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ORIGINAL PAPER

Tillage system affects fertilizer-induced nitrous oxide emissions

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Abstract Since the development of effective N_2O mitigation options is a key challenge for future agricultural practice, we studied the interactive effect of tillage systems on fertilizer-derived N_2O emissions and the abundance of microbial communities involved in N_2O production and reduction. Soil samples from 0–10 cm and 10–20 cm depth of reduced tillage and ploughed plots were incubated with dairy slurry (SL) and manure compost (MC) in comparison with calcium ammonium nitrate (CAN) and an unfertilized control (ZERO) for 42 days. N_2O and CO_2 fluxes, ammonium, nitrate, dissolved organic C, and functional gene abundances (16S rRNA gene, *nirK*, *nirS*, *nosZ*, bacterial and archaeal *amoA*) were regularly monitored. Averaged across all soil samples, N_2O emissions decreased in the order CAN and SL (CAN = 748.8 ± 206.3, SL = 489.4 ± 107.2 μg kg^{-1}) followed by MC (284.2 ± 67.3 μg kg^{-1}) and ZERO (29.1 ± 5.9 μg kg^{-1}). Highest cumulative N_2O emissions were found in 10–20 cm of the reduced tilled soil in CAN and SL. N_2O fluxes were assigned to ammonium as source in CAN and SL and correlated positively to bacterial *amoA* abundances. Additionally, *nosZ* abundances correlated negatively to N_2O fluxes in the organic fertilizer treatments. Soils showed a gradient in soil organic C, 16S rRNA, *nirK*, and *nosZ* with greater amounts in the 0–10 than 10–20 cm layer. Abundances of bacterial and archaeal *amoA* were higher in reduced tilled soil compared to ploughed soils. The study highlights that tillage system induced biophysicochemical stratification impacts net N_2O emissions within the soil profile according to N and C species added during fertilization.

Keywords Nitrous oxide · Nitrification · Denitrification · Fertilization · Reduced tillage · Soil organic carbon

Maike Krauss and Hans-Martin Krause contributed equally to this work.

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Introduction

Nitrous oxide (N_2O) is a major greenhouse gas and the predominant ozone depleting substance in the stratosphere, estimated to account for 6 % of global warming (Montzka et al. 2011; Ravishankara et al. 2009). Concentration of atmospheric N_2O has risen by 20 % since the preindustrial period mostly due to anthropogenic interventions in the N cycle (Davidson 2009). Around 60 % of anthropogenic N_2O emissions originate from microbial processes in agriculturally managed soils (Syakila and Kroeze 2011). To develop effective mitigation strategies for N_2O emissions, a detailed understanding of microbial responses on agricultural management practices is needed (Venterea et al. 2012). Reduced tillage (RT) or no tillage (NT) practices are widely used in cereal-based cropping systems due to their beneficial effects regarding the prevention of soil erosion and water conservation (Derpsch et al. 2010; Powlson et al. 2014). Stratification of soil organic matter increases with reduced tillage intensity (Luo et al. 2010) which

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impacts soil physicochemical properties like soil aeration and C availability within the soil profile. This can affect microbial community composition and functioning (Wallenstein et al. 2006). For reduced tillage, changes in PLFA profiles were already reported for bacteria, archaea, and fungi (Kuntz et al. 2013). Studies investigating the impact of tillage systems on N cycling microbial communities in relation to N_2O emissions are however scarce and mainly focus on denitrifiers solely (Baudoin et al. 2009; Melero et al. 2011). In relation to fertilizer-induced N_2O emissions, knowledge is lacking about nitrifiers affected by tillage system, but is important as many fertilizers are ammonium based.

Fertilizer types can influence N_2O emissions due to different N species (NH_4^+, NO_3^-, and N_{org}) and amounts of available C added (Butterbach-Bahl et al. 2013). In a simplified view, ammonium (NH_4^+) is the major source for N_2O emissions via nitrification under oxic conditions while under suboxic conditions N_2O is mostly produced by reduction of nitrate (NO_3^-) in the process of denitrification. Under moderate moisture conditions, both denitrification and nitrification appear simultaneously in different microsites (Butterbach-Bahl et al. 2013). Denitrification thereby shows a higher potential for N_2O production as N_2O is an obligate intermediate during this process. Furthermore, the addition of C during organic fertilization was repeatedly shown to increase denitrification and N_2O emissions (Flessa and Beese 2000). In contrast, C addition under nitrate shortage was also shown to promote N_2O reduction to dinitrogen (N_2), thereby lowering N_2O emissions (Miller et al. 2009; Senbayram et al. 2012; van Groenigen et al. 2004).

To assess the abundance of functional communities involved in nitrification and denitrification, functional gene quantification via qPCR presents the most widely used approach. Bacterial and archaeal *amoA* are used as marker genes for nitrification, while *nirK* and *nirS* genes are often used to assess denitrifier abundance (Philippot et al. 2011). The *nosZ* gene serves as a marker for nitrous oxide reduction, the only known process that acts as a sink for N_2O (Butterbach-Bahl et al. 2013). As not all denitrifier possess the complete set of denitrifying enzymatic systems, the genetic potential to reduce N_2O and the N_2O/N_2 product ratio also depends on denitrifier community composition (Domeignoz-Horta et al. 2015; Graf et al. 2014). Especially the recently discovered clade of bacteria bearing atypical *nosZ* genes were found to lack antecedent denitrifying enzymatic systems (Jones et al. 2014).

Although the overall impact of reduced tillage systems on N_2O emissions based on annual budgets is reported to be similar to plowing systems and emissions even tend to decrease when RT is applied in the long run (Rochette 2008; Six et al. 2004; van Kessel et al. 2013), tillage systems may still respond differently to mitigation options. For example, fertilization methods offer an opportunity for system optimization. Banded placement of mineral fertilizers at depths >5 cm lowered N_2O emissions significantly under NT/RT (van Kessel et al. 2013). However, few studies exist regarding the impact of organic fertilizer types and their placement on N_2O emissions under contrasting tillage strategies. As various techniques exist for the application of organic fertilizers, stratification of soil microbial communities as a result of tillage system change could affect N_2O emissions.

The objective of this study was therefore to gain basic knowledge about N_2O processes for fertilizers with different composition of N and C species in two tillage systems and soil depths. We therefore used laboratory experiments to simulate a fertilizer application to a clayey soil from the long-term organic tillage trial in Frick, Switzerland (Berner et al. 2008; Gadermaier et al. 2012). We hypothesized that (1) tillage systems affect soil properties and abundance of N cycling microbial communities within the soil profile, (2) fertilizer types determine N_2O emissions and predominant N cycling processes, and (3) tillage system–induced changes in biophysicochemical soil properties affect N_2O emissions in dependency of fertilization strategy.

Materials and methods

Site conditions, and soil and fertilizer sampling

Soil samples were taken from the long-term organic tillage trial in Frick, Switzerland (47°30′N, 8°1′E, 350 m a.s.l.). Tillage treatments include plowing to a depth of 15–18 cm (CT) and reduced tillage with a skim and chisel plough (RT) to 5–10 cm. The soil was classified as Vertic Cambisol with a texture of 45 % clay, 33 % silt, and 22 % sand. Samples from the upper (0–10 cm) and lower (10–20 cm) topsoil were taken across all four field replicates in March 2013, homogenized, air-dried to a gravimetric water content of 17.9 (±0.9) %, and sieved to 5-mm aggregates before storage at 4 °C. In order to assess the impact of fertilizer type on N_2O emissions and N cycling microbial communities, two organic fertilizers also used in the field trial, liquid dairy slurry (SL) and dairy manure compost (MC, stable manure composted for 18 weeks), were compared with calcium ammonium nitrate (CAN, 27 % N) and an unfertilized control (ZERO). Basic physicochemical properties and nutrient contents of soils and fertilizers are given in Tables 1 and 2.

Experimental setup

After preincubation for 1 week at room temperature, the equivalent of 100 g dry soil was filled in 250 ml DURAN wide neck glass bottles (Schott AG, Mainz, Germany) which served as microcosms. The soil aggregates were evenly compacted to a bulk density of 1.25 g cm^{-3}. In order to mimic a moderate fertilization event, fertilizer N addition was

Table 1 Means and standard deviations of C and N contents of fertilizers ($n = 4$)

	Dry matter		Total C	Dissolved organic C	Total N	Nitrate	Ammonium	Organic N	C/N	pH (H_2O)
Fertilizer	%	mg applied	mg applied		mg applied					
Calcium ammonium nitrate (CAN)	100	41.0	–	–	11.07[a]	5.54[a]	5.54[a]	–	–	7.81 (0.01)
Slurry (SL)	2.43	198.9	63.92 (1.31)	7.95 (0.54)	11.02[b]	–	3.33 (0.02)	7.69 (0.18)	5.8	7.34 (0.01)
Manure compost (MC)	20.02	284.3	89.52 (3.66)	3.63 (0.41)	11.05[b]	3.00 (0.07)	0.01 (0.01)	8.04 (0.07)	8.1	8.09 (0.01)

Inputs refer to the amount of fertilizer applied to each microcosm

[a] Total N refers to the manufacture specifications of 27 % N as ammonium nitrate

[b] Total N was calculated as the sum of nitrate, ammonium, and N_{org}

normalized to 35 kg N ha^{-1} (11 mg N_t per microcosm). Slurry (SL), CAN solution, and H_2O_{demin} (ZERO) were evenly spread superficially in its liquid form, while particles of fresh manure compost were homogenized with the dry soil before compaction. This procedure assured homogeneous physicochemical soil conditions in all treatments to focus on the reaction of microbial communities under simulated conditions. Water-filled pore space (WFPS) was adjusted to 60 % to account for moisture conditions during fertilization in the field. Microcosms were incubated at constantly 20 °C in the dark in a completely randomized order. Swelling of soil samples due to the high clay content and loss of water during incubation were compensated by added H_2O_{demin} on a daily base. Analysis of greenhouse gases (N_2O, CO_2) was carried out daily within the first week after fertilizer application and weekly thereafter for 42 days. Parallel microcosm sets were set up and stored in the same way and destructively sampled for soil analysis after 1, 3, 7 and 42 days of incubation.

Physicochemical analysis

Soil pH was determined in a 1:2.5 (*w*/*v*) dilution with H_2O_{demin}. Soil organic C (SOC) and fertilizer C_t (60 °C dried samples) were analyzed by dry combustion (multi N/C2100S + HT1300; Analytik Jena AG, Jena, Germany). SOC was determined by the subtraction of 105 °C (C_t) by 500 °C (inorganic C) treated and then at 1000 °C combusted samples. Total soil N (N_t) was also determined by combustion (CN Vario Max; Elementar Analysensysteme GmbH, Hanau, Germany). Microbial biomass C and N (C_{mic}, N_{mic}) were assessed with the chloroform fumigation extraction method with 0.5 M K_2SO_4 as described in Fließbach et al. (2007). Dissolved organic C (DOC) was extracted using 0.01 M $CaCl_2$ filtered through a 0.45-μm membrane filter (Porafil® CM; Macherey-Nagel, Düren, Germany) with a vacuum device (SM; Sartorius AG, Göttingen, Germany). Extracts were determined with a TOC/TNb analyzer (DIMA-TOC 100; Dimatec Analysentechnik GmbH, Essen, Germany).

Kjehldahl wet digestion (2020 Digestor; Foss Tecator AB, Höganäs, Sweden) was employed to quantify organically bound N (N_{org}) in both organic fertilizers. Ammonium contents in liquid slurry were analyzed by direct distillation (Büchi 315; Büchi AG, Flawil, Switzerland) whereas ammonium and nitrate of fresh manure compost and soil samples were determined by $CaCl_2$ (0.01 M $CaCl_2$ at 1:4 *w*/*v*) extraction. After filtration (MN 619EH; Macherey-Nagel, Düren, Germany), ammonium and nitrate contents were determined spectrophotometrically (SAN-plus Segmented Flow Analyzer; Skalar Analytical B.V., Breda, Netherlands).

Table 2 Means and standard deviations of physicochemical properties for soil samples from conventional (CT) and reduced tillage (RT) and two soil depths (0–10, 10–20 cm) before incubation ($n = 4$)

	Soil organic C (g kg^{-1})			Microbial biomass C (mg kg^{-1})			Total N (g kg^{-1})			Microbial biomass N (mg kg^{-1})			pH (H_2O)		
Treatment	Tillage^{ns}, depth***			Tillage†, depth***			Tillage***, depth***			Tillage**, depth**			Tillage*, depth***		
CT, 0–10 cm	26.82	b	(0.10)	667.9	b	(12.7)	2.87	b	(0.05)	62.65	bc	(3.42)	7.22	ab	(0.09)
CT, 10–20 cm	24.04	c	(0.32)	581.8	c	(22.2)	2.67	c	(0.05)	58.95	c	(2.90)	7.24	a	(0.03)
RT, 0–10 cm	28.56	a	(0.39)	892.0	a	(52.7)	3.30	a	(0.04)	76.57	a	(4.23)	7.10	b	(0.02)
RT, 10–20 cm	22.22	d	(0.14)	655.5	b	(7.6)	2.65	c	(0.06)	65.58	b	(1.78)	7.22	a	(0.01)

Significant differences (ANOVA) within tillage and depth factors are indicated in the headline. Values with different letters are statistically different at $p < 0.05$ (Tukey test). Level of significance for tillage and depth factors: †$p < 0.1$, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, *ns* not significant

Greenhouse gas analysis

Constant temperature conditions during GHG sampling were assured by a temperature-controlled tray (20 °C) directly placed at an autosampler (MPS 2XL; Gerstel AG, Sursee, Switzerland). Microcosm headspaces were gently fanned and sealed with a lid containing a rubber septum before sampling. Three gas samples of 5 ml were directly taken every 20 min and analyzed by gas chromatography (7890A; Agilent Technologies, Santa Clara, CA). To avoid a vacuum effect, 5 ml of helium gas was injected and mixed in the microcosm headspace prior to sampling. CO_2 concentrations were determined with a flame ionization detector (FID) and N_2O with an electron capture detector (μECD).

Molecular analysis

DNA extraction of soil samples was performed using Fast DNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the instructions given by the manufacturer. Quality and quantity of DNA extractions were determined spectrophotometrically (NanoDrop 2000 UV–vis Spectrometer; Thermo Fisher Scientific, Wilmington, DE, USA). Yields of extracted DNA ranged from 73.0 to 138.4 ng/μl, and no treatment specific bias was detected. Functional genes were quantified using SYBR green approach (Kapa SYBR® Fast qPCR Kit Master Mix (2×) Universal; Kapa Biosystems, Wilmington, MA) on a Rotor-Gene Q platform (Rotor-Gene Q; QIAGEN, Venlo, Netherlands). Master Mix compositions, temperature profiles, and gene specific primers are listed in Supplement Table S1 and S2. For qPCR analysis, biological triplicates were used, of which each sample was analyzed twice. Measurement of a sample was repeated when C_t values differed by more than 0.5. In each qPCR run, negative controls as well as a serial dilution of plasmids containing a fragment of the respective target gene were included. Concentration of standard plasmids was determined spectrophotometrically (NanoDrop 2000 UV–vis Spectrometer; Thermo Fisher Scientific, Wilmington, DE, USA) and gene copy numbers of standard curves (ranging from 10^1 to 10^8 gene copies/μl) were calculated using molecular weight of the standard plasmids according to Behrens et al. (2008). For each gene, a joined standard curve was constructed with C_t values from the serial dilution of standard plasmid from six independent qPCR runs. Efficiencies of qPCR reactions ranged from 88 to 96 % for bacterial *amoA* (AOB), 91–99 % for archaeal *amoA* (AOA), 92–99 % for *nosZ*, 88–90 % for *nir*K, 92–95 % for *nirS*, and 92–97 % for 16S rRNA gene. R^2 was above 0.999 for all qPCR runs.

Data transformation and statistics

All data preparation and statistical analyses were performed in R (R Core Team 2013). Gas fluxes were calculated using a linear model considering the He dilution. Cumulative gas emissions (μg kg^{-1}) were integrated according to the trapezoidal integration method Eq. 1:

$$\text{cumulative } flux = \Sigma_i^n (t_{i+1} - t_i) * \left(f_i + f_{i+1}\right)/2 \quad (1)$$

with t = sampling time (h) and f = gas flux (μg kg^{-1} h^{-1}) and n = number of sampling dates.

Treatment effects on initial soil and gene data (ANOVA) as well as linear regressions were assessed with a linear model. Log-transformed cumulative gas data were assessed with a linear mixed effect model using the nlme package with microcosm replicates as random effect (Pinheiro et al. 2014). Post hoc pairwise comparisons (Tukey test) were calculated with the multcomp package (Hothorn et al. 2008). Linear regressions of physicochemical and gene time series data with N_2O-N fluxes were calculated with generalized least square models considering the temporal autocorrelation in a compound symmetry correlation structure. Normality and homoscedasticity of residuals were assessed graphically.

Results and discussion

Effects of tillage system on soil biophysicochemical parameters

Stratification of soil organic C and N was more pronounced in RT compared to CT (Table 2) in line with results of a recent meta-analysis (Luo et al. 2010). In RT, soil organic C and total N contents in the upper soil layers were 28.5 and 24.5 % higher, respectively, compared to the lower soil layers. In CT, the increase accounted only for 11.5 and 7.5 %, respectively (Table 2). The overall effect of tillage system was significant for total N but not for soil organic C content as soil organic C was 6.5 % higher in the upper soil layer but 7.6 % lower in the lower soil layer in RT compared to CT. Microbial biomass showed a significant effect of soil depth and tillage system with higher concentrations in the upper soil layer and the RT system. Microbial C and N showed highest concentrations in the upper soil layer under RT (892.0 and 76.57 mg kg^{-1}) and lowest concentrations in the lower soil layer under CT (581.8 and 58.95 mg kg^{-1}) (Table 2). While an increase of microbial biomass in the upper soil layers due to reduced tillage intensity was regularly reported (Heinze et al. 2010; Kaurin et al. 2015), a generally increased microbial biomass under RT, although occasionally observed (Jacobs et al. 2009), seems not to be a normal case (van Capelle et al. 2012).

Soil depth and tillage system affected the abundance of functional gene markers for nitrification and denitrification differently. While ammonium oxidizing archaea (AOA) and ammonium oxidizing bacteria (AOB) were significantly more abundant under RT and hardly affected by soil depth, the opposite was true for most functional gene markers involved in denitrification. Here, significant effects of soil depth were found for *nirK* and typical *nosZ* but not for *nirS* abundances (Table 3). However, it should be noted that the primer pairs we used for *nirK* and *nirS* quantification are limited to alpha-, beta-, and gamma-proteobacteria and do not cover all phylogenetic groups detected by recently established primers (Wei et al. 2015). Similar to our study, declining abundance of denitrifiers with the increase of soil depth had been observed across a variety of agroecosystems (Boz et al. 2013; Marhan et al. 2011; Melero et al. 2011; Regan et al. 2011). Only abundance of *nirK* bearing denitrifiers was affected by tillage system with 148 and 143 % increased gene copy numbers under RT in the upper and lower soil layer. This confirms increased denitrifier abundances as observed elsewhere for no-till (Baudoin et al. 2009; Melero et al. 2011; Tellez-Rio et al. 2015) and minimum tillage systems (Tellez-Rio et al. 2015). 16S rRNA gene copy numbers were significantly increased under RT and in the upper soil layers. 16S rRNA gene copy numbers declined in the order RT 0–10 cm (9.1×10^9) and CT 0–10 cm (7.4×10^9), RT 10–20 cm (6.4×10^9) and CT 10–20 cm (5.8×10^9) confirming results from microbial biomass data. Abundances of denitrifiers were highly collinear to 16S rRNA gene copy numbers and also correlated to soil organic C contents (Supplementary Table S3). It was shown that AOA dominate in agriculturally managed soils with AOA/AOB ratios of up to 232 (Leininger et al. 2006). In our study, AOA also exceeded AOB abundances by more than one order of magnitude, with mean AOA/AOB ratios of 39 and 8 for CT and RT, respectively. RT thereby enhanced abundances of AOA (+65 % in 0–10 cm and +55 % in 10–20 cm) and AOB (+60 % in 0–10 cm and +38 % in 10–20 cm) compared to CT. In line with our data, enhanced AOA and AOB abundances in no-till had been observed in a paddy rice system (Li et al. 2015). We have found no studies on the long-term impact of reduced tillage systems on nitrifying guilds in aerobically managed agricultural soils. Yet, higher contents of mineralizable N in topsoils was frequently reported for NT in comparison with ploughing systems (Balesdent et al. 2000) which suggests an enhanced NH_4^+ supply and the promotion of nitrifier abundances.

Impact of fertilizer type on N_2O emissions and abundance of N cycling microbial communities

In our setup, fertilization showed a greater impact on cumulative N_2O emissions and N_2O fluxes compared to tillage system which, however, showed an interactive effect. Discussing

Table 3 Means and standard deviations of the general bacterial marker gene 16S rRNA and the functional genes *amoA* (bacterial and archaeal), *nirK*, *nirS*, and *nosZ* for soil samples from conventional (CT) and reduced tillage (RT) and two soil depths (0–10, 10–20 cm) before incubation ($n = 3$)

	16S rRNA (copies g^{-1})		Archaeal *amoA* (AOA) (copies g^{-1})		Bacterial *amoA* (AOB) (copies g^{-1})		*nirK* (copies g^{-1})		*nirS* (copies g^{-1})		*nosZ* (copies g^{-1})	
Treatment	Tillage*, depth*		Tillage*, depth^{ns}		Tillage***, depth^{ns}		Tillage*, depth*		Tillage^{ns}, depth^{ns}		Tillage^{ns}, depth**	
CT, 0–10 cm	7.4×10^9	ab	(3.6×10^8)	b	(1.1×10^8)	b	(5.8×10^5)	b	(1.4×10^7)	a	(1.9×10^8)	a
CT, 10–20 cm	5.8×10^9	b	(5.1×10^8)	ab	(1.3×10^8)	b	(2.9×10^5)	b	(1.3×10^6)	a	(1.3×10^8)	ab
RT, 0–10 cm	9.1×10^9	a	(1.4×10^9)	ab	(7.0×10^7)	a	(1.5×10^6)	a	(2.9×10^7)	a	(3.9×10^8)	a
RT, 10–20 cm	6.4×10^9	b	(8.3×10^8)	a	(5.8×10^7)	a	(2.3×10^6)	a	(7.7×10^6)	a	(2.6×10^7)	a
	3.0×10^8		(1.9×10^8)		1.9×10^8		(8.9×10^6)					
	3.2×10^8		(1.3×10^8)		1.5×10^8		(5.4×10^6)					
	4.7×10^8		(3.9×10^8)		1.8×10^8		(2.1×10^7)					
	2.4×10^8		(2.6×10^7)		1.2×10^8		(2.3×10^7)					

Significant differences (ANOVA) within tillage and depth factors are indicated in the headline. Values with different letters are statistically different at $p < 0.05$ (Tukey test). Level of significance for tillage and depth factors: $^{\dagger}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$, *ns* not significant

fertilizer impacts on N_2O emissions and predominant N-transforming processes first is therefore a prerequisite to evaluate implications of tillage systems later on.

Averaged per fertilizer treatment and highest cumulative N_2O emissions over the 42-day period were observed in CAN and SL (CAN = 748.8 ± 206.3, SL = 489.4 ± 107.2 μg kg^{-1}) followed by MC (284.2 ± 67.3 μg kg^{-1}) in contrast to ZERO (29.1 ± 5.9 μg kg^{-1}) (Fig. 1). In a similar incubation study with 65 % WFPS, the same trend in cumulative N_2O emissions (CAN = 2.7, organic cattle slurry = 2.4, and ZERO = 0.6 mg N_2O-N kg^{-1} soil, 98 days) on a sandy soil has been found (Velthof et al. 2003). Thus, ammonium addition induced more climate-relevant N_2O emissions than nitrate application under oxic conditions. Additionally, increasing nitrate concentrations in all fertilization treatments suggest nitrification to be the predominant N transforming process under the chosen conditions.

In SL and CAN, N_2O fluxes correlated positively to decreasing ammonium concentrations and AOB abundances (Table 4). This highlights ammonium oxidation as controlling

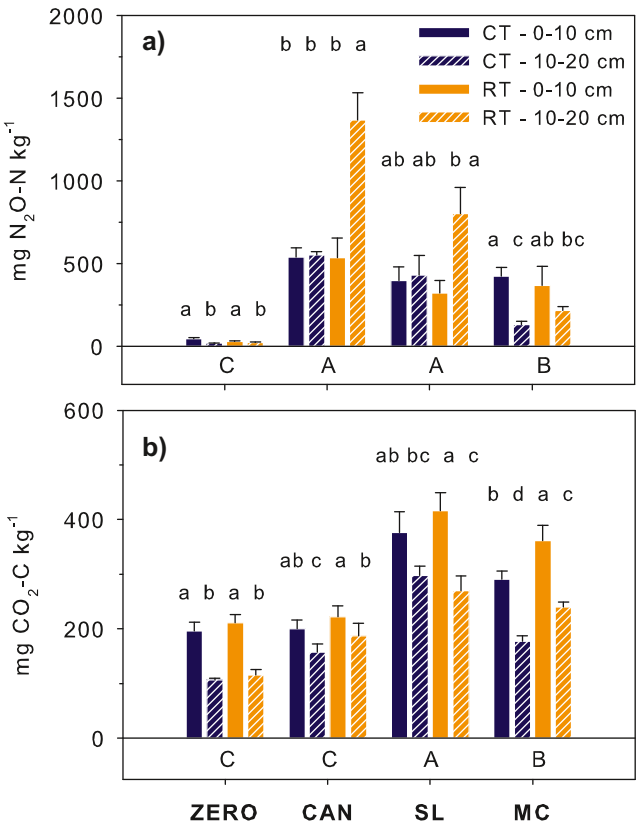

Fig. 1 Cumulative emissions of (**a**) N_2O-N and (**b**) CO_2-C of soil samples from conventional tillage (*CT*) and reduced tillage (*RT*) systems and two soil depths (0–10, 10–20 cm) after application of demineralized water (*ZERO*), calcium ammonium nitrate (*CAN*), slurry (*SL*), and manure compost (*MC*) during 42 days of incubation. *Bars* represent means and standard errors (n = 4). *Capital letters* indicate significant differences between and *small letters* within fertilizer treatments (ANOVA, Tukey test, $p < 0.05$)

factor for N_2O fluxes after addition of ammonium and confirms the findings of Di et al. (2009) who suggested AOB rather than AOA to drive ammonium oxidation rates under ammonium excess. In accordance, Kool et al. (2011) demonstrated ammonium to be the major source of N_2O emissions at 50 and 70 % WFPS through the processes of nitrification and nitrifier-denitrification. Both processes likely occurred also in our case although we could not distinguish them in our setup. In contrast to AOB, AOA abundances correlated positively to N_2O fluxes in ZERO but negatively in SL and CAN (Table 4). Furthermore, an increased growth of AOA in SL was detected compared with CAN (Supplement Fig. S1). This further suggests AOA growth rather to be attributable to N_{org} addition, as already proposed by Taylor et al. (2012). For the abundances of *nirK* and *nirS* bearing bacteria, we could observe few significant relationships with N_2O fluxes. Miller et al. (2009) investigated denitrifier abundances after application and could not find significant relationships with N_2O fluxes. Similar to our study, missing relationships between N_2O fluxes and the abundance of denitrifying communities might be caused by the limited phylogenetic diversity covered by the used primers (Wei et al. 2015).

After organic fertilization in SL and MC, we observed an increase in typical *nosZ*-bearing bacteria that significantly negatively correlated to N_2O fluxes (Table 4). This correlation indicates that denitrifiers with the genetic potential to reduce N_2O played a major role in determining net N_2O fluxes after organic fertilization. This is most likely linked to increased availability of C substrates and the formation of anoxic microsites after organic fertilizer addition by increased soil respiration (Butterbach-Bahl et al. 2013; Miller et al. 2009). Significantly elevated CO_2 emissions in SL and MC compared with ZERO and CAN add to this interpretation (Fig. 1). Also, faster declining N_2O fluxes in SL compared to CAN support the hypothesis of increased N_2O reduction due to organic fertilization.

In MC, about 30 mg nitrate-N kg^{-1} were added to each microcosm (Table 2). Still, nitrate contents after 1 day of incubation did not significantly rise above initial background concentrations (28.4 ± 2.4 mg NO_3-N kg^{-1} across all soil samples, Fig. 2). This suggests instant denitrification or immobilization of manure-derived nitrate in MC despite prevailing nitrifying conditions. The massive but short-lived N_2O peaks directly after fertilization further hint towards denitrification as a major N_2O-producing process in MC (Fig. 2). Significant negative correlation of N_2O fluxes with changes in *nirS* and typical *nosZ* abundances in MC (Table 5) may represent growth of respective heterotrophic microorganisms after N_2O emissions terminated and may be associated with the addition of C-rich material. Generally, N_2O produced in the course of denitrification can exceed those of nitrification by some orders of magnitude (Braker and Conrad 2011; Canfield et al. 2010). This might explain highest N_2O flux rates in MC

Table 4 Regressions of gas fluxes with soil biophysicochemical properties during the 42 days of incubation after application of demineralized water (ZERO), calcium ammonium nitrate (CAN), slurry (SL), and manure compost (MC)

	Coefficients (β) and significance levels (F test)											
	CO_2-C		N_2O-N									
Treatment	16S rRNA		Ammonium		Archaeal *amoA* (AOA)		Bacterial *amoA* (AOB)		*nirK*		*nirS*		*nosZ*	
ZERO	3.9×10^{-8}	***	0.004	ns	1.3×10^{-10}	**	1.5×10^{-9}	ns	1.4×10^{-11}	ns	6.7×10^{-10}	ns	4.2×10^{-11}	ns
CAN	2.0×10^{-8}	*	1.26	**	-1.9×10^{-8}	†	1.9×10^{-7}	†	-1.0×10^{-8}	†	-1.8×10^{-7}	†	-2.4×10^{-8}	ns
SL	-1.2×10^{-7}	***	1.73	***	-4.0×10^{-9}	*	2.6×10^{-7}	*	-3.2×10^{-9}	ns	-9.8×10^{-8}	ns	-3.5×10^{-8}	*
MC	-4.6×10^{-9}	ns	1.58	ns	-2.1×10^{-9}	ns	-2.2×10^{-8}	ns	5.6×10^{-9}	ns	-4.9×10^{-7}	**	-9.9×10^{-8}	*

Timelines of CO_2-C fluxes were correlated with 16S rRNA gene abundances and N_2O-N fluxes with soil ammonium concentrations and functional gene abundances across all soil samples and for each fertilization treatment. The temporal correlation was considered in the generalized least square model. Level of significance: †$p < 0.1$, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, *ns* not significant

compared to the other fertilization treatments reaching up to 23.8 μg N_2O-N kg^{-1} h^{-1} in the first hours of incubation.

Whether functional gene quantification can be linked to process rates is currently debated (Bier et al. 2015; Rocca et al. 2015). Although relationships between functional gene abundances and process rates are not straightforward, a recent meta-study showed that provision of nutrients by fertilization increased reliability of functional gene analysis as an indicator for process rates in the agricultural context (Rocca et al. 2015). By time series regression, we could link N_2O fluxes to changes in abundances of AOB after addition of ammonium, while abundances of typical *nosZ*-bearing bacteria were significantly correlated to N_2O fluxes after the addition of organic fertilizers. This shows that fertilizer type not only affects N_2O fluxes but also the abundance of N-transforming microbial communities.

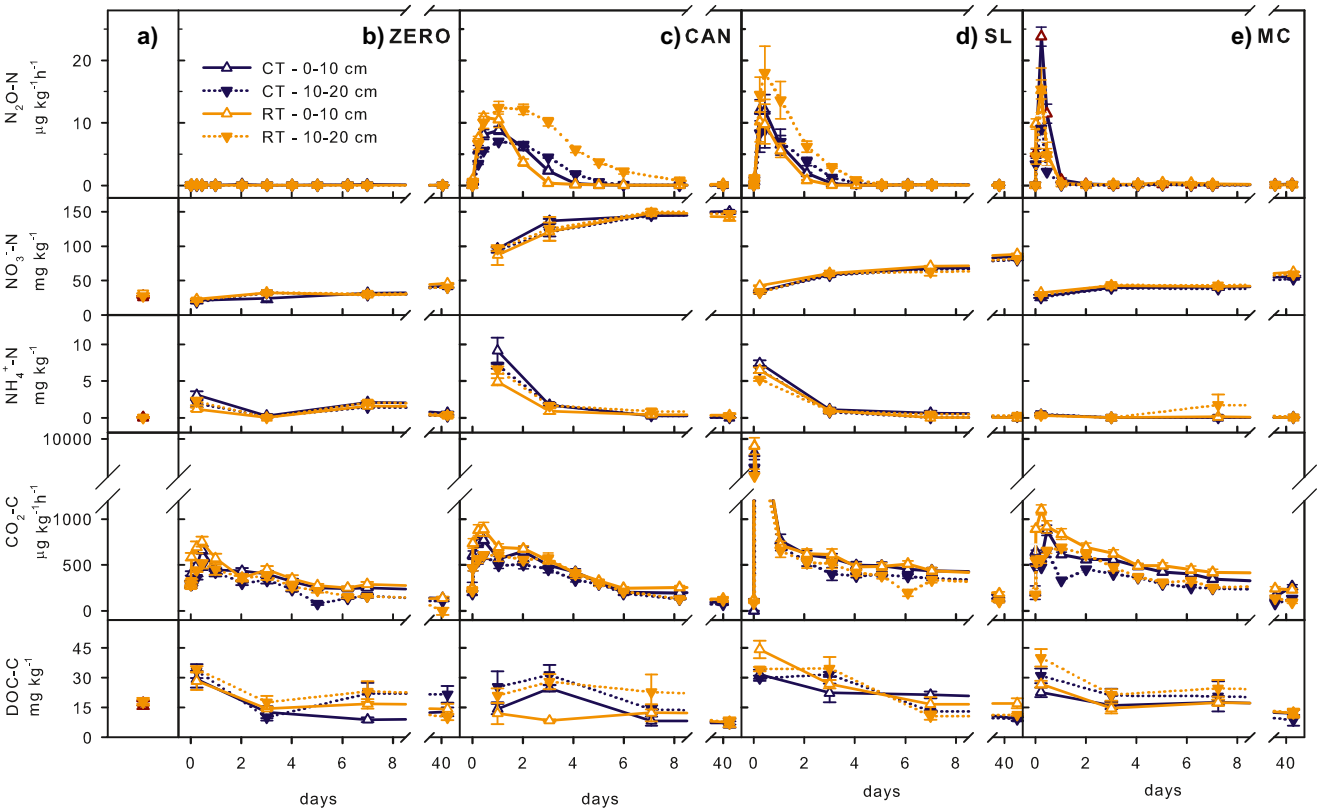

Fig. 2 Fluxes of N_2O-N, soil nitrate (NO_3^--N) and ammonium (NH_4^+-N) contents, CO_2-C fluxes, and dissolved organic C (DOC) contents of soil samples from conventional tillage (CT) and reduced tillage (RT) systems and two soil depths (0–10, 10–20 cm). Panel (**a**) shows soil physicochemical parameters before incubation. Panels (**b**)–(**e**) show soil physicochemical parameters, N_2O-N and CO_2-C emissions after application of demineralized water (*ZERO*), calcium ammonium nitrate (*CAN*), slurry (*SL*), and manure compost (*MC*) during 42 days of incubation. *Error bars* show the standard error of the mean of each treatment ($n = 4$)

Table 5 Linear regression of cumulative CO_2-C (mg kg^{-1} soil) and N_2O-N (μg kg^{-1} soil) emissions with initial soil organic C contents (g kg^{-1} soil) for each fertilization treatment

	Coefficients (β), significance levels (*F* test) and R^2					
Treatment	CO_2-C			N_2O-N		
ZERO	17.9	***	0.73	2.3	ns	0.17
CAN	7.0	†	0.18	−111.7	*	0.46
SL	23.9	**	0.56	−65.9	*	0.36
MC	22.5	**	0.56	36.9	*	0.30

Treatments include the application of demineralized water (ZERO), calcium ammonium nitrate (CAN), slurry (SL), and manure compost (MC). Level of significance: †$p < 0.1$, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, *ns* not significant

Tillage system–induced stratification in biophysicochemical soil properties affects N_2O emissions in dependency of fertilization strategy

Enhanced soil respiration was observed in the upper compared to the lower soil layers regardless of fertilizer treatment. An effect of tillage system was only detected in MC with 24.6–35.4 % higher cumulated CO_2 emissions in RT compared with CT in the upper and lower soil layers, respectively (Fig. 1). Cumulative soil respiration was therefore positively correlated to soil organic C contents (Table 5). Across all fertilization treatments, dynamics of nitrate concentration as an indicator of ongoing net nitrification did not differ between soils during incubation (Fig. 2). Increased nitrifier abundances in RT were thus not directly translated into a higher net nitrification. Yet, average cumulative N_2O emissions were 8 % lower in the 0–10 cm layer and 81 % higher in the 10–20 cm layer of RT compared with CT. This effect was far greatest in CAN and SL with significantly higher cumulative N_2O emissions in the lower soil layer in RT compared to all other soils (Fig. 1). In addition, cumulative N_2O emissions correlated significantly with soil organic C contents in the fertilized treatments (Table 5). This correlation was positive for MC and negative for CAN and SL. N_2O emissions in ZERO were too low to show a distinct effect. These observations suggest that the long-term effect of tillage systems on C distribution and microbial communities within the profile influenced fertilizer-induced soil respiration and related heterotrophic processes more than nitrification. Ammonium-derived N_2O emissions were therefore conversely affected rather than nitrate-derived N_2O emissions in our experimental setup.

Positive correlation in MC can be explained by heterotrophic activity and denitrification due to fertilizer C addition besides soil organic C availability and the affiliated higher abundance of denitrifiers in the respective soil layers. As cumulative N_2O emissions cannot be explained by differing response of nitrification between soil layers in CAN and SL, negative correlation with soil organic C is a hint towards an increased N_2O reduction in C- and denitrifier-rich layers. The prolonged phase of N_2O fluxes after ammonium addition in the lower soil layer in RT also suggests lower N_2O reduction. N_2O fluxes thereby lasted 3 days longer in CAN compared with SL (Fig. 1). Provision of labile C in SL seemed to enhance N_2O reduction in addition. The fact that the tillage-induced soil organic C effect on N_2O emissions was not entirely masked by the addition of labile fertilizer C emphasizes the important role of soil organic C on N_2O formation. This was not reported yet for tillage systems but for long-term fertilization experiments. For sandy and C-poor soils, no soil organic C impact on N_2O emissions was reported (Jaeger et al. 2013). In contrast, a long-term fertilization effect was found for silt loam soils where increased soil organic C contents enhanced denitrification rates, such as in our case (Dambreville et al. 2006; Tatti et al. 2013). The marked C effect in our study could therefore be associated with the high clay content and associated high soil organic C concentrations (22–28 g kg^{-1}). Clay soils are known to have a high potential of binding carbon (von Luetzow et al. 2006) which was mirrored by a fast soil organic C accumulation in this soil already after some years of management change (Gadermaier et al. 2012). Besides the impact of tillage systems on soil organic C stratification, other specifically tillage-related biochemical effects could explain the marked differences between tilled and untilled soil layers. It was shown that tillage operations disrupt soil aggregates, increasing soil organic matter accessibility for microorganisms and creating new surfaces for microbial colonization (von Luetzow et al. 2006; Wiesmeier et al. 2014). Vogel et al. (2014) found hotspots for microbial activity to be located at existing colonized organic-mineral complexes. Tilled soil layers may have therefore provided better conditions for denitrifiers and N_2O reduction than the untilled lower soil layer in RT. Our experimental setup therefore offered the opportunity to track the influence of tillage systems on fertilizer-induced N_2O emissions with regard to a range of microbial and physicochemical soil properties. Under real field conditions, soil physical conditions like, e.g., constraints in diffusion (Petersen et al. 2008) will additionally regulate microbial N_2O production and reduction.

Conclusions

Our study showed the interactive effect of tillage system impact on soil properties on fertilizer-induced N_2O emissions. It is one of the first studies that detected higher nitrifier abundances in reduced compared to ploughed soils. Soil organic C and fertilizer C and N species helped explain net N_2O emissions within the soil profile, while functional gene abundances partly explained microbial processes. Nitrification was shown to be an important driver of N_2O emissions in conditions close to fertilizer field applications. Additionally, indications for

increased N_2O reduction after organic fertilization and in soil layers with high soil organic C contents were found. The role of N_2O reduction after organic fertilization requires further investigation by addressing atypical *nosZ*-bearing denitrifiers and quantifying N_2 emissions in a stable isotope approach.

Increased N_2O emissions in lower soil layers may be compensated through higher N_2O reduction in the topsoil of reduced tilled systems. Yet, our results suggest that placing ammonium in lower soil depths may increase N_2O production considerably. Higher N_2O emissions have already been observed after injection of slurry into deeper soil layers in the field (Montes et al. 2013), and there is a need to clarify if increased availability of N due to decreased NH_3 loss or microbial responses to C availability as seen in our study are the main drivers.

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