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Mineral and organic fertilisation influence ammonia oxidisers and denitrifiers and nitrous oxide emissions in a long-term tillage experiment

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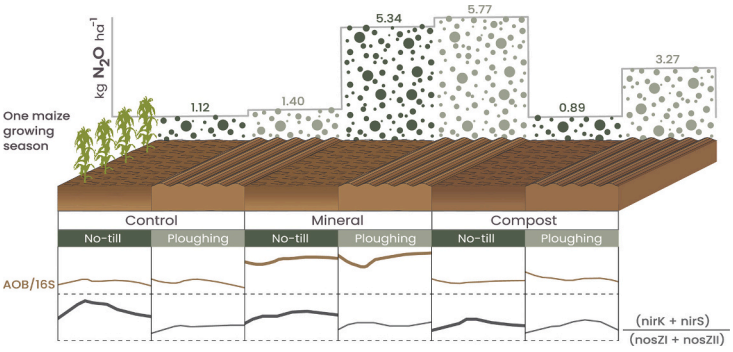
HIGHLIGHTS

- Rain events were the main triggers for N_2O emissions over maize growing season.
- Cumulative emissions were highest in mineral, followed by organic and no fertilisation.
- Mineral fertilisation increased share of bacterial ammonia oxidizers within the bacterial community.
- N_2O genetic consumption potential was higher in no-till organic-fertilised than mineral or unfertilized plots.

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrous oxide (N_2O) emissions from different agricultural systems have been studied extensively to understand the mechanisms underlying their formation. While a number of long-term field experiments have focused on individual agricultural practices in relation to N_2O emissions, studies on the combined effects of multiple practices are lacking. This study evaluated the effect of different tillage [no-till (NT) vs. conventional plough tillage (CT)] in combination with fertilisation [mineral (MIN), compost (ORG), and unfertilised control (CON)] on seasonal N_2O emissions and the underlying N-cycling microbial community in one maize growing season. Rainfall events after fertilisation, which resulted in increased soil water content, were the main triggers of the observed N_2O emission peaks. The highest cumulative emissions were measured in MIN fertilisation, followed by ORG and CON fertilisation. In the period after the first fertilisation CT resulted in higher cumulative emissions than NT, while no significant effect of tillage was observed cumulatively across the entire season. A higher genetic potential for N_2O emissions was observed under NT than CT, as indicated by an increased (*nirK* + *nirS*)/(*nosZI* + *nosZII*) ratio. The mentioned ratio under NT decreased in the order CON > MIN > ORG, indicating a higher N_2O consumption potential in the NT-ORG treatment, which was confirmed in terms of cumulative emissions. The AOB/16S ratio was strongly affected by fertilisation and was higher in the MIN than in the ORG and CON treatments, regardless of the tillage system. Multiple regression has revealed that this ratio is one of the most important variables explaining cumulative N_2O emissions, possibly reflecting the role of bacterial ammonia oxidisers in minerally fertilised soil. Although the AOB/16S ratio aligned well with the measured N_2O emissions in our experimental field, the higher genetic potential for denitrification expressed by the (*nirK* + *nirS*)/(*nosZI* +

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nosZII) ratio in NT than CT was not realized in the form of increased emissions. Our results suggest that organic fertilisation in combination with NT shows a promising combination for mitigating N_2O emissions; however, addressing the yield gap is necessary before incorporating it in recommendations for farmers.

1. Introduction

Tillage and fertilisation affect soil physicochemical properties which in turn affect different biological processes. Biologically emitted nitrous oxide (N_2O) gas, is one of the most studied global warming and ozone layer depletion related substances (Ravishankara et al., 2009; IPCC, 2013). Agriculture via its nitrogen (N) inputs is one of the biggest anthropogenic N_2O contributors (Tian et al., 2020), although implementing practices that promote C sequestration and limit N_2O emissions hold great promise for climate change mitigation (Minasny et al., 2017; de Vries, 2018; IPCC, 2022). Furthermore, owing to the interconnectedness of the C and N cycles, an increase in soil carbon content could increase microbiological activity and promote higher N_2O emissions (Guenet et al., 2021).

Soil water content, temperature, pH, N and C availability, soil organic matter content, and the microbial community composition are important determinants of N_2O production in soil (Wallenstein et al., 2006; Braker and Conrad, 2011; Butterbach-Bahl et al., 2013; Wrage-Mönnig et al., 2018). Different agricultural practices such as tillage, fertilisation, crop rotations, cover cropping etc. affect most of the above-mentioned parameters and thus, it is important to understand the relationship between agricultural practice and its effect on the mentioned parameters, especially when implementing a combination of practices. In a recent meta-analysis, Guenet et al. (2021) reviewed different agricultural management practices and their impact on C storage and N_2O emissions. Interestingly, reduced tillage intensity negatively affected the trade-off between C sequestration and N_2O emissions, whereas other practices such as agroforestry, inclusion of cover crops, and use of organic amendments proved to be net positive in terms of C sequestration and greenhouse gas (GHG) balance, or even improved both (i.e. addition of biochar or non-pyrogenic organic amendment). However, the same authors also noted that the mentioned practices are mostly examined separately (Mei et al., 2018; Skinner et al., 2019; Bhattacharyya et al., 2022) and highlighted the need to study potential synergistic or antagonistic effects of combination of practices between N_2O emissions and C sequestration. For example, although some temporal/partial studies assessing the effect of fertilisation and tillage on N_2O emissions in long-term experiments exist, they are either focused only on N-fertiliser application rates (Liu et al., 2005; Pelster et al., 2011; Pareja-Sánchez et al., 2020), follow short term application strategies (Venterea et al., 2005; Bayer et al., 2015), do not include mineral-organic fertilisation comparisons (Krauss et al., 2017b), have a shorter duration (Plaza-Bonilla et al., 2014), or are a combination of the mentioned (MacKenzie et al., 1998). Furthermore, there is still a need for deciphering the mechanisms underlying the impact of tillage and fertilisation on soil microbial communities responsible for N_2O emissions.

N_2O can be produced through several biotic and abiotic pathways. However, biotic pathways including denitrification, nitrification, nitrifier-denitrification, and dissimilatory nitrate reduction to ammonia (DNRA), are more significant than abiotic pathways in terms of their contribution to the total N_2O production (Braker and Conrad, 2011; Butterbach-Bahl et al., 2013). In agricultural systems, nitrification and denitrification act as the main sources of N_2O emissions, and their contributions vary depending on the conditions (Khalil et al., 2004; Bateman and Baggs, 2005). Denitrification is promoted under high water-filled pore space (WFPS) and low oxygen availability, as well as increased organic C and NO_3^- availability; whereas nitrification is favoured under lower WFPS and increased oxygen availability conditions. Recently it was shown that not only nitrification but nitrifier-denitrification can account for a large part of N_2O emissions in the WFPS range of approximately 50 % (Kool et al., 2011; Wrage-Mönnig et al., 2018). This makes N_2O emissions source identification even more challenging, especially in fields where soil heterogeneity plays an important role in different habitat conditions in addition to soil structural heterogeneity (Szoboszlay and Tebbe, 2021). Various methods can be applied to explore microbial communities responsible for N_2O emissions, one of them being the quantification of N-cycle genes and interpreting emission patterns using environmental and soil physicochemical data (Wang et al., 2019; Wang et al., 2021a, b). N-cycle genes utilized for this purpose include gene markers for archaeal and bacterial ammonia monooxygenase (*amoA*) (Leininger et al., 2006; Tourna et al., 2008), nitrite reductases *nirK* (Henry et al., 2004) and *nirS* (Throbäck et al., 2004), and nitrous oxide reductases *nosZI* (Henry et al., 2006) and *nosZII* (Jones et al., 2013).

In our long-term field experiment the combined effects of tillage (no-till vs. conventional plough tillage) and fertilisation (mineral-, compost-, and no- fertilisation) are studied. Previous results have shown that NT resulted in the highest bacterial biomass and abundance associated with an increased SOC (Govednik et al., 2023). Furthermore, NT and MIN treatments exhibited a greater N_2O emission potential compared to other treatments, as indicated by higher *nosZI/nosZII* ratios in NT than in CT and the increased bacterial *amoA*/16S ratio in MIN fertilisation. The aim of the present study was to investigate the link between functional community composition and N_2O emissions in relation to soil physicochemical parameters over the course of a maize-growing season. To the best of our knowledge, this is the first study to examine the effect of the combination of long-term tillage with different fertilisation regimes on N_2O emissions and N functional gene abundances over an entire maize growing season.

We hypothesised the following: (i) no-till will promote denitrifier community abundance consequently contributing to higher N_2O emissions in this system compared to CT; (ii) addition of mineral fertiliser will increase N_2O emissions compared to compost fertilisation, as a consequence of an increased abundance of bacterial ammonia oxidisers in those treatments; and (iii) cumulative N_2O emissions will be associated with the abundance ratios of soil nitrifying and denitrifying microbial communities.

2. Materials and methods

2.1. Site description and experimental design

Soil was sampled in a long-term field experiment established in 1999 at the Biotechnical Faculty in Ljubljana (TillComp:46 °3 ‘N, 14 °30 ‘E) (Bai et al., 2018; Bongiorno et al., 2020; Bongiorno et al., 2019; Govednik et al., 2023). Two different tillage systems were applied in the experimental field: conventional plough tillage (CT) to a depth of 25–28 cm and non-inversion minimum tillage to a maximum of 10 cm depth which was replaced by a no-till system (NT) in 2017. Both tillage systems were combined with three different fertilisation regimes: no fertilisation control (CON), mineral fertilisation (MIN), and compost fertilisation (ORG). The soil was classified as Eutric Gleysol (IUSS Working Group WRB, 2022). The local 10-year mean annual temperature and rainfall was 11.3 °C and 1383 mm, respectively (SURS, 2022). The field experiment was divided into two main plots, each corresponding to the respective tillage system. Within the main plot, each of the three fertilisation regimes was replicated in three subplots (6 × 8 m), resulting in 18 subplots. The following crops were rotated in the following order during the study period: maize, barley, oilrape, oats, sunflowers (biomass), alfalfa, winter wheat, buckwheat, and soybeans.

In May 2021, maize was sown as the main crop. Before seedbed preparation, fertilisation was carried out as follows: CON plots did not receive any fertiliser, MIN plots were fertilised with mineral NPK (15:15:15; at a rate of 50 kg N ha^{-1}), and ORG plots with compost (120 kg N ha^{-1}). Only the MIN plots received a second fertilisation at the end of June, with CAN (calcium ammonium nitrate) at a rate of 100 kg N ha^{-1}. Seedbed preparation in 2021 in CT plots involved ploughing to a depth of 25 cm and rotary hoeing prior to seeding (10,000 seeds ha^{-1}), whereas NT plots were seeded directly using a special NT drill (Great Plains 3P1006NT). In June 2021, a combination of Banvel (Syngenta) and FOCUS Ultra (BASF), herbicides containing the active ingredients dicamba and cycloxydim, respectively, was applied at the recommended doses to control weeds.

2.2. Soil sampling and analysis

Soil samples were collected from 21 sampling dates at 0–10 soil depth during the maize-growing season from May to October 2021. Eight to ten soil cores (1 cm in diameter) were collected from each plot as a composite sample. Homogenised samples were divided into three parts: (i) approximately 2 g of soil was stored in cryovials which were flash-frozen in dry ice and later stored at −20 °C until use for DNA extraction, (ii) approximately 15 g was used for gravimetric water determination, and (iii) the remaining part was dried at 40 °C for 24 h for soil physicochemical analyses (ISO 11464, 2006).

2.3. Physicochemical analyses

Soil bulk density was determined directly using core sampling method (Kopecky cylinders) and calculated as the ratio of the dried mass of soil to its total volume. Soil water gravimetric content was evaluated in each homogenised soil sample by drying until constant mass at 105 °C (ISO 11465, 1993). WFPS was calculated from soil bulk density and water gravimetric content as previously described (Yanai et al., 2007). Soil pH was measured using a 0.01 M calcium chloride ($CaCl_2$) suspension (ISO 10390, 2005). Dissolved organic carbon (DOC), dissolved total nitrogen (TDN), nitrate-nitrogen (NO_3-N), and ammonium-nitrogen (NH_4-N) concentrations were determined by extraction using a 1:10 (*w/v*) soil/0.01 M $CaCl_2$ solution (ISO 14255, 1998). DOC and TDN were evaluated by oxidation and gas analysis using NDIR and EC detectors, respectively (Vario TOC Cube, Elementar, Germany). The extracted NO_3-N and NH_4-N were determined using an automated discrete photometric system at 540 and 655 nm (Gallery Plus, Thermo Scientific, Waltham, Massachusetts, USA), respectively (ISO 7150-1, 1984).

2.4. Gas measurements and analysis

Soil N_2O emissions were measured using closed static chambers (Mosier and Hutchinson, 1981). Gas samples were collected at 21 dates during the maize growing season, focusing around agricultural practices (tillage and fertilisation) and rain events. Sampling was consistently performed between 9 and 11 a.m. CET. To collect gas samples, one ring with a diameter of 30 cm was permanently installed per plot between the maize rows and was removed only during tillage. During sampling, protruding rings were covered by a 9 cm high chamber for 45 min. Gas samples were collected using a syringe at regular intervals of approximately 15 min, with the first sample being collected immediately upon chamber closure. This resulted in four gas samples per chamber per sampling event which were stored in evacuated 12 mL Exetainer vials (Labco Ltd., UK). Soil temperature at 0–10 cm depth was monitored over the entire sampling season using in situ temperature sensors (Pečan et al., 2023), whereas the chamber temperature during each sampling event was monitored using a temperature data logger (Voltcraft DL-210TH, Germany). N_2O content of gas samples was analysed in CREA Centro di ricerca Viticoltura ed. Enologia in Gorizia using a gas chromatograph (7890A, Agilent Technologies, CA) equipped with an electron capture detector (ECD). Peak areas were integrated using the Open Lab ChemStation Software (Agilent Technologies, CA). Three standard concentrations (0.200, 0.700, and 2.500 ppm of N_2O) were used for calibration, measured every 25 samples.

N_2O flux calculations were performed in R (R Core Team, 2022) using the *gasfluxes* package (Fuss, 2020) which calculates and selects the most suitable model (linear, robust linear, or HMR) for each concentration curve based on the kappa max parameter (Hüppi et al., 2018). Cumulative emissions were obtained using the same package by trapezoidal integration of the emission curves using agg.fluxes function. They were divided in two emission periods, first lasting between 10.5.2021 and 24.6.2021 and the second between 24.6.2021 until the end of the growing season on 22.10.2021.

2.5. Molecular analyses

Out of 21 soil sampling dates 10 were chosen for molecular biological analysis based on implementation of agricultural management practices and evolution of N_2O emissions (Fig. 2). This resulted in the selection of following sampling dates (Fig. 1): (i) before any disturbance in the system; (ii) after tillage and fertilisation and beginning of the first emission period; (iii) peak N_2O emissions; (iv) end of first emission period; (v) directly before second fertilisation; (vi and vii) two consecutive peaks in the second emission period; (viii, ix, x) evenly spaced over the remaining of the season when negligible N_2O emissions were observed. DNA from selected soil samples was extracted using a DNeasy PowerSoil Pro Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. The purity and concentration of DNA extracts were determined spectrophotometrically (NanoDrop 2000 UV–vis Spectrometer; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and fluorometrically (Qubit 4, Thermo Fischer Scientific, Waltham, Massachusetts, USA), respectively. Target genes were quantified by SYBR green qPCR using a standard curve on the Quantstudio 5 (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Each reaction consisted of 2 μL of 150× diluted DNA extracts (~2–5 ng DNA per reaction), 7.5 μL ABsolute Blue qPCR SYBR Green Low Rox (Thermo Fischer Scientific, Waltham, Massachusetts, USA), and 1 μM for amplifying bacterial 16S, ITS and thaumarchaeal 16S, archaeal and bacterial *amoA* and 2 μM primers for amplifying *nirK*, *nirS*, *nosZI*, *nosZII* and *nrfA* gene markers. 250 ng of T4gp32 protein was added to qPCR reactions for the *amoA*, *nirK*, *nirS*, *nosZI*, *nosZII*, and *nrfA* genes. Primer sequences and thermal cycling conditions are listed in Table S1. Analysis of each biological replicate was performed in duplicate within the same qPCR run. Run efficiencies ranged between 80 and 100 % for 16S rRNA, ITS, bacterial *amoA*, *nirK*, *nirS*, *nosZI,* and approximately between 75 and 69 % for archaeal *amoA* and *nosZII*, respectively. All R^2 values of the runs were equal to or higher than 0.999. No PCR inhibition was detected as demonstrated by spiking samples with a known amount of external pGEM-T plasmid (Promega, Madison, Wisconsin, USA) and comparing its C_q values with the positive control containing only the plasmid. The standard for thaumarchaeal 16S rRNA was obtained by cloning a PCR product from an environmental sample in a pGEM-T Easy plasmid (Promega, Madison, Wisconsin, USA), whose gene sequence was confirmed by Sanger sequencing. Several gene ratios were calculated for the following purposes: (i) to illustrate relative abundances such as AOA/Thaumarcheal 16S, AOB/16S, *nirS*/16S, *nirK*/16S, *nosZI*/16S, *nosZII*/16S, *nrfA*/16S; (ii) to identify dominant genes within processes such as AOA/AOB for nitrification, *nirS/nirK* for nitirite reduction and *nosZI/nosZII* for N_2O reduction, and (iii) to estimate genetic potential ratio for denitrifier N_2O production and reduction (*nirK* + *nirS*)/ (*nosZI* + *nosZII*).

2.6. Statistical analysis

Statistical analyses were performed in R environment software 4.0.3

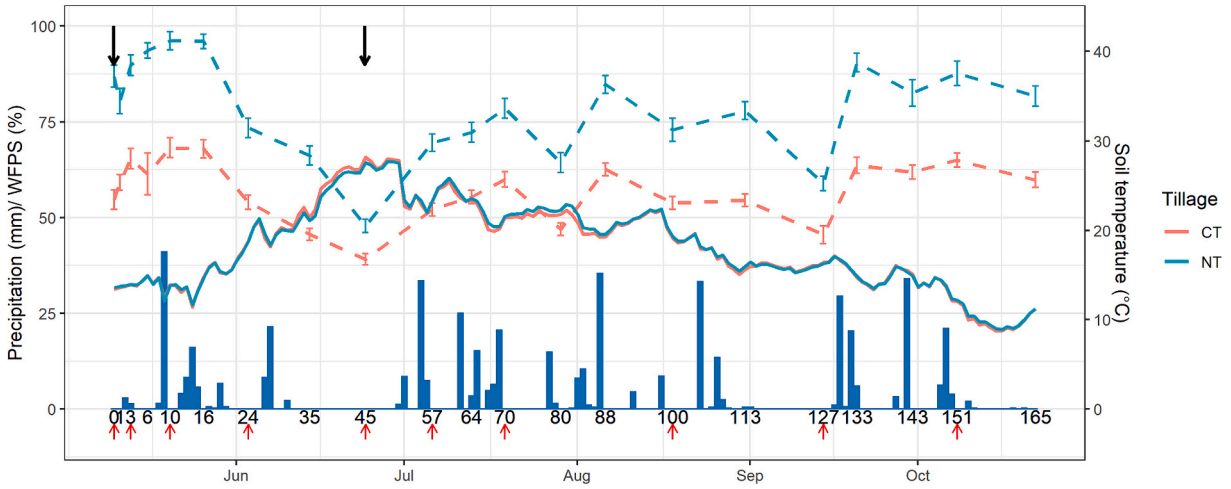

Fig. 1. Seasonal dynamics of daily precipitation (bars), water-filled pore space (WFPS) (dashed line) and daily average (n = 3) soil temperature (solid line) in the top 10 cm of soil under no-till (NT) and conventional tillage (CT). Numbers on the x-axis represent gas sampling events (days since the beginning of the experiment), while red arrows represent selected sampling dates for molecular analyses. Upper black arrows indicate fertilisation events. For WFPS means and standard errors per tillage system are presented (n = 6). Please note different y axes on separate sides.

(R Core Team, 2022) and the ggplot2 package (Wickham, 2016) was used for producing graphs. A linear mixed-effects model was used to assess the significance of the tillage system, fertilisation, and their interactions on the measured seasonal soil parameters using nlme package (Pinheiro et al., 2020). The plot was included as a random effect factor. The homogeneity of variance across treatments was tested using Levene's test. Data showing heteroscedasticity were log transformed. For cumulative N_2O emissions, two-way analysis of variance (ANOVA) was used to test tillage, fertilisation, and their interaction effects. Tukey *Post-hoc* test was performed to evaluate differences between the levels of different treatments at 0.05 significance level using emmeans package (Lenth, 2022). Contrast analysis was used to assess the differences in cumulative emissions within the same treatments in different periods using multcomp package (Hothorn et al., 2008). Additionally, multiple regression analysis was performed to explain the cumulative N_2O emissions in relation to the measured soil biological and physicochemical parameters. It was performed in R environment using the 'step' function starting with minimal model (cumulative N_2O emissions ~1) and then building towards the biggest model including all gene ratios and measured physicochemical parameters. The best-forward-selected model was determined based on AIC criteria.

3. Results

3.1. Environmental conditions

N_2O emissions were monitored during the 2021 maize growing season (May–October). Precipitation was evenly distributed during the season, except for a longer dry period in the second part of June when the soil temperature in the upper 10 cm was highest (Fig. 1). Water-filled pore space (WFPS) was higher under NT compared to CT over the entire season, with the highest values observed at the beginning of the season (Table 1, Fig. 1). Dissolved nitrogen (total (TDN), NH_4-N, and NO_3-N) across the entire season showed similar distribution patterns, being higher in the NT than in the CT system and following the MIN > ORG > CON order within the individual tillage systems (Table 1, Fig. S1). Easily available NH_4-N, NO_3-N, and TDN peaks were observed in the MIN treatment following the second fertilisation in both tillage systems, whereas at the same time a slight increase in NO_3-N was observed in CON and ORG, which was more pronounced in NT (Fig. S1). Across season, DOC was higher in NT than in CT, with decreasing gradient from ORG fertilisation followed by MIN and CON in both tillage systems (Table 1, Fig. S2).

3.2. Dynamics of N_2O emissions

N_2O emission peaks were observed during two distinct periods (Fig. 2). The first period was immediately after tillage and the first fertilisation followed by a rain event, and the second occurred after the second fertilisation, also after a rain event. It must be noted that the second fertilisation was only performed in MIN, while emission peaks were observed in all fertilisation treatments (CON, MIN, and ORG). Emission peaks in the CT were higher in the first period than in the second, although cumulative differences between the two periods were only significant within the CT-MIN (analysis with contrasts, $p < 0.01$). NT-MIN exhibited comparable peaks in both periods, whereas the NT-CON and NT-ORG treatments exhibited detectable emission peaks only in the second period.

Fertilisation had a stronger effect on cumulative N_2O emissions than tillage, which was apparent in both emission periods (after the first and the second fertilisation) and during the entire season. The highest emissions were observed in the MIN treatment, followed by ORG and CON treatments (Table 2). The tillage system affected cumulative emissions of N_2O only during the first period, with higher emissions in CT than in NT, whereas no significant effect of tillage on cumulative emissions was observed across the entire growing season (Table 2). Total N_2O emissions were significantly higher in MIN than in CON in the CT system in the first period (after the first fertilisation), whereas the same pattern occurred in NT only in the second period (after the second fertilisation) (Table 2). Between the tillage systems over the entire season, CON and MIN fertilisations displayed comparable cumulative emissions, whereas NT-ORG exhibited noticeably lower emissions than CT-ORG, although this difference was not statistically significant. Despite maize yields being affected by tillage and fertilisation (Table S2), yield-scaled emissions were not affected by either and were comparable among the treatments, ranging from 101 to 352 g·N_2O-N Mg^{-1} dry biomass for the NT-ORG and NT-MIN treatments, respectively (Table 2).

3.3. N cycling genes abundance in soil

The total extracted DNA per gram of dry soil ranged between 60 and 100 μg in NT, and 34 and 61 μg in CT. This difference in the amount of extracted DNA in different tillage treatments clearly affected the distribution of the different genes (Table S3, Fig. S3). Therefore, to evaluate

Table 1
Results of the linear mixed effects model testing the effect of tillage [no-till (NT) vs conventional tillage (CT)] and fertilisation [unfertilised control [(CON), mineral (MIN) and organic (ORG)] and their interaction on bulk density, pH, water-filled pore space (WFPS), NH_4-N, NO_3-N, total dissolved nitrogen (TDN) and dissolved organic carbon (DOC) across the growing season. Number of observations for individual parameters is stated in parentheses in cases where it differs from the total number of observation (N = 378). The Tukey *Post-hoc* test was used to differentiate between classes of different treatments.

Factor		Bulk density (g cm^{-3}) (N = 18)		pH (N = 90)		Soil water content (g g^{-1})		WFPS (%)		NH_4-N (mg 100 g $dry\ soil^{-1}$)		NO_3-N (mg 100 g $dry\ soil^{-1}$)		TDN (mg 100 g $dry\ soil^{-1}$)		DOC (mg 100 g $dry\ soil^{-1}$)	
Tillage		***		n.s.		***		***		***		***		***		***	
Fertilisation		n.s.		n.s.		n.s.		n.s.		**		***		***		***	
Tillage × Fertilisation		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
CT	CON	1.13	(0.05) b	6.32	(0.08) a	0.21	(0.003) bc	53.82	(1.26) b	0.18	(0.02) d	0.41	(0.03) d	1.27	(0.04) d	9.98	(0.17) d
	MIN	1.21	(0.03) ab	6.40	(0.09) a	0.21	(0.003) c	58.34	(1.23) b	0.40	(0.07) bc	1.13	(0.16) b	1.92	(0.14) c	10.43	(0.19) cd
	ORG	1.16	(0.01) ab	6.51	(0.06) a	0.22	(0.004) bc	58.53	(1.26) b	0.21	(0.02) cd	0.49	(0.03) cd	1.50	(0.05) cd	11.29	(0.18) c
NT	CON	1.30	(0.04) ab	6.41	(0.05) a	0.23	(0.004) ab	76.06	(1.69) a	0.33	(0.03) ab	0.66	(0.04) bc	2.13	(0.06) b	13.95	(0.23) b
	MIN	1.30	(0.02) ab	6.29	(0.07) a	0.23	(0.004) ab	76.71	(1.77) a	0.56	(0.07) a	1.53	(0.17) a	3.06	(0.19) a	15.13	(0.23) a
	ORG	1.33	(0.05) a	6.60	(0.06) a	0.24	(0.004) a	83.36	(1.87) a	0.39	(0.03) a	0.93	(0.06) ab	2.61	(0.08) a	15.60	(0.26) a

n.s. = not significant *p < 0.05. **p < 0.01. ***p < 0.001.

the shifts and possible niche differentiation within the microbial community responding to tillage and fertilisation related environmental conditions, the ratios of different mutually exclusive genes were calculated and statistically analysed (Table 3).

The average proportion of bacterial ammonium oxidisers in the bacterial community estimated by the bacterial *amoA*/16S ratio (AOB/16S) was distinctly higher in the MIN fertilisation than in CON and ORG in both tillage systems during the season (Table 3, Fig. 3). The same pattern was observed for the ratio of bacterial to archaeal *amoA* (AOB/AOA) (Fig. S4). Across season, the percentage of AOA in Thaumarchaeal 16S (AOA/Thaumarchaeal 16S) was not affected by either tillage or fertilisation. However, an increasing trend of this percentage was observed over the growing season (Fig. S4).

The denitrifier community composition, assessed by the abundance of *nirK*, *nirS*, *nosZI*, and *nosZII* genes, was seemingly determined by tillage rather than by fertilisation. Specifically, *nirS*/16S, *nirK*/16S, and *nosZI*/16S ratios were higher in NT than in CT across season, whereas the *nosZII*/16S ratio showed the opposite trend, being higher in CT than in NT contributing to the higher *nosZI*/*nosZII* ratio under NT than under CT (Table 3 and Figs. 3 and S5). The (*nosZI* + *nosZII*)/16S ratio was not affected by either tillage or fertilisation. A fertilisation effect was observed in the *nosZII*/16S ratio, which was the highest in ORG fertilisation, followed by CON and then MIN for both tillage systems (Fig. S5). The (*nirK* + *nirS*)/(*nosZI* + *nosZII*) ratio was higher in NT than in CT. Within the NT system, this ratio was lowest in ORG fertilisation, followed by MIN and CON, whereas in CT, it was comparable across fertilisation regimes (Fig. 3, Table 3). Genetic potential for DNRA (as expressed by the *nrfA*/16S ratio) was not affected by tillage or fertilisation (Table 3, Fig. S4).

3.4. Multiple regression

Multiple regression analysis including physicochemical and biological parameters was performed to explain the cumulative N_2O emissions. The best forward-selected model explained 58 % of the variability in overall cumulative emissions. The model included multiple biological and physicochemical variables, as shown in Table 4. The variables which had an increasing cumulative N_2O emission effect in the model were the ratios AOB/16S, (*nirK* + *nirS*)/*nosZI*, *nirK*/16S, NO_3-N, and bulk density, whereas *nirS*/16S, WFPS, temperature, and DOC/NO_3^- had a decreasing effect.

4. Discussion

In the present study, we investigated the effect of more than two decades of differential tillage, in combination with a mineral, organic, or no-fertilisation, on seasonal N_2O emissions in conjunction with the soil microbial community functional composition. Emission peaks, consistent with previous reports (Tellez-Rio et al., 2015a; Krauss et al., 2017b; Wang et al., 2021b), were primarily triggered by rain events throughout the season. However, tillage and fertilisation also modulated these emissions, depending on the period (Figs. 1 and 2).

Generally, there is divergence in findings regarding the N_2O emissions of various tillage systems. While some meta- and experimental studies have shown higher emissions in systems with reduced tillage intensities compared to conventional tillage (Mangalassery et al., 2014; Huang et al., 2018; Mei et al., 2018; Sandén et al., 2018; Autret et al., 2019; Sanaullah et al., 2020; Guenet et al., 2021; Shakoor et al., 2021), other studies debate that factors such as climate, duration of the differential tillage, and N placement modulate the effect of tillage on N_2O emissions (Six et al., 2004; van Kessel et al., 2013; Zhao et al., 2016). Supporting the theory of higher emissions under NT systems, a meta-analysis by Wang and Zou (2020) revealed a higher soil genetic potential for N_2O production in the NT systems, as indicated by an increased (*nirK* + *nirS*)/*nosZ* ratio. A similar increase of (*nirK* + *nirS*)/ (*nosZI*+ *nosZII*) ratio in NT compared to CT was demonstrated in our

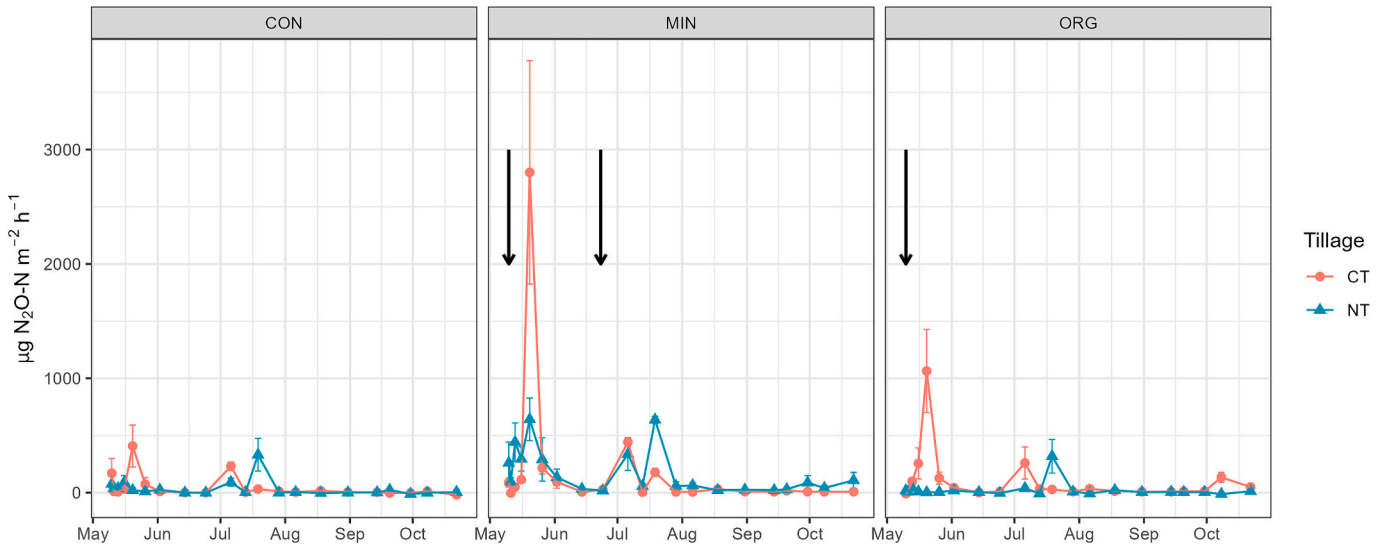

Fig. 2. Seasonal dynamics of N_2O emissions in no-till (NT) and conventional tillage (CT) systems in combination with unfertilized control (CON), mineral (MIN) and organic (ORG) fertilisation regimes. The means are presented with standard errors (n = 3). Black arrows indicate fertilisation events.

Table 2
Cumulative emissions in different tillage [no-till (NT) and conventional tillage (CT)] and fertilisation [unfertilised control [(CON), mineral (MIN) and organic (ORG)] treatments in different periods (after first and second fertilisation and across the entire season) including yield scaled cumulative emissions per total maize biomass. The means are presented with standard errors (SE) (n = 3). Significant factors of ANOVA testing on the effect of tillage, fertilisation and their interaction on cumulative N_2O emissions are presented in the bottom row. The Tukey *Post-hoc* test was used to differentiate between classes of different treatments.

		First fertilisation period (kg N_2O-N ha^{-1})		Second fertilisation period (kg N_2O-N ha^{-1})		Entire growing season (kg N_2O-N ha^{-1})		Yield scaled (g N_2O-N Mg^{-1} dry total biomass)	
Tillage	Fertilisation	Mean (SE)	Tukey HSD	Mean (SE)	Tukey HSD	Mean (SE)	Tukey HSD	Mean (SE)	Tukey HSD
CT	CON	0.69 (0.34)	b	0.71 (0.11)	b	1.40 (0.45)	ab	121.20 (29.76)	a
	MIN	4.13 (1.35)	a	1.67 (0.07)	ab	5.77 (1.40)	a	252.97 (61.08)	a
	ORG	1.87 (0.64)	ab	1.40 (0.65)	ab	3.27 (1.22)	ab	190.07 (68.37)	a
NT	CON	0.22 (0.08)	b	0.89 (0.29)	b	1.12 (0.35)	b	226.84 (105.84)	a
	MIN	2.22 (0.81)	ab	3.12 (0.80)	a	5.34 (1.33)	ab	352.49 (105.67)	a
	ORG	0.10 (0.02)	b	0.79 (0.35)	b	0.89 (0.35)	b	101.47 (44.74)	a
Significant factors:		Tillage*, Fertilisation**		Fertilisation*		Fertilisation**			

n.s. = not significant *p < 0.05. **p < 0.01. ***p < 0.001.

experimental field (Table 3, Fig. 3), however, this potential was not realized on the level of N_2O emissions. Notably, in the period following the first fertilisation event, CT exhibited more pronounced N_2O peaks and higher cumulative N_2O emissions than NT (Table 2). This suggests that despite existing genetic potential, various other factors influenced the individual processes leading to N_2O emissions (Wang et al., 2021a). Our results align with the findings of van Kessel et al. (2013), who showed that long-term adoption of NT/RT tillage practices leads to a reduction of N_2O emissions in comparison to CT. Six et al. (2004) attributed this effect to an increase in soil organic matter, which enhances soil structure, thereby reducing anaerobic microsites favourable for N_2O formation. In our experiment, based on aggregate stability measurements conducted a decade ago (Kaurin, 2015), alongside data on bulk density and gravimetric water content (Table 1), we may assume that soil structure is better under NT than CT. Additionally, lower N_2O emissions in NT may also be attributed to higher bulk density in combination with higher seasonal soil water content in NT than CT (Table 1), resulting in distinctly higher WFPS across the season in NT (Fig. 1). Namely, conditions with WFPS above 90 % could favour the final reduction of N_2O to N_2, as the NosZ enzyme is inhibited even at low O_2 concentration (Butterbach-Bahl et al., 2013; Hu et al., 2015) therefore increasing the N_2O sink capacity. Notably, soil air-water conditions and available C and N contents changed differentially between the treatments throughout the season (Table 1, Figs. 1, S1 and S2). For example, ploughing in CT at the beginning of the study (Day 0) likely increased soil porosity and unsaturated hydraulic conductivity (Schwen et al., 2015), allowing for temporary higher water capture capacity. In combination with the initial rain event this possibly influenced increased nutrient turnover and microbial respiration while also displacing gases captured in the pores contributing to the observed peak of N_2O emissions in CT. An alternative explanation for the lower-than-expected N_2O emissions in NT could be the increased weed cover in NT (Table S4), leading to enhanced competition between plants and microbes for mineral nitrogen, thereby possibly steering the microbial pathways towards complete denitrification for increased nitrate use efficiency (Felgate et al., 2012) or limiting microbial N_2O production overall due to a lack of substrate. These latter assumptions could be supported in studies where higher emissions were observed in fallow land than in cropped land (Tellez-Rio et al., 2015b; Han et al., 2017).

Tillage was a significant factor influencing N_2O emissions in the first period after fertilisation; however, fertilisation was the main factor affecting the overall cumulative N_2O emissions in our study, with emissions decreasing in the gradient from MIN over ORG to CON (Table 2). This aligns with the application N dose, which followed the same trend, with 150 kg and 120 kg N ha^{-1} added in MIN and ORG treatments, respectively, whereas no nitrogen was added to CON. Surprisingly, despite a higher N dose was added to MIN at the second fertilisation event than at first (100 vs. 50 kg N ha^{-1}), the emissions in NT-

Table 3
Results of the linear mixed effects model testing the effect of tillage [no-till (NT) vs conventional tillage (CT)] and fertilisation [unfertilised control [(CON), mineral (MIN) and organic (ORG)] and their interaction on microbial community functional genes ratios: abundances of archaeal *amoA* (AOA)/thaumarchaeal 16S; bacterial *amoA* (AOB), *nirS*, *nirK*, *nosZI*, *nosZII* and *nrfA* relative to 16S rRNA genes; and abundance ratio within and between nitrite (*nirK* and *nirS*) and N_2O reducers (*nosZI* and *nosZII*). The Tukey *Post-hoc* test was used to differentiate between classes of different treatments.

Factor		AOA/thaumarch. 16S	AOB /bact. 16S	AOA/AOB	*nirS*/16S	*nirK*/16S	*nirS/nirK*	*nosZI*/16S	*nosZII*/16S	*nosZI*/ *nosZII*	(*nirK* + *nirS*)/ (*nosZI* + *nosZII*)	*nrfA*/16S
Tillage		n.s.	n.s.	n.s.	*	***	n.s	**	***	***	***	n.s
Fertilisation		n.s.	***	***	n.s.	n.s.	n.s.	n.s.	*	n.s.	*	n.s.
Tillage × Fertilisation		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
CT	CON	0.98 (0.03) a	0.00080 (0.00003) b	17.61 (0.74) ab	0.0185 (0.0007) a	0.078 (0.002) b	0.239 (0.009) a	0.0138 (0.0003) a	0.0083 (0.0002) a	1.69 (0.05) bc	4.37 (0.07) c	0.047 (0.001) a
	MIN	0.97 (0.04) a	0.00139 (0.00005) a	10.41 (0.40) c	0.0183 (0.0007) a	0.079 (0.002) ab	0.233 (0.009) a	0.0137 (0.0003) a	0.0080 (0.0001) ab	1.71 (0.04) abc	4.51 (0.08) bc	0.048 (0.002) a
	ORG	0.99 (0.03) a	0.00093 (0.00004) b	14.62 (0.62) abc	0.0170 (0.0004) a	0.079 (0.002) ab	0.217 (0.006) a	0.0132 (0.0003) a	0.0087 (0.0002) a	1.54 (0.05) c	4.38 (0.08) c	0.045 (0.001) a
NT	CON	0.96 (0.03) a	0.00074 (0.00003) b	20.41 (0.76) a	0.0217 (0.0004) a	0.088 (0.002) a	0.248 (0.005) a	0.0145 (0.0003) a	0.0071 (0.0001) b	2.06 (0.04) ab	5.11 (0.10) a	0.052 (0.001) a
	MIN	0.93 (0.03) a	0.00130 (0.00004) a	11.65 (0.36) bc	0.0200 (0.0004) a	0.088 (0.003) ab	0.233 (0.005) a	0.0148 (0.0003) a	0.0070 (0.0001) b	2.15 (0.06) a	4.93 (0.11) ab	0.050 (0.001) a
	ORG	0.92 (0.04) a	0.00087 (0.00003) b	15.78 (0.59) abc	0.0196 (0.0005) a	0.083 (0.002) ab	0.238 (0.006) a	0.0149 (0.0003) a	0.0077 (0.0002) ab	1.95 (0.04) ab	4.57 (0.10) bc	0.051 (0.001) a

n.s. = not significant $^*p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

MIN were comparable in both periods. Furthermore, emissions in CT-MIN were even significantly lower in the second period than in the first. This observation could be perhaps attributed to the later crop stage with greater root development and thus plant uptake but also to a higher contribution of nitrification to the total N_2O emissions in the second period. Namely, previous incubation studies have demonstrated that, in terms of absolute cumulative emissions, nitrification produces lower amounts of N_2O than denitrification (Bateman and Baggs, 2005; Kool et al., 2011). The increased nitrification contribution to N_2O emissions is supported by two observations: (i) WFPS at emission peaks after the second fertilisation was lower in both tillage systems (at 52 % in CT and 70 and 79 % at two consecutive peaks in NT) (Fig. 1), and (ii) increased nitrate concentrations in CON and ORG fertilisations (which were not fertilised in the second period) following rain events in late June and the beginning of July, indicating a nitrification process (Fig. S1). In MIN fertilisation, this effect was masked because both NH_4^+ and NO_3^- were supplied in the form of mineral fertiliser, leading to substantial increases of both. However, a broader and longer-lasting peak for NO_3-N than for NH_4-N was observed, which supports our claim (Fig. S1). These findings could also explain the similar cumulative emissions in both fertilisation periods in the NT-MIN treatment, because 70–80 % WFPS levels in the second period (Fig. 1) suggest optimal conditions for denitrification (Butterbach-Bahl et al., 2013), which was considered as the main source of N_2O emissions in the first period as well.

Denitrification, nitrifier denitrification, and nitrification are known to be the most important microbial sources of N_2O (Philippot et al., 2007; Butterbach-Bahl et al., 2013; Hallin et al., 2018; Wrage-Mönnig et al., 2018). These processes, although they can occur simultaneously at different microsites (Stevens et al., 1997), are performed under different conditions by specific microbial communities (Braker and Conrad, 2011). Based on this fact and the observed changes in environmental and nutrient conditions over the growing season (Figs. 1, S1, and S2), we can assume that different microbial processes contributed to the total measured N_2O emissions at different sampling dates throughout the season. For example, we can assume higher contribution of denitrification in the period after first fertilisation due to increased WFPS in the range between 68 and 95 % (Figs. 1 and 2) (Bateman and Baggs, 2005; Kool et al., 2011), however distinct N_2O fluxes between the CT and NT tillage systems were observed in mentioned emission period possibly indicating tillage driven soil structural and nutritional changes. Namely, with tillage we are incorporating applied organic fertiliser, disrupting soil aggregates and re-distributing nutrients across the ploughing depth, making them more accessible to microorganisms possibly causing hot spots (Kuzyakov and Blagodatskaya, 2015) illustrated by narrower and higher N_2O peaks in CT system as compared to lower and longer lasting peak observed in NT-MIN (Fig. 2). This goes well with the fact that significantly higher dissolved N was observed in NT compared to CT in the sampled 0–10 cm upper layer (Fig. S1, Table 1), as ploughing distributes and consequently dilutes nutrients over the 0–25 cm depth. While peaks in the CT system in the first emission period corresponded to the added dose of easily available mineral N with the highest emissions in CT-MIN followed by CT-ORG and CT-CON the trend showed a different pattern (although insignificant) in the NT system. Namely, higher emissions in NT-MIN and comparably low in NT-CON and NT-ORG were observed (Fig. 2), despite the comparable contents of dissolved N in NT-MIN and NT-ORG (Fig. S1). One explanation for these N_2O patterns could be attributed to the consistent (*nirK* + *nirS*)/(*nosZI* + *nosZII*) ratio observed across fertilisation treatments in CT system, but which showed a decreasing gradient from CON over MIN to ORG in NT system (Table 3, Fig. 3), primarily driven by an increase in *nosZII* and, to a lesser extent, a decrease in *nirK* and *nirS* abundances (Fig. S5). This aligns well with the fact that a higher proportion of the *nosZII* community lacks *nir* genes than *nosZI* community (Jones et al., 2014), resulting in a higher N_2O consumption potential in *nosZII* prevalent communities (Domeignoz-Horta et al., 2016). Similar relationship between (*nirK* + *nirS*)/(*nosZI* + *nosZII*) ratio and N_2O emissions was already observed in

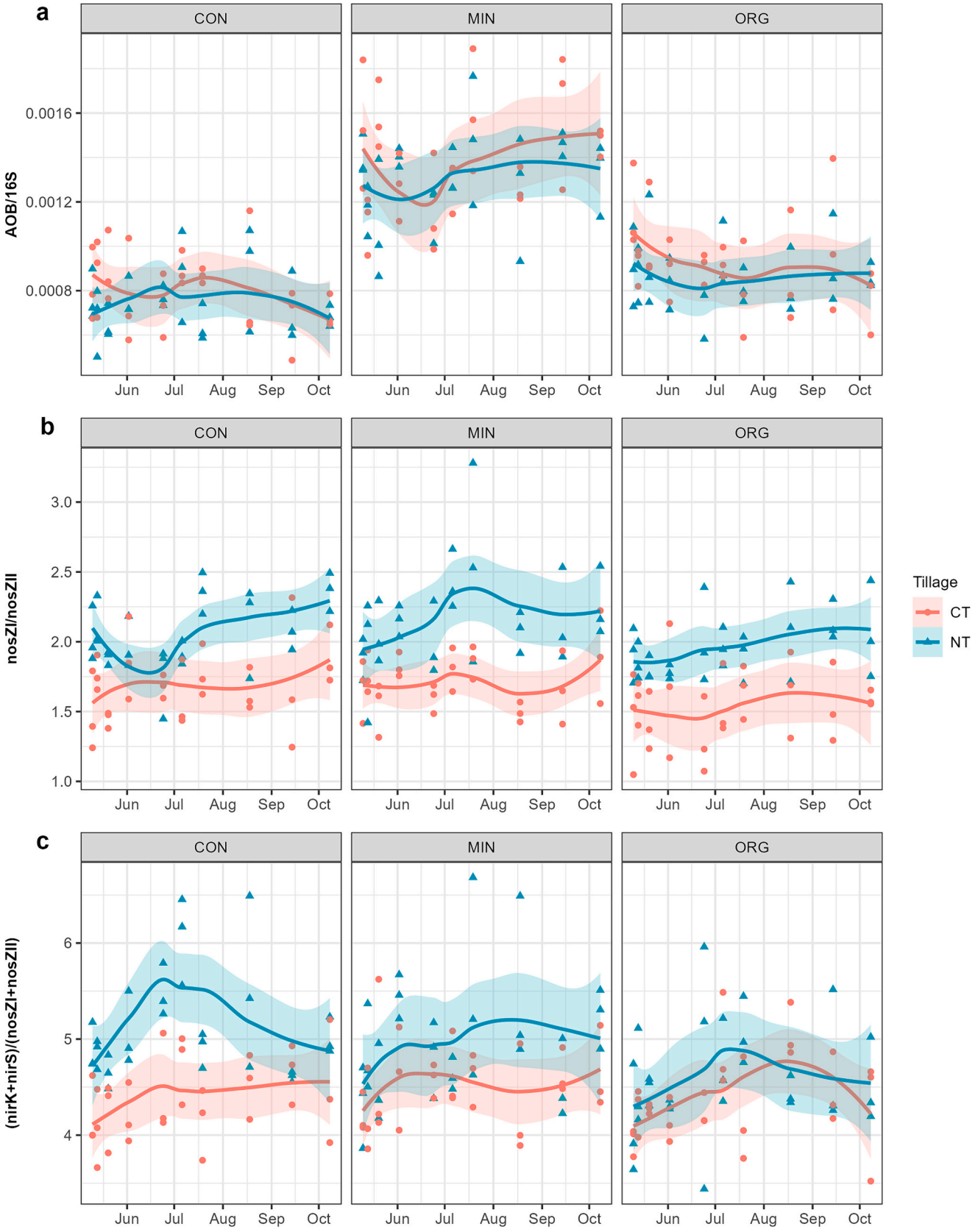

Fig. 3. Smoothed conditional means with local polynomial regression fitting for (a) AOB/16S, (b) *nosZI/nosZII* and (c) (*nirK* + *nirS*)/(*nosZI* + *nosZII*) ratios over the 10 sampling dates in no-till (NT) and conventional tillage (CT) systems in combination with unfertilized control (CON), mineral (MIN) and organic (ORG) fertilisation regimes. Shaded areas represent 95 % confidence intervals.

Table 4
Variables and respective coefficients with *p* values of forward selected multiple regression model for explaining N_2O cumulative emissions.

Variable	Coefficient	p value
AOB/16S	4.14×10^{3}	***
nirS/16S	-2.69×10^{2}	***
(*nirK* + *nirS*)/*nosZI*	2.40×10^{-1}	n.s.
NO_3-N	6.28×10^{-1}	***
WFPS	-7.52×10^{-2}	***
Temperature	-1.65×10^{-1}	***
Bulk density	6.53×10	**
DOC/NO_3^-	-1.14×10^{-2}	*
nirK/16S	2.36×10^{1}	n.s.

n.s. = not significant *p < 0.05. **p < 0.01. ***p < 0.001.

arable soil in the past either based on absolute abundance data (Wang et al., 2019) or inferred from metagenomic data (Wang et al., 2021b).

In our study we observed distinct niche partitioning between *nosZ* clades, indicated by higher *nosZI/nosZII* ratio in NT than CT (Fig. 3). The two tillage systems were shown in the past and in our study to differ in soil properties including soil structure, bulk density, organic matter type and content, nutrient levels, soil water content, and weed dominance (Page et al., 2020), possibly affecting *nosZI* and *nosZII* bearing communities. For example, recently the association between *nosZI* clade and nutrient and labile C rich rhizosphere was shown, while *nosZII* community was more predominant in oligotrophic bulk soil environment (Ai et al., 2020; Graf et al., 2022). This aligns well with our findings since across the season more oligotrophic conditions are expected in the CT system due to regular soil mixing while the carbon that remains in CT system is characterised as more degraded and therefore less labile (Ding et al., 2002). In accordance with this a tendency towards a higher percentage of *nosZII* was observed also in ORG fertilisation (Table 3, Fig. S5) characterised by increased input of stable organic C in the form of compost. It should be acknowledged however that due to taxonomically broadly distributed *nosZ* gene (Hallin et al., 2018) environmental drivers could select rather for different lineages within individual *nosZ* clades than drive *nosZI/nosZII* partitioning (Maheshwari et al., 2023) therefore highlighting the need to avoid oversimplified interpretation of different niche partitioning of *nosZ* communities.

The gene proportion within the total bacterial community that was most strongly affected by fertilisation in our study was AOB/16S, and was significantly higher in MIN than in ORG and CON fertilisations (Table 3, Fig. 3). Increased AOB/16S aligns well with the cumulative emission pattern (Table 2), rendered also by the results of the multiple regression analysis as one of the most important variables explaining cumulative N_2O emissions (Table 4). This observation could indicate an important contribution of AOB to the cumulative emissions of MIN fertilisation in our study, a claim supported by Sterngren et al. (2015) and Rütting et al. (2021). These authors showed that AOB tend to dominate the nitrification process over AOA under high ammonium conditions (Verhamme et al., 2011) in case that other environmental conditions are favourable as well (Nicol et al., 2008; Prosser and Nicol, 2012). This is noteworthy, given the fact that archaeal ammonia oxidisers (AOA) are more abundant than AOB in agricultural soils (Leininger et al., 2006). Similar findings were shown in a study where selecting for mineral over mineral-organic mixture fertiliser led towards higher contribution of nitrification, as determined by N_2O isotopocule deltas (Lin et al., 2020). In addition to nitrification, AOB are the main group of organisms capable of nitrifier-denitrification (Wrage-Mönnig et al., 2018), a trait especially advantageous under variable field water conditions over the season, as it has been shown that a substantial part of N_2O emissions can be sourced back to nitrifier-denitrification under suboptimal conditions for denitrification and nitrification (Kool et al., 2011; Zhu et al., 2013). This could indicate a broader functioning niche for AOB and could support their important contribution to cumulative N_2O emissions over the season in our study.

Correlations between environmental factors and functional microbial community abundance were proven on multiple occasions, however, there is an ongoing debate on how well the gene abundance data correlate with microbial processes and their end products. While a perspective paper by Rocca et al. (2015) showed a weak correlation between gene abundances and respective processes, specific studies focusing on denitrifiers and N_2O production show differential results, linking N_2O emissions with gene abundances (Assémien et al., 2019; Wang et al., 2019; Wang et al., 2021b; Krauss et al., 2017a; Snider et al., 2015) and gene diversity (Domeignoz-Horta et al., 2015). Although specific conclusions among these studies vary, identified relationship between one or more studied genes and N_2O rates are usually found. These differences in conclusions, can on one hand, be driven by different pedo-climatic conditions of each individual study and, on the other hand, can also be related to methodological biases. Namely, DNA extraction, PCR inhibition and efficiency, and selected primers can all influence the results of an individual study (Sáenz et al., 2019; Wang et al., 2017; Pérez et al., 2013; Brankatschk et al., 2012; Wei et al., 2015). Nonetheless, through the application of thoroughly documented methods and conducting extensive laboratory examinations like PCR inhibition testing, it remains possible to critically evaluate meaningful microbial community patterns and connect them to occurring biological processes.

5. Conclusions

Our study showed that fertilisation was the main factor driving N_2O emissions, while microbial community partitioning and yields were influenced by both tillage and fertilisation. In particular, AOB responded strongly to mineral fertilisation, and *nosZI* and *nosZII* dominated NT and CT systems, respectively.

The combination of NT and organic (ORG) fertilisation favoured the abundance of *nosZII* and affected the (*nirK* + *nirS*)/(*nosZI* + *nosZII*) ratio, indicating an increased N_2O sink capacity. This finding is consistent with the lower cumulative N_2O emissions observed in the NT-ORG treatment.

The abundance of individual *nosZ* clades seems to be driven by soil and environmental factors influenced by tillage and, in the case of *nosZII* community, to some extent by fertilisation. Further elucidation of niche partitioning within *nosZ* communities and their role in the N_2O sink could be achieved by investigating *nosZ* community structure and its correlation with potential process rates.

Although the NT-ORG treatment demonstrated a positive trade-off between soil organic carbon content and N_2O emissions, concerns arise due to the observation of one of the lowest maize yields in this treatment. Therefore, trade-offs between C stocks and total GHG emissions should be assessed in combination with other synergistic approaches aimed at improving yields, prior to considering the widespread adoption of this combination of agricultural practices.

CRediT authorship contribution statement

Anton Govednik: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Klemen Eler:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Rok Mihelič:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization. **Marjetka Suhadolc:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172054.

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