess soil's potential for N_2O production and reduction, we combined direct measurements of denitrification end products N_2O and N_2 with molecular analysis of functional denitrifying communities involved in NO_2^- and N_2O reduction on DNA and mRNA levels. In order to monitor N cycling processes under the chosen conditions, stable isotope tracing was employed to quantify nitrification and NO_3^- consumption rates. Results revealed increased NO_3^- consumption and greatest potential for N_2O emissions in BIOORG as a result of increased soil organic carbon contents. Production of N_2 was similar in BIOORG and CONMIN and significantly lower in NOFERT, most likely due to significantly decreased pH inhibiting N_2O reduction. This caused the greatest $N_2O/(N_2O + N_2)$ ratios in NOFERT (0.88 ± 0.02) followed by BIOORG (0.79 ± 0.01) and CONMIN (0.68 ± 0.02) ($p < 0.001$). Lowest $N_2O/(N_2O + N_2)$ ratios in CONMIN were reflected by lowest N_2O emissions and coincided with elevated *nosZ* transcript copies in the beginning of incubation. Although highest N_2O emissions in BIOORG were detected, the incubation setup cannot directly be translated to field conditions. Nevertheless, our results emphasize that farming system induced changes on soil geochemical parameters like soil pH and soil organic carbon affect microbial N_2O production and reduction processes during denitrification.

1. Introduction

Nitrous oxide (N_2O) is a major greenhouse gas, contributing to radiative forcing of Earth's climate. It is also the most important anthropogenic compound involved in the catalytic breakdown of stratospheric ozone (Kanter et al., 2013). Its current atmospheric concentration is 328 ppb and mostly due to anthropogenic interventions in the nitrogen cycle, it rises linearly by 0.25% $year^{-1}$ (Forster et al., 2007; IPCC, 2013). The global warming potential of N_2O exceeds that of CO_2 298-fold and because of its long atmospheric lifetime of 114 years, reducing atmospheric N_2O

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concentrations will be a long term issue (Forster et al., 2007). Mitigation of N_2O emissions is especially challenging for the agricultural sector which accounts for ~60% of anthropogenic N_2O emissions (Syakila and Kroeze, 2011). Within the agricultural sector, fertilization is the most important source of N_2O emissions (Stern, 2006). The amount and speciation of N added during fertilization directly affects N availability and N_2O emissions (Stehfest and Bouwman, 2006; Shcherbak et al., 2014) and large N_2O fluxes often occur directly after fertilization events (Gregorich et al., 2005; Thangarajan et al., 2013).

Different N species can serve as substrates for N_2O formation from a variety of N transforming processes depending on soil aeration status. Under oxic conditions, NH_4^+ serves as a substrate for nitrification. In this process N_2O can be formed as a byproduct via NH_2OH oxidation (Schreiber et al., 2012). Under oxygen limiting conditions NO_3^- reduction leads to N_2O formation via denitrification. The separation of nitrification and denitrification as sources for N_2O is a simplification as a variety of microbial metabolic pathways (e.g. nitrifier-denitrification, co-denitrification) and abiotic reactions (chemodenitrification, chemical decomposition of NH_2OH) can form N_2O and might occur within the same soil aggregate (Kool et al., 2011; Butterbach-Bahl et al., 2013). Denitrification is generally considered a major source of N_2O production and the last step in denitrification; the reduction of N_2O to N_2, is the only known biological sink for N_2O (Thomson et al., 2012; Butterbach-Bahl et al., 2013).

Most N transformations are carried out biologically and a detailed understanding of the drivers regulating denitrification and N_2O production from the soil microbial communities is a prerequisite to develop effective mitigation strategies. Denitrification is a modular pathway which involves four enzymatic systems in the subsequent reduction of NO_3^-, NO_2^-, NO and N_2O (Tiedje et al., 1982). The most widely used genetic marker system targeting denitrification are the functional genes *nirK* and *nirS* encoding for copper- and heme-bearing NO_2^- reductases (Jones et al., 2008). The last step of denitrification, the reduction of N_2O to N_2, is catalyzed by the nitrous oxide reductases encoded by the functional genes *nosZ* and *nosZ-II* (Jones et al., 2013). Not all microbes involved in denitrification necessarily possess the whole set of denitrifying genes (Graf et al., 2014). Around one third of bacteria involved in NO_2^- reduction lack the genetic capability for N_2O reduction and thus are likely to produce N_2O as a denitrification end product (Philippot et al., 2011). On the contrary, other microbes bearing the functional genes *nosZ* and especially *nosZ-II* lack antecedent enzymatic system and thus might act as a sink for N_2O (Graf et al., 2014). Recently there have been several studies investigating mechanisms of N_2O emissions via gene abundance and/or expression with variable results (Miller et al., 2008; Henderson et al., 2010; Morales et al., 2010; Dandie et al., 2011; Harter et al., 2014; Németh et al., 2014; Chen et al., 2015). Yet, most studies lacked quantification of N_2 and the process of N_2O consumption was not directly assessed.

Due to its high atmospheric background concentrations, quantification of N_2 as a denitrification end product is extremely challenging (Groffman et al., 2006) and all approaches to measure N_2 have to deal with inherent drawbacks (Saggar et al., 2013). Recent development of simultaneous tracing of ^{15}N in N_2O and N_2 from the same gas sample has significantly increased the efficiency and reliability of ^{15}N tracing experiments (Lewicka-Szczebak et al., 2013). This method is especially valuable in the agricultural context, since provision of additional N is an intrinsic part of fertilization research. However, ensuring homogenous distribution of added ^{15}N remains challenging in ^{15}N tracing experiments (Spott et al., 2006). Quantification of N_2 provides valuable information about N_2O consumption and $N_2O/(N_2O + N_2)$ product ratios are an important measure for soils performance as sink for N_2O. The $N_2O/(N_2O + N_2)$ product ratio can be affected by C and N availability (Senbayram et al., 2012) as well as soil pH (Cuhel et al., 2010) and the denitrifying community composition (Philippot et al., 2009; Jones et al., 2014). While the impact of single parameters on denitrification processes is quite well studied, there is still a knowledge gap when it comes to complex farming systems.

In the last decades, organic farming systems gained attention due to positive effects on numerous soil quality indicators (Maeder et al., 2002), soil organic carbon stocks (Gattinger et al., 2012) and N_2O and CH_4 fluxes (Skinner et al., 2014). It was also shown that organic farming systems increased richness and diversity of the microbial community (Hartmann et al., 2014). However, it is unclear how the legacy of farming systems affects soil denitrification and especially N_2O reduction. In order to assess the impact of farming system on denitrification and $N_2O/(N_2O + N_2)$ product ratio, we performed an incubation trial with soil samples originating from the DOK long term field trial (D: bio-dynamic, O: bio-organic, K: german “konventionell” integrated). This field trial compares farming systems in place since 1978.

The object of this study was to assess the effect of farming system induced changes in soil geochemical parameters on N cycling under oxygen limited conditions with a special emphasis on N_2O and N_2 emissions. We also aimed to identify the functional impact of denitrifier gene abundance and expression on N_2O production and reduction processes.

2. Material and methods

2.1. Sampling site

Soils were collected from the DOK system comparison trial in Therwil/BL, Switzerland. Soil sampling took place in autumn 2013 and soil was classified as Haplic Luvisol on deposits of alluvial loess. Composite soil samples originated from 4 replicate parcels of BIOORG, CONMIN and NOFERT treatments, and were collected to a depth of 20 cm. Samples were sieved to 2 mm and stored at 4 °C. Soil sampling was carried out during the 6th crop rotation after cultivation of *Zea mays* L. The previous cropping consisted of two years cultivation of a grass clover ley. All farming systems are subjected to the same 7 year crop rotation. Details on the experimental setup of the field trial are described in Fließbach et al. (2007). Briefly, BIOORG represents a livestock-based organic farming system with farmyard manure fertilization without pesticides and did not receive liming treatment. CONMIN is characterized by a livestock-free system comprising mineral fertilization and chemical pest control. This treatment received 4.7 t ha $CaCO_3$ in the 4th crop rotation from 1999 to 2005 (Oberholzer et al., 2009). NOFERT did not receive any fertilization, pest control or liming treatment. The 5th crop rotation started in 2006 with maize, followed by winterwheat, soja, potato, winterwheat and two years of grass clover. Soil samples were taken after harvest of the first crop in the 6th crop rotation which was maize. In the vegetation period before sampling CONMIN received 170 kg N ha^{-1} in the form of calcium ammonium nitrate, while BIOORG received 136 kg N ha^{-1} as rotted manure and 46 kg N ha^{-1} in the form of cattle slurry.

2.2. Incubation setup

For each microcosm, the equivalent of 150 g dry soil was placed in 250 ml DURAN wide neck glass bottles (Schott AG, Mainz, Germany) and compacted to a bulk density of 1.20 ± 0.02 gcm^{-3} by tapping the glass bottles on a soft surface. After pre-incubation for 7 days at a water filled pore space (WFPS) of 50%, an N containing solution equivalent to a moderate fertilization event (40 kg N ha^{-1} or 11 mg N per microcosm) was added in the form of 60 at%

enriched $NH_4^{15}NO_3$. Deionized and autoclaved water was added to adjust to 90% WFPS in order to stimulate denitrifying conditions and enhance distribution of added N solution. Swelling properties of the soil samples resulted in a WFPS of 87.5 ± 1.5% with no farming system specific bias. To assure constant conditions, water content was checked gravimetrically and corrected every second day by adding evaporated water to each microcosm. Microcosms were incubated with open bottles in order to mimick conditions after a heavy rain event in the field. Each treatment was incubated in triplicate at 20 ± 1.2 °C in the dark. In total, 63 microcosms (three treatments X three reps X seven sampling dates) were prepared to enable destructive sampling for geochemical and molecular biological analysis after 0, 2, 5, 8, 11, 14 and 17 days. After destructive sampling, soil was homogenized and divided into subsamples for subsequent analysis.

2.3. Geochemical analyses

2.3.1. pH, soil organic carbon and total N

At the beginning of the incubation, key soil parameters were assessed in triplicate. Soil pH was determined in a 1:2.5 (w/v) suspension with demineralized H_2O. Soil organic carbon (SOC) was analyzed by dry combustion (multi N/C2100S + HT1300, Analytik Jena AG, Jena, Germany) and total N in soils was determined by combustion (CN Vario Max, Elementar Analysensysteme GmbH, Hanau, Germany).

2.3.2. Mineral nitrogen (N_{min}) and dissolved organic carbon (DOC)

For determination of mineral N (NH_4^+ and NO_3^-) and DOC, 80 ml of 0.01 M $CaCl_2$ was added to 20 g soil sample and shaken for 1 h at 130 rpm (SM-30, Edmund Bühler GmbH, Hechingen Germany). The soil solution was filtered through a folded filter (MN619EH, Machery-Nagel, Düren, Germany) and stored at 20 °C until analysis. Concentrations of NH_4^+ and NO_3^- were quantified by continuous flow analyses (San Plus, Skalar Analytical B.V., Breda, Netherland) while DOC was determined via TOC Analyzer (multi N/C 2100S, analyticJena, Jena, Germany). All parameters were quantfifed at each time point but changes in DOC over time were negligible. Therefore only initial DOC contents are reported.

2.3.3. Greenhouse gas (GHG) fluxes

Before destructive soil sampling, production of CO_2 and N_2O were measured after closing each microcosm with a gas-tight lid equipped with a rubber septum for up to 40 min. A cooling tray with circulatory water flow assured constant temperature (20 ± 0.8 °C) of the microcosms during gas sampling. Gas samples of 5 ml were taken from the headspace of the microcosms and directly injected into a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA) after 0, 20 and 40 min by using an autosampler (MPS 2XL, Gerstel, Baltimore, MD). In order to avoid underpressure, 5 ml He was injected into the headspace of the microcosm prior to gas sampling. CO_2 and N_2O concentrations in gas samples were determined via flame ionization detector (FID) linked to a methanizer and electron capture detector (ECD), respectively. Calibration curves for N_2O ($r^2 > 0.99$) and CO_2 ($r^2 > 0.99$) were obtained by a threefold analysis of 3 standard gases with 0.308, 2.94 and 90 ppm N_2O and 300, 2960 and 9000 ppm CO_2 before and after each sampling. For flux calculation, a linear enrichment of gases in the headspace was assumed. Gas samples for $^{15}N_2$ and $^{15}N_2O$ analysis were taken with a syringe directly before destructive sampling after a prolonged enrichment period of 1 h and stored in 12.5 ml gas tight vials. $^{15}N_2$ and $^{15}N_2O$ concentrations in the gas samples were quantified via isotope ratio mass spectrometry, as described in Lewicka-Szczebak et al. (2013). Prior to analysis, in a part of the sample N_2O is frozen in a liquid N trap which enables the quantification of ^{29}R ($^{29}N_2/^{28}N_2$) and ^{30}R ($^{30}N_2/^{29}N_2$) of N_2, N_2O and $N_2O + N_2$ from the same sample (Lewicka-Szczebak et al., 2013). The fraction of NO_3^- derived N_2O and/or N_2 (f_p) were calculated according to Spott et al. (2006) using eq. (1):

$$f_p = \frac{a_m - a_{bgd}}{a_p - a_{bgd}} \quad (1)$$

where a_{bgd} is the ^{15}N abundance of the atmospheric background, a_p is the ^{15}N abundance of the active NO_3^- pool and a_m is the ^{15}N abudance of N_2 and/or N_2O. a_m and a_p were calculated using eqs. (2) and (3):

$$a_m = \frac{29_R + 2^* 30_R}{2\,(1 + 29_R + 30_R)} \quad (2)$$

$$a_p = \frac{30_{x_m} - a_{bgd}^* a_m}{a_m - a_{bgd}} \quad (3)$$

In which 30_{x_m} is the measured fraction of m/z 30 in N_2 and/or converted N_2O calculated as $^{30}R/(1+^{29}R+^{30}R)$. ^{15}N enrichment of the active NO_3^- pools for N_2O and N_2 formation are shown in Fig. S1. The denitrification product ratio (pr) was calculated according to eq. (4):

$$pr = \frac{f_{N_2O}}{f_{N_2O+N_2}} \quad (4)$$

NO_3^- derived N_2O and N_2 fluxes were assessed according to eqs. (5) and (6):

$$N_2O_{NO_3}flux = f_{N_2O}{}^* N_2O_{total}flux \quad (5)$$

$$N_{2NO_3}flux = \left(\left(\frac{1}{pr}\right)^* N_2O_{NO3}flux\right) - N_2O_{NO_3}\,flux \quad (6)$$

In which $N_2O_{total}flux$ was obtained by flux calculation from GC measurements. Hybrid N_2O and N_2 was determined as described in Spott and Stange (2011), but found to be irrelevant.

2.3.4. Isotopic analysis of $^{15}NH_4^+$ and $^{15}NO_3^-$ and calculation of N transforming processes

The ^{15}N abundance in NH_4^+ and NO_3^- was determined according to the procedure described in Stange et al. (2007), whereby NO_3^- was reduced to NO by vanadium chloride ($V^{III}Cl_3$) and NH_4^+ was oxidized to N_2 by sodium hypobromite (BrNaO). The NO and N_2 obtained were then analyzed using a quadrupole mass spectrometer (GAM 200, InProcess Instruments, Bremen, Germany). The analytical precision was determined by repeated measurements of standards (1 at%, 5 at%, 50 at%, 75 at%) and was consistently around 1.2%. Gross nitrification and NO_3^- consumption rates were assessed using the pool dilution approach according to eq. (7) and eq. (8) provided by Davidson et al. (1991) and Stark (2000).

$$p = ((M_0 - M_1)\,/\,t)^* \frac{\log(H_0M_1/\,H_1M_0)}{\log(M_0/M_1)} \quad (7)$$

$$c = p - ((M_0 - M_1)\,/\,t) \quad (8)$$

where p and c are the nitrification and NO_3^- consumption rates (mg N kg^{-1} day^{-1}), M is the amount of NO_3^--N (mg N kg^{-1} dry soil), H the ^{15}N atom fraction of NO_3^-, subscripts 0 and 1 mark first and second time point, respectively and t represents the incubation interval between first and second time point (days). For all

calculations a homogeneous distribution of the labeled pool and negligible immobilization of the ^{15}N tracer into the organic N pool within incubation intervals were assumed. Cumulative values had been quantified by summing the amount of N transformed during all incubation intervals.

2.4. Molecular biological analyses

Subsamples for DNA and RNA extraction were collected and stored at −80 °C. DNA and RNA were co-extracted from 0.5 g soil samples via phenol chloroform extraction as described in Griffiths et al. (2010). In order to assess for individual DNA and RNA recovery rates, 2.50^{10} copies of a linearized plasmid (pJET1.2, CloneJET PCR Cloning Kit, Thermo Scientific, Waltham, MA) carrying a fragment of cassava mosaic virus (APA9, gene accession Nr. AJ427910) (Thonar et al., 2012) and 2.85^{10} transcripts (MEGAscript® T7 transcription KIT, Invitrogen, Carlsbad, CA) were added to the soil samples before bead beating. DNA and RNA concentrations were assessed fluorimetrically with Qubit 2.0 (Qubit dsDNA HS Assay Kit and Qubit RNA HS Assay Kit, Invitrogen, Carlsbad, CA) directly after extraction. Absolute yield for DNA and RNA ranged from 108.4 to 325.2 ng g dry soil^{-1} and 22.4–78.8 ng g dry soil^{-1}, respectively, without treatment specific bias. Reverse transcription was conducted using QuantiTect Reverse Transcription Kit (Qiagen, Venlo, Netherlands) with an integrated removal step for genomic DNA. Successful removal of genomic DNA was assured by the performance of negative controls without addition of reverse transcriptase. Quantitative PCR of functional genes was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA) and a Rotor-Gene-Q (QIAGEN, Venlo, Netherlands). Each 10 µl reaction volume included ~1 ng of template DNA or cDNA. Primers and thermal protocols used for functional gene quantification are listed in Tables S1 and S2. Standard curves were constructed by running a serial dilution with concentrations ranging from ~10^8 to ~10^2 gene copy numbers per reaction of a plasmid bearing a copy of the respective gene. Specifications of vector plasmids and host genes are given in Table S3. Concentrations of standards were measured fluorimetrically with Qubit 2.0 (Qubit dsDNA HS Assay Kit, Invitrogen, Carlsbad, CA). Efficiencies of qPCR yielded 94–96% for APA9 gene fragment, 86–92% for *nirK*, 89–92% for *nirS*, 77–82% for *nosZ* and 82–86% for *nosZ-II* assays and specificity of the amplification was tested via melt curve and agarose gel analysis. Each treatment was assessed with three biological replications and each reaction was performed in analytical duplicates. Analysis was repeated if Ct values differed by more than 0.5. Raw data was analyzed via LinReg PCR by assessing enzyme kinetics for each reaction individually (Ramakers et al., 2003). Additional to normalization of functional gene abundances per g dry soil, DNA and cDNA recovery rates obtained by APA9 quantification were used to correct functional gene and transcript copy numbers. Recovery rates averaged 51.4 ± 12.5% for DNA and 2.3 ± 0.8% for RNA. The absolute values of gene and especially transcript numbers exceeded reported values from other studies, as a consequence of calculation integrating the recovery rate of the internal standard (Tatti et al., 2013; Chen et al., 2015; Snider et al., 2015).

2.5. Statistics

For all parameters measured the effect of farming systems at a specific time point were examined by one way analysis of variance (ANOVA) with a post hoc Tukey test. Differences were considered as significant at $P < 0.05$. In the same way, time integrated N transformation rates, and cumulated N_2O and N_2 production as well as $N_2O/(N_2O + N_2)$ product ratios were tested for effects of treatments. For each parameter the effect of incubation time was assessed by performing a repeated measures ANOVA followed by a post hoc Tukey test. The interaction of treatment and time was assessed by a two-way ANOVA. All statistical analysis was performed with SPSS 25.0. Detailed results are displayed in the supplementary information.

3. Results

3.1. Basic soil properties

Quantification of basic geochemical parameters in soils from different farming systems prior to incubation revealed significantly increased SOC content in BIOORG, followed by CONMIN and then NOFERT. Initial DOC did not show any effect of farming system, while pH and total N contents were significantly decreased in NOFERT (Table 1). Soil C/N ratio was significantly increased in BIOORG compared to NOFERT, while CONMIN did not differ significantly from the other treatments.

3.2. CO_2, N_2O and N_2 fluxes

Across the incubation period, cumulative CO_2 emissions were significantly enhanced in BIOORG compared to CONMIN. Cumulative CO_2 emissions in NOFERT did not differ significantly from the other farming systems. After addition of $NH_4^{15}NO_3$, the greatest N_2O fluxes appeared at 5 days with mean N_2O emissions of 74.01 ± 23.47, 56.91 ± 2.90 and 42.79 ± 2.84 µg N_2O-N kg dry soil^{-1} h^{-1} for BIOORG, NOFERT and CONMIN, respectively (Fig. 1A). Thereafter, N_2O emission declined and reached stable levels after 11 days. In the last phase of incubation CONMIN showed significantly decreased N_2O emissions at days 14 and 17 as compared to BIOORG and NOFERT. N_2O emissions in NOFERT soil emerged slowly and after 2 days of incubation N_2O emissions in NOFERT were significantly lower compared to CONMIN and BIOORG. From day 8 on, N_2O emissions reached similar high levels like BIOORG. N_2 emissions in NOFERT were detectable after 5 days of incubation but remained at constantly low levels throughout the incubation (Fig. 1B). In BIOORG and CONMIN, N_2 emissions increased until day 8 and slightly declined thereafter. Highest N_2 emissions occurred at the end of incubation reaching 24.02 ± 6.40, 24.25 ± 3.35 and 7.44 ± 1.07 µg N_2-N kg dry soil^{-1} h^{-1} in BIOORG, CONMIN and NOFERT, respectively (Fig. 1B). $N_2O/(N_2O + N_2)$ product ratio constantly declined in BIOORG and CONMIN but remained at high levels throughout the incubation time in NOFERT (Fig. 1C). At days 2, 8, 11 and 17 the $N_2O/(N_2O + N_2)$ product ratios were significantly higher in BIOORG compared to CONMIN meaning that significantly lower portion of N_2O was further reduced to N_2. This resulted in distinct $N_2O/(N_2O + N_2)$ product ratios of cumulated fluxes for the different soils that decreased in the order NOFERT, BIOORG to CONMIN (Table 2). Yet, cumulated NO_3^- derived N_2O emissions were the highest for BIOORG (16.18 ± 1.66 mg N_2O-N kg^{-1}) followed by NOFERT (13.87 ± 0.46 mg N_2O-N kg^{-1}) and the lowest in CONMIN (9.59 ± 0.15 mg N_2O-N kg^{-1}) (Table 2). Interestingly, cumulated N_2

Table 1
Basic soil properties before incubation. Data shows means and standard errors (n = 3). Values not followed by the same letter differ significantly at $P < 0.05$.

	BIOORG		CONMIN		NOFERT	
pH (H_2O)	6.13 ± 0.02	a	6.15 ± 0.03	a	5.54 ± 0.04	b
DOC (mg C kg^{-1})	36.00 ± 1.60	a	34.09 ± 1.75	a	32.14 ± 2.17	a
SOC (g kg^{-1})	14.15 ± 0.05	a	13.66 ± 0.05	b	11.74 ± 0.08	c
Total N (g kg^{-1})	1.70 ± 0.01	a	1.72 ± 0.06	a	1.65 ± 0.03	b
C/N	8.33 ± 0.01	a	7.94 ± 0.25	ab	7.11 ± 0.13	b

Abbreviations: DOC – dissolved organic carbon; SOC- soil organic carbon.

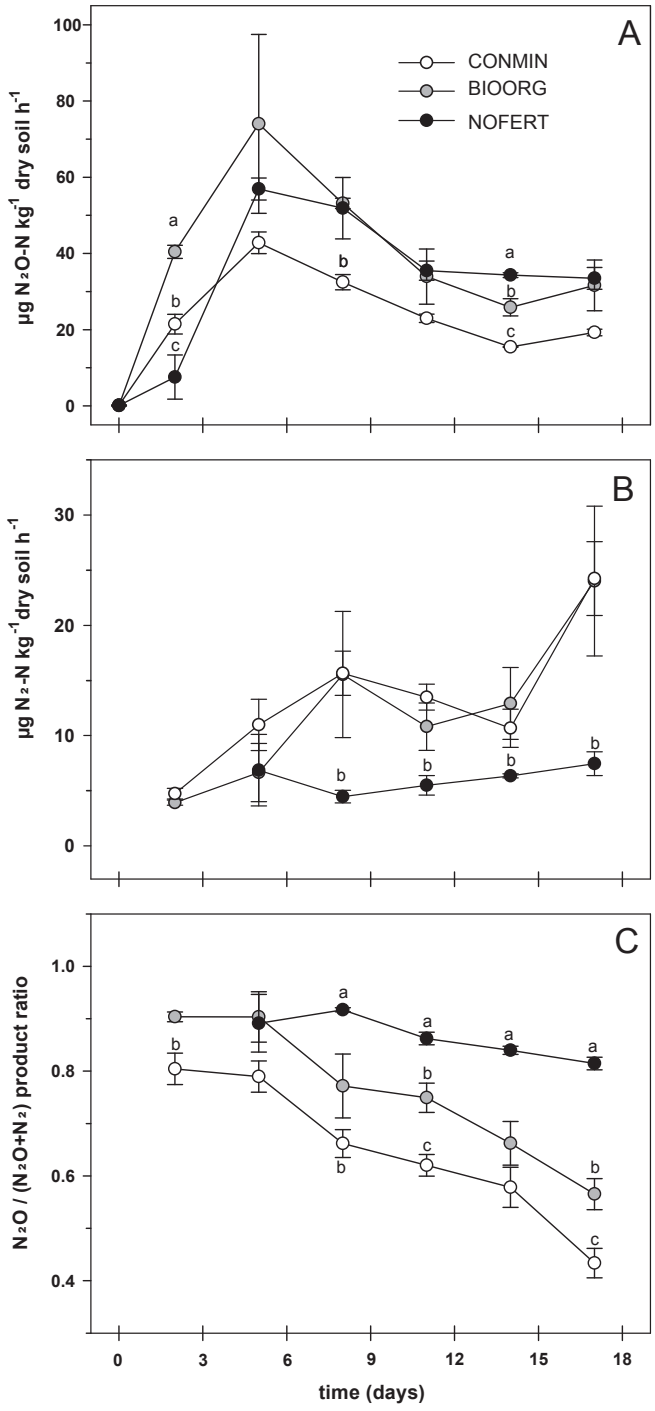

Fig. 1. N_2O emissions from incubated soil samples originating from different soil management practices (CONMIN- conventional, BIOORG – organic, NOFERT – unfertilized) after fertilization with $NH_4^{15}NO_3$ **(A)**, temporal patterns of N_2 emission **(B)** and $N_2O/(N_2O + N_2)$ product ratio **(C)**. Data are only shown if all replications resulted in measurable amounts of N_2. Small letters indicate significant differences in between treatments at a specific time point at $P < 0.05$. Data points are means ± SD (n = 3).

emissions did not differ between BIOORG and CONMIN but were significantly lower in NOFERT (Table 2).

3.3. N transforming processes

Generally, NO_3^- reduction was the dominating N transforming process in all soils. Across the whole incubation period 21.00 ± 0.24 mg of NO_3^--N kg^{-1} was consumed in BIOORG followed by CONMIN (18.44 ± 0.24 mg NO_3^--N kg^{-1}) and NOFERT (15.33 ± 0.37 mg NO_3^--N kg^{-1}) (Table 2). Cumulated gross NO_3^- consumption was significantly increased in BIOORG from day 11 on, while cumulated gross NO_3^- consumption in NOFERT was significantly lower at day 17 (Fig. 2A). For BIOORG and NOFERT the sum of cumulated N_2 and N_2O fluxes (20.59 and 15.82 mg N kg^{-1}, respectively) was in good agreement with gross NO_3^- consumption, whereas in CONMIN N_2+N_2O fluxes were significantly lower (14.40 mg N) compared to gross NO_3^- consumption (Table 2). Nitrification in CONMIN started slowly and cumulated nitrification was significantly lower after 2 days of incubation compared to NOFERT and BIOORG. Yet, from day 14 on, cumulative nitrification was the highest in CONMIN. Significantly lower cumulated nitrification was observed in NOFERT from day 8 on (Fig. 2B).

3.4. Gene and transcript abundances of denitrifying genes

Abundances of functional genes involved in NO_2^- reduction (*nirK*, *nirS*) did not show strong variations within the incubation period and were not influenced by the farming system (Fig. 3A and C). Only NOFERT showed significantly reduced abundances for *nirS* gene at day 11 and *nirK* gene at day 8 and 11. Generally *nirS* gene abundance exceeded *nirK* gene abundance by around one order of magnitude. In terms of *nirS* and *nirK* gene expression little differences were observed between farming systems (Fig. 4A and C). Gene expression of both nitrite reductases increased after fertilization in the beginning of the incubation. While *nirK* transcripts increased by almost one order of magnitude in CONMIN and NOFERT, increase in *nirS* transcripts was only 2–3 fold. Gene copy numbers of *nosZ* and *nosZ-II* were stable during incubation (Fig. 3B and D). Significant differences between soils were detected for *nosZ* abundance at day 8 and 11 with the lowest gene abundances in NOFERT. *NosZ-II* gene abundance was the lowest in CONMIN at all sampling points, although this was not significant. Transcription of *nosZ-II* and especially *nosZ* fluctuated strongly during incubation (Fig. 4B and D). Directly after fertilization the transcripts of *nosZ* increased by almost one order of magnitude in all soils and reached a first peak after 2 days of incubation. Thereafter, expression declined in all farming systems at day 5 and stayed at stable levels in CONMIN. Significantly increased *nosZ* transcripts in CONMIN could be observed during increasing N_2O emissions until day 5. In that period N_2O emissions and *nosZ* transcripts correlated negatively with N_2O emissions across all soils ($r^2 = 0.81$) (Fig. S2). *NosZ* transcripts in NOFERT and BIOORG peaked a second time at day 11 and were higher compared to CONMIN although this was only significant for BIOORG. For *nosZ-II* no transcripts were detectable until day 2 in CONMIN and BIOORG and day 5 in NOFERT. Afterwards, transcripts increased and peaked at day 8 in CONMIN and at day 11 in BIOORG and NOFERT (Fig. 4B and D). However, no farming system effects were detected for transcripts of *nosZ-II*.

4. Discussion

4.1. Geochemistry of the microcosms and N transforming processes

At the beginning of the experiment microcosms were fertilized with 11 mg N as NH_4NO_3 in correspondence to a moderate fertilization event in the field of 40 kg N ha^{-1}. Despite differing fertilization history, all soils were treated equally in order to assess long-term impact of farming systems on N transformations. Chosen incubation conditions (WFPS of 90%) aimed at favoring denitrification, but occurrence of nitrification proved partial availability of oxygen. This was expected as incubated soil samples were open to the atmosphere and soil pores were not completely water

Table 2
Cumulative gas fluxes, measures of denitrification and cumulative N-transforming processes for soil samples from conventional (CONMIN) and organic (BIOORG) farming system in comparison with an unfertilized control (NOFERT) after 17 days of incubation and fertilization with $NH_4^{15}NO_3$. Data shows means and SE (n = 3). Values not followed by the same letter differ significantly at P < 0.05.

	BIOORG		CONMIN		NOFERT	
gaseous emissions						
CO_2-C mg kg^{-1}	61.49 ± 4.97	a	49.26 ± 3.12	b	53.18 ± 5.30	ab
NO_3^- derived N_2O-N mg kg^{-1}	16.18 ± 1.66	a	9.59 ± 0.15	b	13.87 ± 0.46	a
NO_3^- derived N_2-N mg kg^{-1}	4.41 ± 0.41	a	4.81 ± 0.41	a	1.95 ± 0.33	b
measures of denitrification						
$N_2O/(N_2O + N_2)$	0.79 ± 0.02	b	0.67 ± 0.02	c	0.88 ± 0.01	a
NO_3^- derived N_2O emissions (%)	95.01		94.29		95.52	
N transforming processes						
Gross NO_3^- consumption mg-N kg^{-1}	21.00 ± 0.24	a	18.44 ± 0.24	b	15.33 ± 0.37	c
Nitrification mg-N kg^{-1}	11.31 ± 0.07	b	12.02 ± 0.06	a	6.22 ± 0.02	c

Cumulative data was obtained by subsequently cumulating means of time weighted process rates calculated for each time point. Abbreviations: DNRA – dissimilatory nitrate reduction to ammonium.

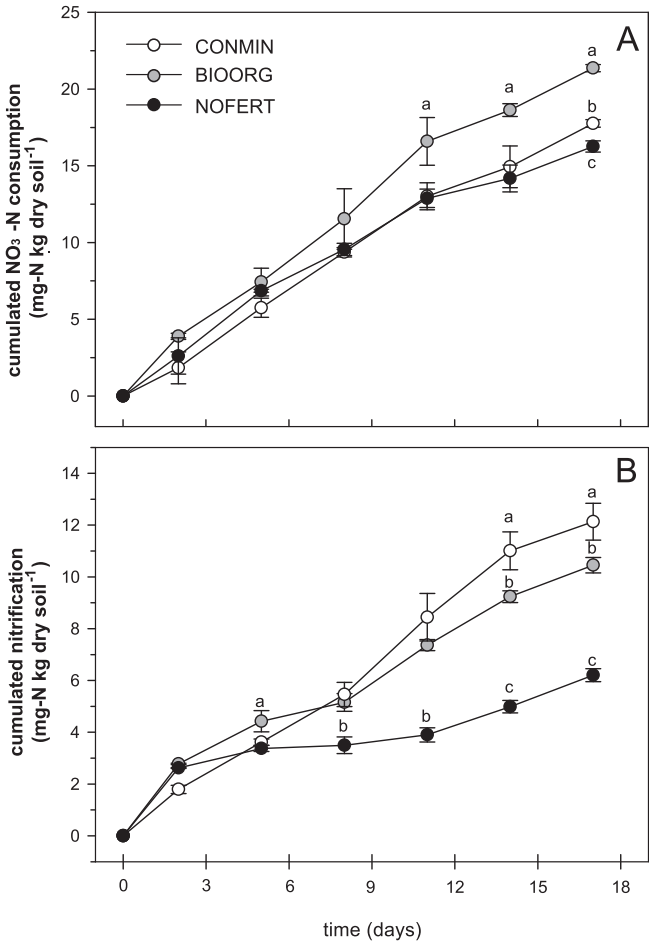

Fig. 2. Cumulative NO_3^- consumption **(A)** and nitrification **(B)** from incubated soil samples originating from different soil management practices (CONMIN- conventional, BIOORG – organic, NOFERT – unfertilized) after fertilization with $NH_4^{15}NO_3$. Small letters indicate significant differences in between treatments at a specific time point at P < 0.05. Data points are means ± SD (n = 3).

saturated, a situation which can occur in the field after a raining event. During most times of the year we would expect soil conditions to be rather oxic and thus nitrification related processes to be the major source for N_2O emissions. Yet, it was frequently reported that highest N_2O emissions in the field occur under conditions when low oxygen concentrations in the soil favor reducing processes like denitrification (Groffman et al., 2009). Not surprisingly the oxygenation status of the soil was reported to be the major control of N_2O emission on a regional scale (Jungkunst et al., 2006).

As distinct SOC contents presented a major distinguishing factor for soils from the different farming systems, no additional C was added during fertilization. Constant bacterial abundances as indicated by stable 16S rRNA gene numbers indicated limited bacterial growth and suggests C-limiting conditions in our setup (Fig. S3). Nevertheless, significantly increased CO_2 emissions in BIOORG compared to CONMIN demonstrate enhanced heterotrophic activity as a consequence of long term organic fertilization and elevated SOC levels (Table 2). This is in agreement with the studies of Hartmann et al. (2014) and Carpenter-Boggs et al. (2000) where increased soil microbial activity and/or abundance in organically managed soil had been described.

Nitrifiers can contribute to N_2O emission directly via NH_2OH oxidation and nitrifier denitrification and indirectly through NO_3^- provision for denitrification (Wrage et al., 2001). The occurrence of nitrification in our setup was not surprising as NH_4^+ oxidation and NO_3^- reduction processes can co-occur over a wide range of soil moisture conditions (Kool et al., 2011). Although the occurrence of nitrification (and possibly nitrifier denitrification) was proven, the contribution of this pathway to N_2O emission seems negligible as NO_3^- derived N_2O emissions accounted for ~95% of N_2O emission in all soils. Similarly, Kool et al. (2011) ascribed ~92% of N_2O emissions to denitrification at a WFPS of 90%.

For the calculation of nitrification and NO_3^- consumption rates and NO_3^- derived N_2O and N_2 emissions with the stable isotope approach homogeneous distribution of the added tracer and negligible N recycling within the incubation time must be assumed (Stark, 2000). The latter point was addressed by limiting incubation intervals to 72 h. Nevertheless, our approach might have overestimated N transformation rates since we could not account for N immobilization within an incubation interval. Although in our case a high WFPS assisted in uniform distribution of the added tracer, assuring homogenous labeling can only be approximated (Stark, 2000; Stange et al., 2007). Good agreement of $^{15}NO_3^-$ and the ^{15}N enrichments of the active NO_3^- pool for N_2O and N_2 formation is an indication for homogeneity in ^{15}N labeling (Stevens et al., 1997). While ^{15}N enrichments in the NO_3^- and the active NO_3^- pool for N_2O formation was almost identical, the active NO_3^- pool for N_2 formation showed higher ^{15}N signatures (Fig. S1). This indicates homogeneous conditions for N_2O formation but increased N_2 formation at isolated microsites, e.g. NO_3^- derived N_2O from deeper soil layers was more likely to be further reduced to N_2 compared to NO_3^- derived N_2O from shallow soil within our soil microcosm. Similarly, distinct active pools for nitrification were already reported during an oxic incubation experiment (Deppe et al., 2017).

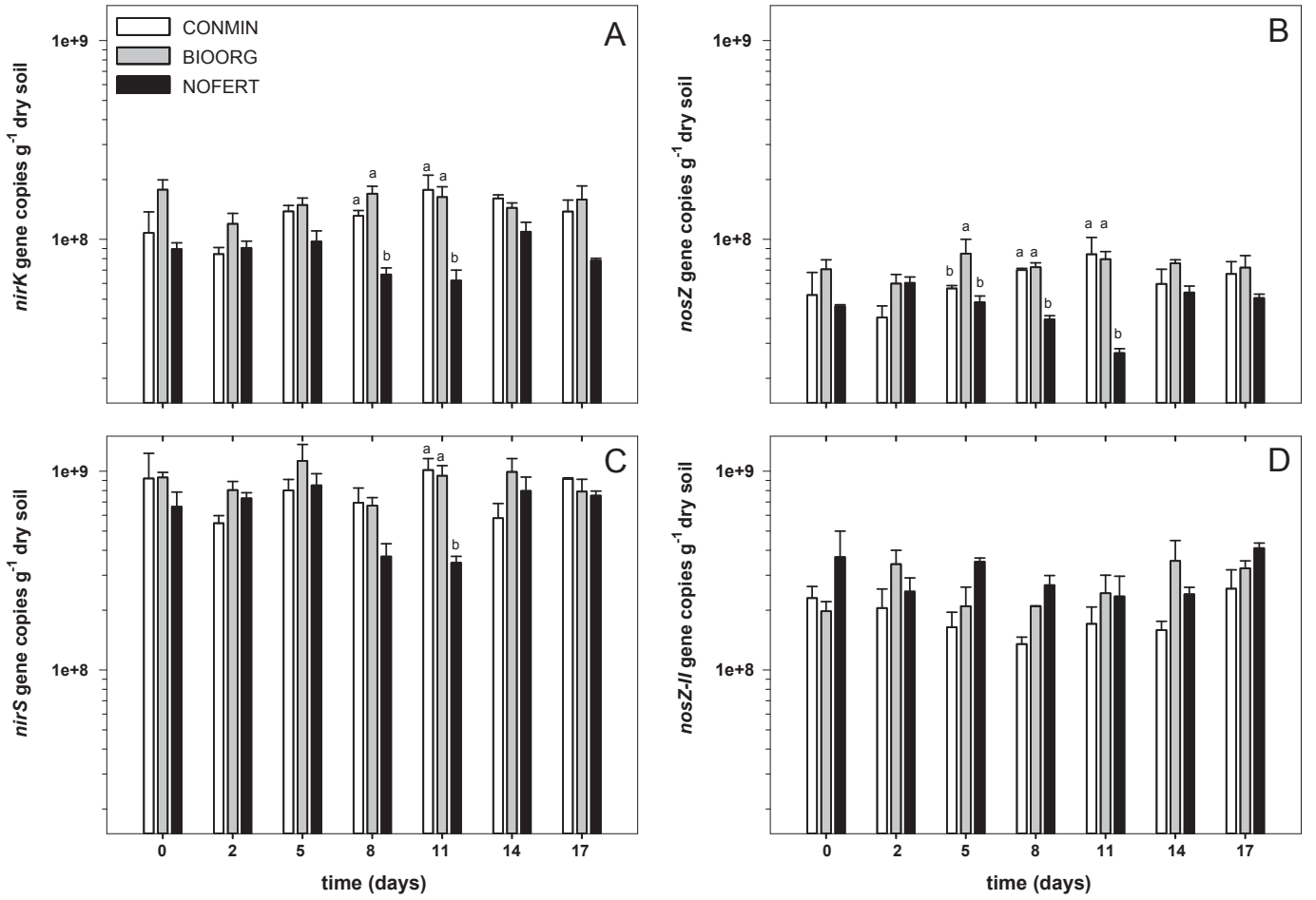

Fig. 3. Gene copies of nitrite reductases (*nirK* and *nirS*, **A** and **C**) and nitrous oxide reductases (*nosZ* and *nosZ-II*, **B** and **D**) communities in DNA of soil samples originating from different farming system (CONMIN- conventional, BIOORG – organic, NOFERT – unfertilized) after fertilization with $NH_4^{15}NO_3$. Letters indicate significant differences at a specific time point in between treatments at $P < 0.05$. Data points are means ± SE ($n = 3$).

4.2. Impact of farming systems on N_2O emissions and $N_2O/N_2O + N_2$ product ratio

The main goal of this incubation study was to assess the long term impact of farming system on soil's potential to perform N_2O production and reduction within the process of denitrification. Generally, N_2 production was low compared to other studies focusing on denitrification end products. In-situ measurements of $N_2O/(N_20 + N_2)$ product ratios in a grassland agroecosystems were reported not to exceed 0.45 (Baily et al., 2012; McGeough et al., 2012; Friedl et al., 2016). Nevertheless, $N_2O/(N_2O + N_2)$ product ratios are known to be largely dependent on the relative availabilities of C and NO_3^- (Senbayram et al., 2009, 2012; Morley and Baggs, 2010) and $N_2O/(N_2O + N_2)$ product ratios of up to 0.94 were reported in an incubation experiment under C limiting conditions with NO_3^- excess (Senbayram et al., 2012). Most likely this was the case in our study where high NO_3^- concentrations reduced the relative importance of N_2O reduction and shifted dominating denitrification end product towards N_2O (Fig. S1). It might also be that O_2 was allowed to penetrate in the soil column, inhibiting functionality of the nitrous oxide reductase. However, this seems unlikely since in a recent incubation study it was observed that N_2O reduction was not inhibited after addition of low molecular C sources at a WFPS of 90% (Giles et al., 2017).

In accordance with enhanced NO_3^- consumption in BIOORG, other studies demonstrated long term addition of farmyard manure to increase denitrification rates (Tatti et al., 2013) as well as N_2O emissions after NH_4NO_3 application (Jäger et al., 2013). Yet, quantification of N_2 additionally revealed N_2O reduction to play a key role affecting total N_2O budgets. Lowest N_2O emissions in CONMIN cannot solely be assigned to decreased NO_3^- consumption but also to high N_2 production. This is reflected by the significantly lowest $N_2O/(N_2O + N_2)$ product ratio at most time points of sampling and proved most efficient reduction of N oxides during the subsequent denitrification steps in CONMIN. There are two possible explanations how legacy of farming systems might have affected $N_2O/(N_2O + N_2)$ ratios in CONMIN and BIOORG. First, lower SOC levels in CONMIN compared to BIOORG might have reinforced C limitation and shifted denitrification end product towards N_2. Additionally, it needs to be considered that fertilization history of CONMIN is characterized by repeated fertilization with NH_4NO_3 without C addition. Therefore, adaptation of the denitrifying community to reoccurring conditions might have led to most efficient use of provided N oxides under C-limiting conditions. Latter hypothesis is supported by significantly increased *nosZ* expression in CONMIN in the beginning of incubation.

It is also known that pH can greatly impact $N_2O/(N_2O + N_2)$ ratios (Cuhel et al., 2010). Most likely low pH was the main driver for high $N_2O/(N_2O + N_2)$ ratios in NOFERT. In this farming system constant low N_2 production was observed, although expression of *nosZ* and *nosZ-II* genes was similar to BIOORG. The study of Bergaust et al. (2010) showed that acidic conditions below a pH of 6 impedes correct folding and thus functioning of the nitrous oxide reductase as a post-transcriptional effect in *Paracoccus denitrificans*. Liu et al.

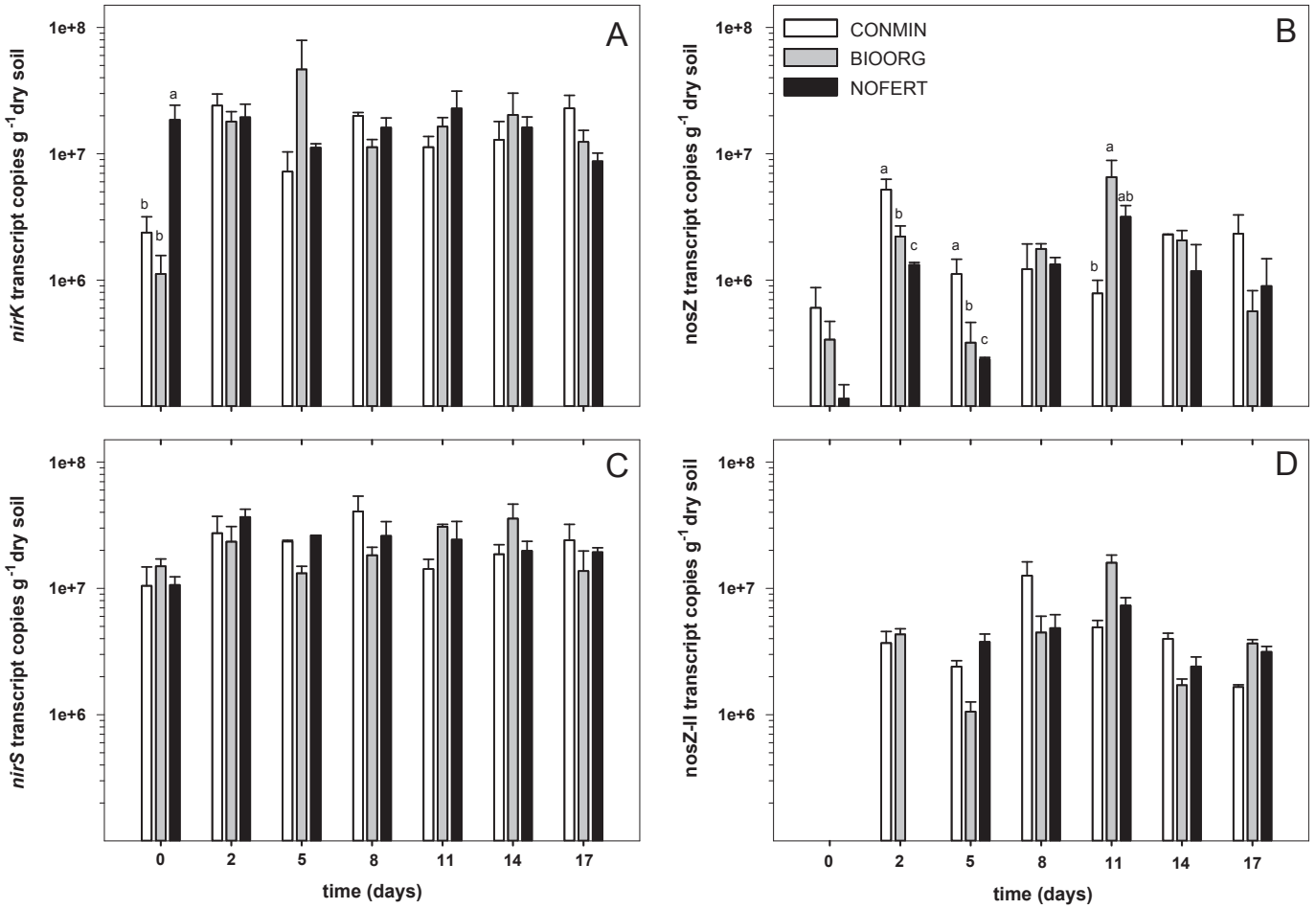

Fig. 4. Transcript copies of nitrite reductases (*nirK* and *nirS*, **A** and **C**) and nitrous oxide reductases (*nosZ* and *nosZ-II*, **B** and **D**) communities in soil samples originating from different farming systems (CONMIN- conventional, BIOORG – organic, NOFERT – unfertilized) after fertilization with $NH_4^{15}NO_3$. Letters indicate significant differences at a specific time point in between treatments at $P < 0.05$. Data points are means ± SE ($n = 3$). Lacking data point indicate transcript copies below detection limit in two or three replicates.

(2014) demonstrated that pH-induced dysfunctional assembly of the nitrous oxide reductase can affect whole communities. Since NOFERT did not receive any liming treatment throughout its management history since 1978, pH was significantly lower and most likely under the functional threshold that enables correct folding of the N_2O reductase. The agricultural practice of maintaining pH above 6 should therefore be considered as an important part of climate friendly farming systems. Unlike CONMIN, BIOORG also did not receive liming treatment throughout the management of the field trial. Nevertheless the pH stayed constant and the functionality of the nitrous oxide reductase was not inhibited. This suggests organic farming systems maintain functionality of N_2O reductase more effectively in the long run compared to conventional farming systems. Although NO_3^- consumption and N_2O emissions were enhanced due to increased SOC levels in BIOORG, increased N_2O fluxes in the field in organic farming systems seem unlikely considering the anomalous addition of NO_3^- in our setup. In fact, a recent meta study showed organic farming systems to decrease area scaled N_2O emissions due to lower inputs of available N (Skinner et al., 2014).

4.3. Influence of denitrifier abundance and activity on N_2O emission dynamics

The approach of predicting microbial processes with functional gene or transcript abundance is currently under debate as correlations between process rates and functional gene or transcript abundances are often missing (Bier et al., 2015; Rocca et al., 2015). In terms of denitrification, functional gene and transcript abundances had been studied extensively with variable results. While there are examples for studies that could successfully link denitrifier gene and/or transcript copies with N_2O emissions (Morales et al., 2010; Tatti et al., 2013; Harter et al., 2014; Chen et al., 2015), in other studies significant relations were missing (Miller et al., 2009; Henderson et al., 2010; Dandie et al., 2011). Therefore, experimental conditions need to be evaluated carefully for each experiment before comparing molecular data with process rates. In our case, C limitation most likely hampered growth of heterotrophic denitrifiers and thus differentiation of functional gene abundance in between farming systems was negligible. Consequently, mRNA analysis as a measure for functional activity becomes more important when comparing the impact of farming systems on denitrifying communities. Expression of *nirK* and *nirS* genes was rather stable, while *nosZ* and *nosZ-II* transcription levels fluctuated throughout the incubation time. This suggests increased susceptibility of *nosZ* and *nosZ-II* regulation to environmental factors compared to genes involved in nitrite reduction. Nevertheless, it needs to be noted that the primers used to quantify *nirK* and *nirS* genes only detect alpha-, beta- and gammaproteobacteria and other phylogenetic groups involved in nitrite reduction that were not accounted for in our analysis can significantly contribute to N_2O emissions (Wei et al., 2015). Still,

correlation between *nosZ* transcripts and N_2O emissions before day 5 ($r^2 = 0.81$) shows the potential use of mRNA analysis as predictor for N_2O emissions during increasing N_2O emission rates. In our case peak expression of *nosZ* genes in CONMIN and BIOORG were followed by increased N_2 emissions indicating the functional impact of increased *nosZ* transcription levels in these soils. This is in line with the study of Chen et al. (2015) who found the transcript of *nosZ* to be correlated with N_2 production in an incubation trial after NO_3^- and glucose addition. Similar to other studies abudance and/or expression of functional genes involved in N_2O reduction showed higher explanatory power for N_2O emission compared to genes involved in nitrite reduction (Chen et al., 2015; Krauss et al., 2016). Still, *nosZ* transcripts and N_2O emissions in our case did not correlate throughout the incubation period which emphasizes limited applicability of this approach and shows that gene expression cannot necessarily be translated to enzyme activity. Also the fact that pH affects N_2O reduction at a post-transcriptional level further limits the explanatory power of this analysis. Nevertheless, understanding environmental and regulatory factors involved in N_2O reduction is crucial for the development of effective N_2O mitigation strategies. While the expression of the denitrification genes is known to be regulated by O_2 and the concentrations of NO, NO_3^- and NO_2^- via a variety of different regulatory proteins (Zumft and Kroneck, 2007; Spiro, 2012) the regulation of *nosZ* seems to be decoupled from antecedent denitrifying enzymatic systems (van Spanning et al., 2007). For the denitrifying model organism *Paracoccus denitrificans* a combined upregulation of *nosZ* due to oxygen depletion (via NNR regulatory protein) and NO concentrations (via FnrP regulatory protein) was demonstrated (Bergaust et al., 2012) which could explain distinct *nosZ* transcription peaks in BIOORG and NOFERT. Yet, undetectable expression of *nosZ-II* at the beginning of incubation further illustrates that distinct evolution of nitrous oxide reductases as described by Sanford et al. (2012) is also reflected in regulation mechanics. There are several indications that these functionally equivalent enzymes are ecologically not redundant. The *nosZ-II* bearing and nitrate-ammonifying microbe *Wollinella succinogenes* was shown to mediate upregulation of *Nap*, *Nrf* and *Nos* genes via nitrosative stress regulator, while lacking the O_2 sensing regulatory proteins (Kern and Simon, 2015; Torres et al., 2016). A population response to high NO concentrations could explain delayed transcription of *nosZ-II* in our setup and would further stress the importance of *nosZ-II* bearing bacteria as a sink for N_2O as a consequence of nitrosative stress regulation. On a DNA level it was already shown that n*osZ-II* dominated microbial communities increase soil N_2O sink capacity (Jones et al., 2014). In our case, however, farming systems only significantly affected gene expression of the typical *nosZ* gene, suggesting differences in N_2O reduction to be mainly driven by this functional group.

5. Conclusions

In conclusion, we showed that at 90% WFPS increased SOC levels due to organic farming increased NO_3^- consumption and susceptibility for N_2O emissions, when NO_3^- is available in excess. Furthermore, pH seemed to the major determining factor for N_2O reduction since a low pH impedes the functionality of the nitrous oxide reductase. Therefore maintenance of a high pH seems to be a crucial part of climate friendly farming systems. The use of functional gene analysis as predictor for N_2O emissions was restricted to *nosZ* gene expression during emerging N_2O fluxes. Nevertheless, significant treatment effects of *nosZ* gene expression in this period stressed the importance of the N_2O reducing functional communities as a regulator of the N_2O sink.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.06.025.

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