Minimizing tillage modifies fungal denitrifier communities, increases denitrification rates and enhances the genetic potential for fungal, relative to bacterial, denitrification

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A B S T R A C T

Nitrous oxide (N_2O) emissions from arable soils are predominantly caused by denitrifying microbes, of which fungal denitrifiers are of particular interest, as fungi, in contrast to bacteria, terminate denitrification with N_2O. Reduced tillage has been shown to increase gaseous nitrogen losses from soil, but knowledge of how varying tillage regimes and associated soil physical and chemical alterations affect fungal denitrifiers is limited. Based on results from a long-term (>40 years) tillage experiment, we show that non-inversion tillage resulted in increased potential denitrification activity in the upper soil layers, compared to annual or occasional (every 4–5 years) conventional inversion tillage. Using sequence-corrected abundance of the fungal *nirK* gene, we further identified an increased genetic potential for fungal denitrification, compared to that caused by bacteria, with decreasing tillage intensity. Differences in the composition and diversity of the fungal *nirK* community imply that different tillage regimes select for distinct fungal denitrifiers with differing functional capabilities and lifestyles, predominantly by altering carbon and nitrogen related niches. Our findings suggest that the creation of organic hotspots through stratification by non-inversion tillage increases the diversity and abundance of fungal denitrifier communities and modifies their composition, and thus their overall relevance for N_2O production by denitrification, in arable soils.

1. Introduction

Agricultural soils are major sources of nitrous oxide (N_2O), a potent greenhouse gas that also contributes to the depletion of the ozone layer in the atmosphere. Denitrification, an anaerobic microbial process that reduces nitrate or nitrite to gaseous nitrogen compounds, including N_2O, is the most important process contributing to N_2O emissions from agricultural soils (Ward, 2013). Denitrification is a common functional trait among bacteria as well as in certain archaea and fungi (Shoun et al., 1992; Philippot et al., 2007; Kim et al., 2009). However, unlike many prokaryotic denitrifiers, all genetically described fungal denitrifiers lack the N_2O reductase (Shoun et al., 2012; Graf et al., 2014; Higgins et al., 2016) which makes them a potential source of N_2O. Representative fungal denitrifiers, mainly from the genera *Fusarium, Aspergillus, Trichoderma,* and *Penicillium* have been isolated from agricultural soils on

multiple occasions, and demonstrated to produce N_2O in pure culture (Maeda et al., 2015; Mothapo et al., 2015). Ectomycorrhizal species have also been reported to produce N_2O (Prendergast-Miller et al., 2011). There are also reports showing the importance of fungal denitrifiers for *in situ* N_2O emissions (Mothapo et al., 2013; Wei et al., 2014; Ibraim et al., 2019).

Denitrification is directly affected by a variety of soil factors, of which carbon (C) and nitrogen (N) content as well as soil aeration are the most important for both bacterial and fungal denitrification (Wagner-Riddle et al., 2020). Fungal denitrification has been shown to be greater than bacterial denitrification at lower soil pH (<5.5), under conditions with more complex C substrates, e.g. lignocellulose, and under sub-oxic rather than anoxic soil conditions that typically promote denitrification (Chen et al., 2015a,b). Many of these factors are altered by soil management practices, and tillage regimes are especially

relevant since they impact the depth-distribution of soil organic matter and affect the soil pore architecture which in turn influences soil aeration (Wuest, 2001; Kautz, 2015; Ramesh et al., 2019). Minimum tillage, often in combination with other practices, has been promoted to improve soil health through enhanced microbial activity and increased soil organic matter (SOM) in the surface layer (Doran, 1980; Six et al., 2002; Blanco-Canqui and Wortmann, 2020; Krauss et al., 2020). However, systems with reduced tillage have been shown to increase gaseous N-losses from soil (Six et al., 2002; Lognoul et al., 2017). It has been proposed that increased N_2O emissions are caused by sub-oxic and anoxic hotspots created by the degradation of crop residues (Kravchenko et al., 2018), thereby linking reduced tillage with increased denitrification activity (Wang and Zou, 2020). Ladan and Jacinthe (2016) used selective inhibitors to distinguish between bacterial and fungal denitrification in differently tilled soils, reporting higher overall fungal denitrification rates in no-till soils. Although soil fungal community structure has been shown to be affected by tillage practices (Hydbom et al., 2017; Legrand et al., 2018; Wang et al., 2016), little is known about the effects of tillage regimes on the composition, diversity, and abundance of fungal denitrifiers. Increased knowledge of fungal denitrifiers and their responses to tillage-induced changes in soil structure and nutrient availability is essential to understand the interplay between sustainable soil management and N_2O emissions, and to provide a mechanistic basis for undertaking actions for N management that may improve terrestrial climate regulation services.

The main objective of this study was to assess long-term (>40 years) effects of three different tillage regimes (inversion tillage, occasional inversion tillage and non-inversion tillage) and associated soil structural and chemical changes in the abundance, diversity, and composition of fungal denitrifier communities. By including both soil chemical and physical properties, we aimed to achieve a comprehensive picture of edaphic factors that may promote the genetic potential of fungal denitrification and specific fungal denitrifier communities. The relative importance of bacterial and fungal denitrifiers was further assessed in terms of their respective genetic potential and correlated with potential denitrification rates. Due to the anticipated differences in the stratification of the vertical soil profile and creation of microhabitats depending on tillage regime, we expected compositional differences in fungal denitrifier communities across the soil layers and increased overall diversity with minimized tillage. Further, we hypothesized that denitrification activity in the upper soil layer would increase under non-inversion tillage because of increasing carbon accumulation, and that the genetic potential of fungal denitrification would increase relative to that of bacteria due to less physical disruption of the mycelia. To investigate whether the observed effects of tillage practices on fungal denitrifiers were due to an overall effect on the soil microbial communities, we also determined the abundance, diversity, and composition of the total bacterial and fungal communities.

2. Materials and methods

2.1. Experiment design, soil sampling and soil physicochemical analysis

Soil samples were collected in November 2017 from a long-term tillage experiment maintained since 1974, and located in Uppsala, Sweden (latitude: 59.82, longitude: 17.64). The soil is classified as a Cambisol (Eutric) with 45.7% clay, 33.9% silt and 20.3% sand. The experiment includes five different tillage treatments in a randomized complete block design with four blocks as previously described (Etana et al., 2009; Arvidsson et al., 2014; Parvin et al., 2014). This study focuses on three treatments with decreasing tillage intensity: conventional tillage with soil inversion (CIT) consisting of yearly mouldboard ploughing to ca. 25 cm depth followed by conventional seedbed preparation with a disc harrow or cultivator; shallow tillage with occasional inversion by mouldboard ploughing every 4–5 years (OIT); and shallow non-inversion tillage (NIT) based on yearly disking/cultivating to ca. 10

cm. In 2017, the plots had been cropped with spring barley. Sampling took place between harvest and autumn tillage (i.e. in undisturbed stubble), and the CIT soils were last ploughed in fall 2016 and the OIT soils in November 2015.

For microbial analyses, five soil cores (30 mm diameter) were taken from the soil surface to a depth of 18 cm at random locations within each plot. The soil cores were then divided into two depths: 2–8 cm (within the tilled layer of all systems) and 12–18 cm (below tillage depth of shallow tillage, but within the ploughed layer of the other systems). The five cores were then combined into separate composite samples for each depth. This resulted in 24 soil samples (three treatments, two depths, four blocks), with four biological replicates (n = 4) per tillage treatment and soil layer. Similarly, composite samples for soil chemical analysis were taken at each depth. For soil physical measurements, three undisturbed soil cores (72 mm diameter, 50 mm height) were sampled in each plot at depths of 3–10 cm and 13–20 cm. Samples for both physical and chemical analysis were stored at 4 °C prior to analysis, and the composite samples were homogenized by sieving (2 mm ⌀). Samples for microbial analysis were placed in −20 °C storage immediately after collection, then thawed and sieved at 4 mm ⌀ before being stored again at −20 °C until further processing.

The water content at sampling was measured gravimetrically by the loss of weight after drying 10 g of each soil at 60 °C for 48 h. Total carbon (C_{tot}), total nitrogen (N_{tot}), pH, total K and P (HCl-extracted), plant available K and P (ammonium-lactate [AL] extracted) were measured at the Soil and Plant Laboratory, SLU Uppsala, Sweden. Three undisturbed soil cores per treatment and block were used for measurements of water retention and metrics derived thereof, as follows. The undisturbed soil cores were slowly saturated from the bottom, and drained stepwise on porous plates (EcoTech Umwelt-Meßsysteme GmbH suction plates) to four different matric potentials (−10, −50, −300 and −600 hPa). The saturated soil cores were weighed before and after oven-drying (24 h at 105 °C) to determine soil water content at each matric potential. Water retention at −15,000 hPa was determined on remoulded soil samples in a pressure plate system. Measured particle densities were used to calculate total porosity. Air-filled porosity was calculated for each matric potential as the difference between total porosity and volumetric water content, and water-filled pore space as the ratio of volumetric water content to total porosity.

2.2. Potential denitrification assays

Potential denitrification rates were measured with the addition of either nitrate (NO_3^-) or nitrite (NO_2^-) as the terminal electron acceptor. Nitrite was used to account for denitrifiers that cannot reduce nitrate, notably fungi (Shoun and Takaya, 2002; Maeda et al., 2015). The experimental setup followed a modified version of the protocol for potential denitrification described by Pell et al. (1996). The assays were performed with 5 g of soil, thawed for 24 h in 125 mL Duran bottles at room temperature. Distilled water was then added to a volume of 20 mL, and the bottles were hermetically sealed before exchanging the headspace with nitrogen gas to create anoxic conditions. After 30 min of incubation at 25 °C and constant agitation (180 rpm), 10 mL acetylene was added prior to adding 1 mL of substrate solution consisting of 25 mM Glucose, 75 mM Na-acetate, and 37.5 mM Na-succinate and 3 mM KNO_3 or 3 mM $NaNO_2$. Bottles were then incubated for 150 min, and a volume of 0.5 mL was sampled from the headspace every 30 min. The N_2O concentration in the headspace was determined by gas chromatography (Clarus-500 with an Elite-Q PLOT phase capillary column and ^{63}Ni electron-capture detector, PerkinElmer, Hägersten, Sweden), with serial dilutions of N_2O as a standard. The potential denitrification rate was then calculated based on the non-linear model described by Pell et al. (1996) utilizing the "*nls2*" package in the R statistical programming environment.

2.3. DNA extraction and quantification of denitrification gene abundance

Soil DNA was extracted from each of the 24 samples using the DNeasy PowerLyzer PowerSoil Kit (Qiagen AB, Kista, Sweden) according to the manufacturer's instructions. The extracted DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham MA, USA). Prior to quantification of specific genes, possible PCR inhibition by co-extracted inhibitors was examined by adding a known amount of pGEM-T plasmid (Promega, Madison WI, USA) to 10 ng of template DNA or distilled water, followed by real-time quantitative PCR (qPCR) with plasmid-specific primers for each sample. No inhibition was detected for the amount of template DNA used in the reactions based on comparing cycle threshold (C_t) values between reactions with DNA template and non-template controls.

Quantification of gene abundance was performed using a CFX Connect Real-Time System (Bio-Rad, Hercules CA, USA). All reactions for bacterial 16S rRNA and denitrification genes were performed in triplicate runs (three technical replicates per biological replicate). The total reaction volume of 15 µL consisted of 1X iQ™ SYBR Green supermix (Bio-Rad), 1 µg/µL bovine serum albumin (New England Biolabs, Ipswich, MA, USA), primers (nirK: 0.5 µM, nirS: 0.8 µM, nosZI: 1.0, nosZII: 2.0 µM, 16S rRNA gene: 0.5 µM) and 10 ng template DNA. Fungal internal transcribed spacer (ITS) 2 and fungal *nirK* abundance were measured in duplicate runs (two technical replicates per biological replicate) with a total reaction volume of 15 µL. These reactions consisted of Takyon Low Rox SYBR MasterMix dTTP Blue (Eurogentec, Seraing, Belgium), 1% T4 gene 32 protein (MP Biomedicals, Strasbourg, France), 2.0 µM primer, and 2 ng template DNA and were run on a QuantStudio TM 5 Real-Time PCR system (Thermo Fisher Scientific). Primer sequences and amplification protocols for all qPCR assays are listed in Table S1. Since the fungal nirK primers also amplify bacterial nirK (Bonilla-Rosso et al., 2016), we corrected the abundance values for each sample individually, using the respective fraction of fungal nirK sequence derived from sequencing analysis. Large sequence similarities between *nirK* in fungi and bacteria make it difficult to target fungal *nirK* exclusively (Chen et al., 2016; Ma et al., 2019); hence corrections based on sequence-data are necessary for reliable quantification.

2.4. Amplicon sequencing and sequence processing

The microbial community composition was analyzed by amplicon sequencing of fungal ITS2, V3–V4 of the bacterial 16S rRNA gene, and fungal *nirK*. Primers and reaction conditions for each target are listed in Supplemental Table S2. Sequencing of all three target regions was performed at the National Genomics Infrastructure (NGI)/Uppsala Genome Center in Uppsala, Sweden.

Amplification of the fungal ITS2 region was performed as described previously (Ihrmark et al., 2012) with two replicate PCR reactions of 50 µL per sample. Each reaction consisted of 1.25 U DreamTaq polymerase (Thermo Fisher Scientific), 1x DreamTaq Reaction Buffer, 2.75 mM MgCl, 0.5 µg/µL BSA (New England Biolabs), 0.2 mM dNTPs, tagged primer mix (0.5 µM gITS2, 0.3 µM ITS4) and 20 ng template DNA. Thermal cycling of the reaction was performed following the protocol of Castaño et al. (2020) with initial optimal cycle number determination for each sample, resulting in 23–29 cycles. The final libraries were sequenced after adapter ligation on a Pacific Biosciences Sequel sequencing platform.

For 16S rRNA gene amplicons, a two-step amplification procedure was performed. Reactions in the first step consisted of 1x Phusion PCR Mastermix (Thermo Fisher Scientific), 1 µg/µL BSA (New England Biolabs), 0.25 µM of primers Pro341F and Pro805R (Takahashi et al., 2014) with adaptors for Illumina Nextera barcoded sequencing primers, and 10 ng template DNA in a total volume of 15 µL. All reactions were prepared in duplicate with 25 cycles of reaction conditions described in Table S2. Replicate reactions for each sample were then pooled and purified with Sera-Mag™ Select purification beads (Cytiva,

Marlborough, MA, USA) according to the manufacturer's protocol. The second PCR step consisted of 1x Phusion PCR Mastermix (Thermo Fisher Scientific), 1 µg/µL BSA, 0.2 µM of Nextera barcoded sequencing primers and 3 µL purified first step PCR product in a total volume of 30 µL. Thermal cycling was performed using the same conditions as the first step but with 8 amplification cycles. The final products were again bead purified, quantified using a Qubit fluorometer, and pooled in equimolar proportions across samples. The final pool was then sequenced on an Illumina MiSeq platform using v2 (2 × 250 bp) chemistry.

Fungal *nirK* amplicons were produced in a two-step protocol similar to that used for 16S rRNA with primers developed by Maeda et al. (2015). Reactions in the first step consisted of 1x Terra PCR Direct Polymerase reaction buffer and 0.025 U Terra polymerase (Takara Bio, Kusatu, Shiga, Japan), 1 µM of primers EunirK-F1 and EunirK-R1 with Nextera adaptors, and 55 ng template DNA in a total volume of 25 µl. Four replicate reactions were performed for each sample, with 30 cycles of thermal cycling conditions as specified in Table S2, followed by pooling of replicates and bead purification with Sera-Mag™ Select purification beads. The second amplification step was performed using DreamTaq DNA Polymerase (Thermo Fisher Scientific) with 1x DreamTaq Reaction Buffer, 0.2 mM dNTPs, 0.2 µM Nextera barcoded sequencing primers, 1 U DreamTaq polymerase, and 6 µL purified first-step PCR product, and was prepared in quadruplicate 30 µL reactions per sample. Thermal cycling conditions were as follows: an initial denaturation step of 3 min at 95 °C followed by 8 cycles of 30 s at 95 °C for, 30 s at 55 °C, 45 s at 72 °C and final elongation of 10 min at 72 °C. The resulting replicate PCR products were pooled, bead purified, and quantified as described above. The final library was prepared by equimolar pooling of samples and sequenced on a MiSeq platform using v3 (2 × 300 bp) chemistry.

2.5. Bioinformatic analyses

For the fungal communities, 238 260 circular consensus ITS2 sequences were filtered and clustered with the SCATA sequence analysis pipeline (https://scata.mykopat.slu.se/). Briefly, sequences shorter than 200 bp or sequences with mean quality scores below 20 or containing bases with a quality score below 10, were removed, as well as sequences with missing or mismatched primer or tag sequences. The remaining 53.8% of total sequences were compared pairwise using the USEARCH algorithm, followed by single-linkage clustering at 98.5% similarity. Removal of singleton reads resulted in 84 400 reads with an average of 3517 reads per sample grouped into 957 operational taxonomic units (OTUs), corresponding to 35.4% of total sequencing reads. Representative sequences for the resulting OTUs were classified utilizing the Protax taxonomic classification tool of the PlutoF biodiversity platform (plutof.ut.ee) as described by Clemmensen et al. (2021).

The 16S rRNA gene sequences were demultiplexed utilizing the MultiQC software (https://multiqc.info/) and subjected to the sequence processing and analysis pipeline DADA2 (Callahan et al., 2016) to obtain amplicon sequence variants (ASV). The taxonomic classification was performed utilizing the implementation of the naïve Bayesian classifier in DADA2 with the provided Silva nr v138.1 training set (obtained June 21, 2021). Based on the classification, all non-bacterial ASVs were excluded, resulting in a total of 1.3 million reads corresponding to 30.8% of total reads, with an average of 54 522 sequences per sample that were further grouped into 8517 ASVs.

The sequences of fungal *nirK* were demultiplexed and processed using DADA2, similar to bacterial 16S rRNA sequences. One sample, representing a biological replicate of conventionally tilled soils at the lower depth, had to be discarded due to an overall low sequence quality and abundance. A total of 21 026 ASVs was obtained from 8 953 982 reads (19.5% of total reads). Classification of fungal *nirK* ASVs was performed with GraftM (Boyd et al., 2018) utilizing a reference alignment and phylogeny for fungal *nirK* and default search and phylogenetic placement parameters. In brief, a seed alignment of *nirK* amino acid

sequences obtained from Bonilla-Rosso et al. (2016) combined with fungal *nirK* sequences from pure culture studies (Maeda et al., 2015; Wei et al., 2015; Chen et al., 2016) was used to create a hidden Markov model (HMM) to search archaeal, bacterial and fungal genome assemblies retrieved from the NCBI GenBank and NGI repositories (September 2019) using the HMMer software (Eddy, 2008). The resulting hits were aligned by amino acids with HMMer, and the alignment was manually curated in the ARB software environment (Ludwig et al., 2004). Multi-copper oxidase proteins were identified as an outgroup (Bartossek et al., 2012), and fungal *nirK* as well as a selection of bacterial and archaeal *nirK* sequences were used to generate a reference phylogeny using the IQ-Tree software (Nguyen et al., 2015). Automatic model selection was used to predict the best model (LG + R6) for the phylogenetic tree. The reference package was then generated from the phylogeny, alignment, and listing of taxonomic affiliation using GraftM. Following classification, 11 223 of the resulting ASVs were determined to be *nirK*, of which 257 were found to be of fungal origin. This corresponded to $0.6 \pm 0.3\%$ (mean $\pm$ SD) and $0.4 \pm 0.2\%$ (mean $\pm$ SD) of the total reads obtained in the upper and lower soil layers, respectively. The resulting classification table for fungal *nirK* ASVs was examined and manually curated by BLAST search (Altschul et al., 1990) against the NCBI nr protein database. Phylogenetic placements of ASVs within the fungal *nirK* tree were visualized using iTOL v5 (Letunic and Bork, 2021). All sequence data can be obtained under NCBI Bioproject accession number PRJNA792806.

2.6. Statistical analyses and community diversity

All statistical analyses were performed in the R environment. Analysis of variance (ANOVA) testing for main and interaction effects of tillage regimes and soil depth on soil properties, gene abundance data and denitrification activity were based on generalized linear models using a gamma-distribution error model with a log link function, whilst diversity of fungal, bacterial and fungal denitrifier communities used a linear model approach. The normality of the residuals was tested with the Shapiro-Wilk test. Pairwise and multiple comparisons among tillage and depth were performed with the Dunn–Šidák correction method and Tukey's HSD post-hoc testing where applicable. Spearman's correlations were used to test for relationships between soil physicochemical properties, gene abundance, diversity measures and potential denitrification.

Analyses of community diversity and structure were based on rarefied OTU and ASV tables for fungal ITS2, bacterial 16S rRNA gene and fungal *nirK* datasets, respectively. Alpha-diversity based on Shannon's

H' was calculated using the R-packages "Phyloseq" (McMurdie and Holmes, 2013) and "microbiome" (Lahti and Shetty, 2017). The effects of tillage practices and sampling depth on community structure were visualized by non-metric multidimensional scaling of Hellinger distances and statistically evaluated utilizing PERMANOVA with 999 permutations. Soil variables correlated with the community structure and OTUs/ASVs driving community separation were identified by significant correlation with the NMDS-axis (soil variables: $P < 0.05$, species: $P < 0.01$, 999 permutations) using the *envfit* function in the "vegan" package (Oksanen et al., 2020).

3. Results

3.1. Effect of tillage regime on soil properties

Total C and N content, as well as the C:N ratio, differed significantly across tillage regimes and with soil depth (Table S3). The soil in plots subjected to non-inversion tillage had the highest total C and N content in the upper layer. However, no differences between tillage systems were observed in the lower layer (Table 1). There was also a significant effect of tillage on total K and P and plant available K at both soil depths. Soil pH and plant available P did not change across the different tillage regimes. Among the physical properties, water content at sampling was significantly affected by soil depth. Interaction effects of tillage and depth were also observed for water content at sampling, bulk density, porosity, and air-filled porosities at -300 and -600 hPa. All other soil physical properties were unaffected by tillage and/or depth.

3.2. Potential denitrification activity with NO_3^- or NO_2^- as a terminal electron acceptor

The effect of tillage regime on potential denitrification activity was dependent on soil depth, with significantly different patterns at each depth (Fig. 1; Table S4). In the upper soil layer, activity was highest in the NIT soil and dropped as tillage intensity increased. The opposite pattern was observed in the lower layer, where activity was lowest in the NIT soil and increased with tillage intensity. This effect was more pronounced when NO_2^- was supplied as the electron acceptor, with activity rates nearly twice as high as those obtained with NO_3^- addition. Correlation analyses revealed that activity increased with increasing available K, total C, porosity, and water content and declined with increasing bulk density (Table S5) regardless of electron acceptor. The NO_3^- induced denitrification rates also correlated with total N content.

Table 1
Effects of conventional inversion (CIT), occasional inversion (OIT) and non-inversion tillage (NIT) on edaphic factors in the upper and lower layer of the topsoil (mean $\pm$ SD, n = 4). Different letters indicate significant differences within tillage treatment and depth based Šidák post hoc testing, P < 0.05.

Edaphic factor	Upper soil layer			Lower soil layer		
	CIT	OIT	NIT	CIT	OIT	NIT
HCl–K (mg·100g^{-1})	460.51 ± 57.39^a	360.51 ± 16.99^b	369.38 ± 24.02^b	458.19 ± 52.94^a	343.88 ± 15.55^b	391.57 ± 15.68 [ab]
HCl–P (mg·100g^{-1})	62.40 ± 0.45^a	53.31 ± 3.04^b	61.26 ± 3.43^a	61.52 ± 2.45^a	56.76 ± 4.30 [ab]	57.41 ± 1.62 [ab]
AL-K (mg·100g^{-1})	15.50 ± 1.94 [ab]	12.71 ± 0.41 [ab]	15.86 ± 1.58^a	15.16 ± 2.66 [ab]	12.60 ± 1.62^b	12.44 ± 0.54^b
AL-P (mg·100g^{-1})	4.10 ± 0.35^a	3.81 ± 0.31^a	4.85 ± 0.76^a	4.02 ± 0.77^a	4.98 ± 2.44^a	4.22 ± 1.00^a
Total C (%)	1.72 ± 0.09^b	1.54 ± 0.10^b	2.29 ± 0.04^a	1.70 ± 0.20^b	1.66 ± 0.11^b	1.68 ± 0.09^b
Total N (%)	0.18 ± 0.01^b	0.16 ± 0.01^b	0.22 ± 0.00^a	0.18 ± 0.02^b	0.17 ± 0.01^b	0.18 ± 0.01^b
C:N	9.59 ± 0.36^b	9.47 ± 0.33^b	10.42 ± 0.20^a	9.43 ± 0.34^b	9.63 ± 0.35^b	9.31 ± 0.20^b
pH	6.12 ± 0.10^a	6.16 ± 0.31^a	5.95 ± 0.03^a	6.15 ± 0.13^a	5.96 ± 0.22^a	6.08 ± 0.04^a
Water content (%)	22.0 ± 0.02 [ab]	22.7 ± 0.01 [ab]	27.4 ± 0.05^a	21.7 ± 0.03^b	21.3 ± 0.02^b	19.9 ± 0.01^b
Bulk Density (g·cm^{-3})	1.42 ± 0.09^a	1.48 ± 0.03^a	1.42 ± 0.04^a	1.46 ± 0.06^a	1.42 ± 0.09^a	1.52 ± 0.03^a
Porosity (cm^{-3}·cm^{-3})	0.46 ± 0.03^a	0.44 ± 0.01^a	0.47 ± 0.02^a	0.45 ± 0.02^a	0.46 ± 0.04^a	0.42 ± 0.01^a
WFPS 50 hPa	0.873 ± 0.05^a	0.933 ± 0.04^a	0.891 ± 0.01^a	0.915 ± 0.05^a	0.901 ± 0.04^a	0.937 ± 0.01^a
WFPS 300 hPa	0.811 ± 0.05^a	0.869 ± 0.05^a	0.836 ± 0.01^a	0.854 ± 0.05^a	0.773 ± 0.14^a	0.879 ± 0.01^a
WFPS 600 hPa	0.781 ± 0.05^a	0.836 ± 0.05^a	0.805 ± 0.01^a	0.824 ± 0.05^a	0.805 ± 0.03^a	0.846 ± 0.01^a
AFP 50 hPa	0.06 ± 0.026^a	0.03 ± 0.02^a	0.051 ± 0.007^a	0.038 ± 0.021^a	0.048 ± 0.02^a	0.027 ± 0.004^a
AFP 300 hPa	0.089 ± 0.027 [ab]	0.059 ± 0.022^a	0.077 ± 0.007 [ab]	0.066 ± 0.021 [ab]	0.111 ± 0.075 [ab]	0.052 ± 0.004^b
AFP 600 hPa	0.102 ± 0.026 [ab]	0.073 ± 0.021 [ab]	0.091 ± 0.008 [ab]	0.08 ± 0.02 [ab]	0.093 ± 0.02 [ab]	0.066 ± 0.005^b

Water content: at the time of sampling, WFPS: Water-filled pore space, AFP: Air-filled porosity.
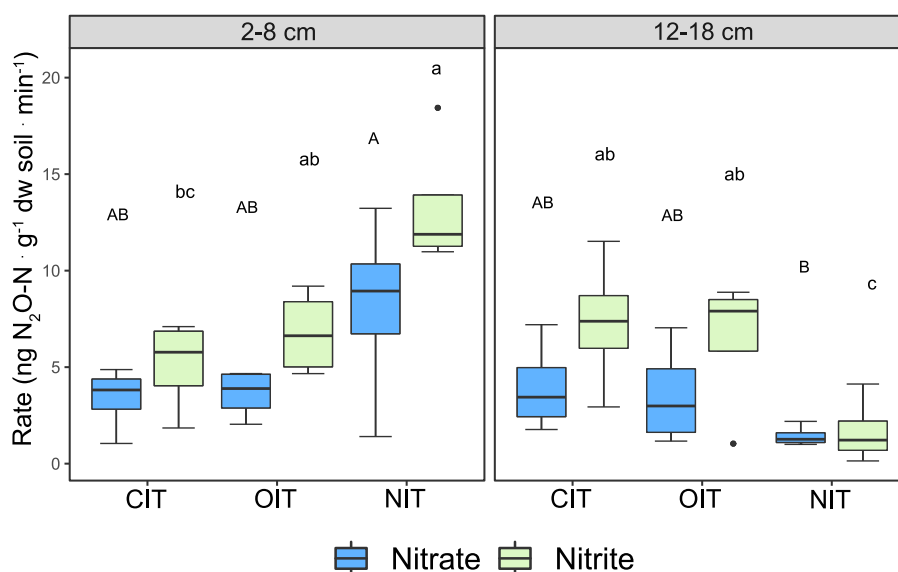
Fig. 1. Potential N₂O production with addition of NO_3^- or NO_2^- in the upper and lower layer of the topsoil subject to conventional inversion (CIT), occasional inversion (OIT) and non-inversion tillage (NIT). Letters above the boxes indicate statistically significant differences among tillage and depth for each substrate (P < 0.05, n = 4). Upper-case letters represent the comparison among measurements with NO_3^- and lower-case letters show the comparisons of measurements with NO_2^- as substrate.

3.3. Abundances of fungal ITS2, bacterial 16S rRNA gene and denitrification genes

Tillage regime affected the total abundance of fungi and bacteria in the upper soil layer, whilst no differences were detected in the lower layer (Table S6). The copy numbers of both ITS2 and 16S rRNA genes were approximately two to three times higher in the upper layer of NIT compared to the other tillage treatments (Table S7). Both groups were positively correlated with soil water content but no relationship to any other physicochemical factor was observed. Fungal ITS2 and bacterial 16S rRNA abundances were further positively correlated with potential activity using either electron acceptor, although a stronger association was observed between ITS2 copy number and activity based on NO_2^- (Table S5).

Total abundance of fungal denitrifiers, based on corrected fungal *nirK* gene copy numbers, was affected by tillage, and the highest abundance was observed in the upper layer of NIT (Fig. 2A, Table S7). However, no general effect of soil depth was observed (Table S6). The

relative abundance of fungal denitrifiers increased with decreasing tillage intensity (Fig. 2B, Table S6), although this effect was only significant in the upper soil layer. The ratio of fungal to bacterial denitrifier abundance increased with decreasing tillage intensity, with a significantly higher ratio in the upper layer of NIT compared with CIT (Fig. 2C, Tables S6 and S7). Nevertheless, bacterial denitrifiers (*nirS* and *nirK*) were about 100 times more abundant than the fungal denitrifiers across all treatments and followed a similar trend to that of the total bacteria, with the highest and lowest abundance of both *nirS*- and *nirK*-type bacterial denitrifiers in the upper and lower layers of NIT, respectively (Fig. S1A; Tables S6 and S7). This was reflected in the significantly lower relative abundance of total bacterial denitrifiers ([*nirS* + *nirK*] per 16S rRNA gene copy) in the lower layer of NIT, whereas no differences were observed between OIT and CIT at either depth (Table S6). The total abundance of both fungal and bacterial *nirK* was positively associated with NO_2^- induced denitrification activity, whereas total *nirS* abundance was correlated to potential activities using either electron acceptor, albeit more strongly to NO_2^- induced activity (Table S5). Like the total
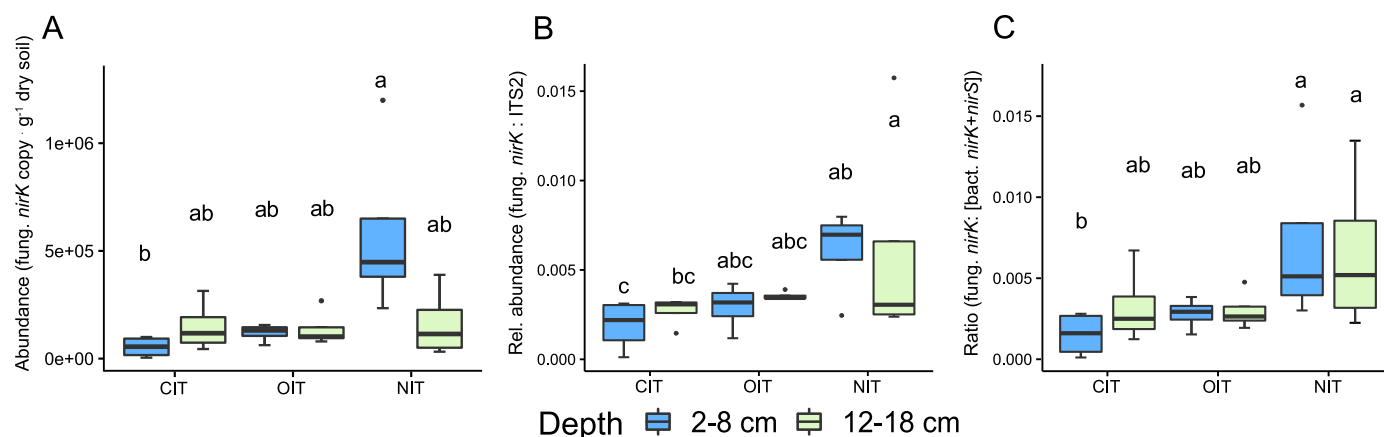

Fig. 2. Abundance of fungal denitrification genes in the upper and lower layer of the topsoil subject to conventional inversion (CIT), occasional inversion (OIT) and non-inversion tillage (NIT). **A)** Absolute abundance of fungal denitrifiers based on fungal *nirK* copy numbers. The abundances were corrected according to the proportion of fungal *nirK* sequences in the *nirK* sequence data set for each sample. **B)** Relative abundance of fungal denitrifiers within the total fungal community calculated as the ratio of fungal *nirK* to the fungal ITS2 copy numbers. **C)** The ratio of fungal to bacterial *nir* gene abundance, calculated as the number of fungal *nirK* divided by the sum of bacterial *nirK* and *nirS*. Different letters above the boxes indicate significant differences (P < 0.05, n = 4).

fungal and bacterial communities, all *nir* gene abundances were positively associated with soil water content. The prokaryotic *nir* genes were further associated with C:N, whereas the fungal *nirK* correlated with N content. The *nirS* abundance was also positively influenced by total C and porosity and negatively by bulk density. Abundance of N₂O reducers (*nosZ*I and *nosZ*II) in the upper soil layer, increased with decreasing tillage intensity (Tables S6 and S7), with *nosZ*II abundance being nearly 100 times greater than *nosZ*I. Total *nosZ*I abundance was significantly correlated with potential denitrification activities, water content, and total C and C:N ratios, whereas no correlations between *nosZ*II and activity or soil properties were found (Table S5).

3.4. Composition and diversity of fungal denitrifier communities

Despite the low percentage of reads remaining after removal of non-fungal *nirK* sequences, the rarefaction curves indicated that the extant diversity of fungal denitrifiers in the samples was well represented in the final dataset (Fig. S2). Most fungal *nirK* sequences placed in the reference phylogeny could be grouped into four fungal classes:

Sordariomycetes, Eurotiomycetes, Leotiomycetes and Tremellomycetes (Fig. 3A). While the majority of ASVs could not be classified to the genus level, those that could were mostly from the genera *Penicillium*, *Chaetomium*, *Fusarium* and *Talaromyces* (Fig. 3B). Amplicon sequence variants belonging to the genus *Trichoderma* were more common in NIT, whereas those belonging to the genus *Chloridium* were more abundant in CIT. *Fusarium* were detected in all treatments and were most abundant in the lower soil layer in NIT (Fig. 3B).

The structure of fungal denitrifying communities was significantly affected by tillage regime and soil depth, with communities in the upper soil layer differentiating along a distinct gradient of tillage intensity (Fig. 3C; Table S8). Communities in the lower layer differed overall from those in the upper layer; however, the separation by tillage regime was less pronounced in this layer. Differences in the structure of the fungal *nirK* community were significantly associated with the abundance of fifteen ASVs (Fig. 3C–D). Those classified as *Trichoderma* and unclassified *Pseudogymnoascus* within Ascomycota were positively associated (P < 0.01) with upper layers of NIT treated soils. In contrast, ASVs within the class Tremellomycetes in the phylum Basidiomycota, and the genus
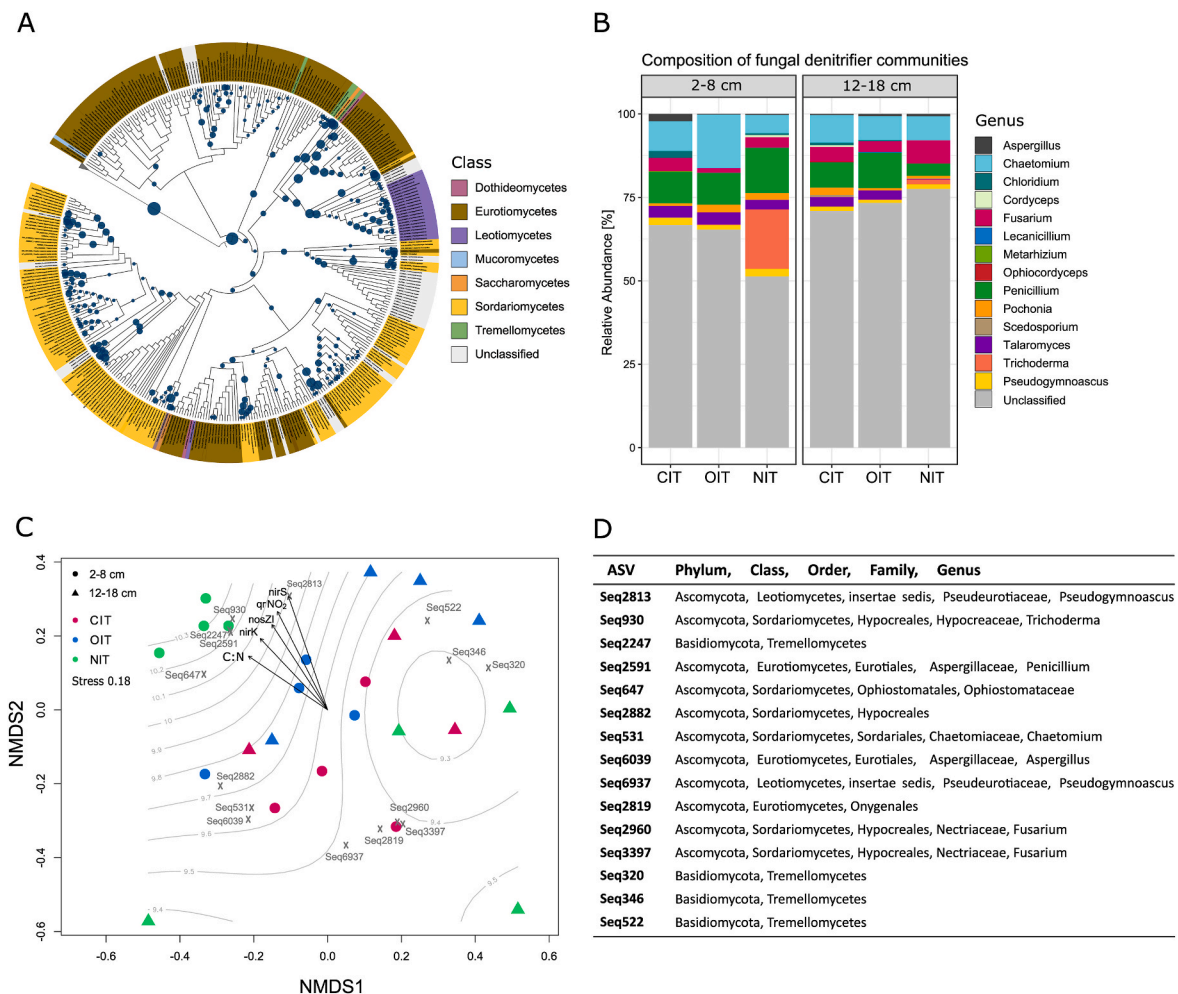

Fig. 3. Fungal denitrifier community structure and composition in the upper and lower layer of the topsoil subject to conventional inversion (CIT), occasional inversion (OIT) and non-inversion tillage (NIT). **A)** Phylogenetic placements of fungal *nirK* amplicon sequence variants within a fungal *nirK* phylogeny of 375 taxa and 42 bacterial, 5 archaeal and 26 multicopper oxidase leaves as the outgroup. The outer ring is coloured according to the taxonomic class. Taxa with unknown taxonomic rank are underlined in white. The majority of fungal *nirK* placements fall in the class of Eurotiomycetes (brown), Sordariomycetes (yellow), Leotiomycetes (purple) and Tremellomycetes (green). **B)** Relative abundances of fungal genera assigned by the placement of *nirK* sequences by tillage treatment and soil depth. 'Unclassified' refers to sequences that could not be assigned at the genus level. **C)** Non-metric multidimensional scaling (NMDS) of the fungal denitrifier community based on fungal *nirK* ASVs with smooth response curves of a distinct gradient of C:N ratio that varied across the treatment depending on depth using the function ORDISURF. Colours indicate tillage regime, and shape depicts soil depth. The arrows show significant correlations (P < 0.01) of denitrification genes and NO₂⁻-induced denitrification rates with the fungal community structure, with the direction indicating a positive correlation, and grey crosses represent significantly correlated ASVs (999 permutations). **D)** Taxonomic lineages of *nirK* ASVs contributing to the sample distribution in the NMDS.

Fusarium were positively correlated with the lower layer samples. The genus *Aspergillus* and a second unclassified *Pseudogymnoascus* were related to the upper soil layer in CIT. Among the measured soil characteristics, only C:N correlated significantly (P < 0.05) with differences in fungal *nirK* community structure along with the abundance of bacterial denitrifiers and NO_3^- related N_2O production (Fig. 3C). The strong clustering of upper NIT soil samples was also observed for overall bacterial and fungal communities (Fig. S3). Along with depth, an additional separation of the samples according to tillage intensity in both 16S rRNA and ITS communities could be observed, though more pronounced for the 16S rRNA communities (Fig. S3).

Alpha diversity (Shannon's *H'*) of fungal denitrifiers was significantly higher in the upper NIT soil layer than in the lower layer. However, no overall effect of tillage regime was observed (Table S9). A similar effect of depth was found for total fungal and bacterial communities. Regarding soil characteristics, fungal *nirK* diversity was significantly correlated with C, N, and the corresponding C:N ratio, whereas fungal ITS diversity was negatively correlated with water-filled pore space.

4. Discussion

Tillage and soil depth had a significant impact on the composition of the overall bacterial and fungal communities, as well as the fungal denitrifier community. Although several studies have reported an increase in diversity of both bacteria and fungi with decreasing tillage intensity (Wortmann et al., 2008; Smith et al., 2016; Legrand et al., 2018; Srour et al., 2020), we did not detect significant differences in diversity between tillage treatments, except for the lower diversity of all communities in the lower layer of the soils without inversion tillage. The pronounced differences in community structure and diversity between the upper and lower layers of soils under this regime are likely explained by the stratification of the soil profile with depth-related effects on the total content of C and N, in contrast to the homogenized plough layer (0–25 cm depth) of soils that were mouldboard ploughed annually. The separation of samples based on the fungal *nirK* community was driven by the presence of members related to *nirK* in Tremellomycetes and *Trichoderma* species in the upper soil layer and Tremellomycetes and *Fusarium* species in the lower soil layer. Positive responses of these organisms to reduced tillage have previously been observed (Bockus and Shroyer, 1998; Meng et al., 2010; Degrune et al., 2017). Members of the genus *Trichoderma* are highly competitive, saprobic, and opportunistic mycoparasitic organisms that produce cellulose- and chitin-degrading enzymes (Harman et al., 2004). Their prevalence in the upper layers of shallow disked-tilled soils could therefore be explained by their association with enriched crop residues that are retained in these soils, resulting in increased amounts of fungal substrates. Tremellomycetes, on the other hand, are yeasts with the ability to degrade complex compounds quickly and overcome anoxic events by fermentation (Yurkov, 2018; Vujanovic, 2021). The greater availability of labile C in less intensely tilled soils (Bongiorno et al., 2019) may promote copiotrophic fermenting yeasts. Similarly, the necessity for enzymatic capacity to degrade complex carbon compounds provides a niche for Tremellomycetes in lower soil layers. By contrast, the presence of *Fusarium* in the lower layers of minimum tilled soils may be caused by extrusion from upper layers, as other decomposers become more competitive over time (Leplat et al., 2013). Combined, this suggests depth-related niche differences between the identified taxa that might be linked to different stages in the decomposition of crop residues in the soil profile under non-inversion tillage. Besides, as microbial SOC decomposition decreases with depth, the C:N ratio decreases concomitantly (Hicks Pries et al., 2018), confirming our observation of the separation of the fungal denitrifier community with respect to variation in C:N. Our results are consistent with previous reports on changes in the composition of fungal and bacterial communities in response to differences in the vertical distribution of carbon (Sun et al., 2018; Zhang et al., 2020) along with

higher nutrient levels in the upper layers of less intensely tilled soils (Smith et al., 2016). Such changes in community composition are often reflected by modifications of functional guilds involved in carbon and nitrogen cycling (Waldrop et al., 2000; Wang et al., 2017; Hui et al., 2018; Srour et al., 2020), which may have consequences for ecosystem functioning and services such as soil fertility and climate regulation.

The frequency of all measured N-cycling genes increased with decreasing tillage intensity in the upper soil layers, suggesting that non-inversion tillage intensifies N cycling transformations leading to gaseous N losses. This is likely because the higher C content, coupled to higher N content, in the upper soil layers supported growth of these N-cycling guilds. However, the increase in bacterial denitrification genes could also be explained by an overall increase in bacterial abundance. By contrast, we observed a proportionally greater impact of non-inversion tillage on fungal denitrifiers than on the size of the total fungal community. The relative change of fungal *nirK* to the bacterial *nir* genes across the tillage gradient also indicates an increasing genetic potential for fungal denitrification compared to bacterial denitrification when tillage is shallow without inversion. Nevertheless, the primers used to detect prokaryotic *nirK* and *nirS* miss certain clades, indicating an underestimation of prokaryotic *nir* gene abundance (Wei et al., 2015; Bonilla-Rosso et al., 2016; Ma et al., 2019). However, this does not affect our conclusion that the genetic potential for fungal denitrifiers increases in NIT upper layers, because fungal denitrifiers remain much less abundant than their bacterial and archaeal counterparts (Table S7). The fungal to prokaryotic *nir* gene ratio was positively correlated with the soil N content, as also observed by Wei et al. (2014). Similar effects of tillage practices on bacterial denitrifier abundance have been reported previously (Kaurin et al., 2018; Wang and Zou, 2020), underpinning the potentially problematic aspects of non-inversion tillage regarding greenhouse gas emissions.

Fungal *nirK* abundance was positively correlated with NO_2^- associated denitrification rates, and potential denitrification rates almost doubled when NO_2^- was used as the terminal electron acceptor. By utilizing NO_2^- as the electron acceptor, microorganisms that start denitrification with NO_2^- can contribute to the process (Zumft, 1997; Philippot, 2002; Jones et al., 2008; Maeda et al., 2015), which is especially relevant for fungal denitrifiers as most of them lack nitrate-reductases (Higgins et al., 2018). Thus, the increased denitrification rates could in part be due to an increased fungal contribution, although NO_2^- addition may also lead to elevated levels of chemo-denitrification (Heil et al., 2016; Liu et al., 2019) or cellular detoxification rather than denitrification (Higgins et al., 2018; Shan et al., 2021). However, without data based on ^{15}N-approaches, we can only speculate on these explanations.

Non-inversion tillage increased potential denitrification in the upper soil layers, whereas the opposite trend was found in the lower soil layers. Our study shows that infrequent soil inversion is sufficient to homogenize C and N contents throughout the topsoil, whereas avoiding inversion by mouldboard ploughing instead causes accumulation in the top layer. This stratification of C and N through accumulation of organic material in the upper layers, can lead to anoxic hotspots due to high microbial activity, that in turn promote denitrification activity and thus increase emissions of N_2O (Six et al., 2002; Kravchenko et al., 2018). Furthermore, the observed differences in community composition between layers could also have played a role in the increased denitrification potentials in non-inverted soils, as physiological properties, and thus functional diversity of the denitrifier community, also determine denitrification rates (Philippot and Hallin, 2005). Increased denitrification activity as a response to decreased tillage intensity, particularly no-till, has been described earlier (Six et al., 2000; Baggs et al., 2003). Our findings underline the importance of soil organic matter related resources and genetic potential for increased denitrification, which were overall higher in the upper layer of non-inverted soils. The importance of C, N, and potassium availability was more pronounced in the potential denitrification assay with NO_3^-, and correlated with *nirS* and *nos*ZI

abundance, which are linked to organisms that contribute to complete denitrification, either alone or in cooperation within the denitrifier community.

5. Conclusion

Different tillage intensities profoundly affected the abundance and composition of fungal *nirK* communities. Non-inversion tillage stratified the soil profile and created more niches occupied by fungal denitrifiers with different capacities and lifestyles. The significant increase in the genetic potential for fungal denitrification, based on the sequence-corrected fungal *nirK* abundance, in relation to that of bacteria in soils without mouldboard ploughing, indicates that the role of fungal denitrifiers for N₂O emissions becomes more prominent under non-inversion tillage. Irrespective of the relative contributions of fungi and bacteria, non-inversion tillage increased potential denitrification activity, suggesting a negative impact of decreased tillage intensity on gaseous N losses. Based on the obtained results, shallow tillage combined with occasional ploughing, might be a viable option to maintain soil structure and suppress weeds, while restraining the denitrifying microbial communities and losses of N and possible emissions of N_2O.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108718.

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