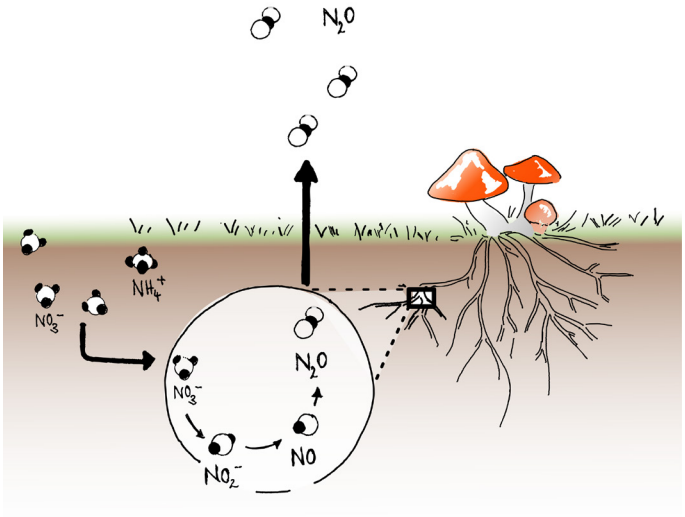
Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2022:51
Faculty of Natural Resources and Agricultural Sciences

Ecology of fungal denitrifiers in terrestrial ecosystems

Global patterns and effects of management in agricultural soils

Yvonne Bösch



Ecology of fungal denitrifiers in terrestrial ecosystems

Global patterns and effects of management in agricultural soils

Yvonne Bösch
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Plant Pathology
Uppsala

SLU
SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

DOCTORAL THESIS
Uppsala 2022

Acta Universitatis Agriculturae Sueciae
2022:51

ISSN 1652-6880
ISBN (print version) 978-91-7760-977-3
ISBN (electronic version) 978-91-7760-978-0

Uppsala
Print: SLU Grafisk Service, Uppsala 2022

Ecology of fungal denitrifiers in terrestrial ecosystems

Abstract

Denitrification is the dominant source of nitrous oxide (N_2O) from terrestrial ecosystems, a potent greenhouse gas and stratospheric ozone-depleting agent. Fungi can perform denitrification and terminate the process with N_2O, making them a potentially important source of N_2O. This thesis aimed to broaden the understanding of the ecology of this understudied group in the nitrogen cycle by assessing their global abundance and distribution in terrestrial ecosystems and evaluating the effects of soil management practices on their abundance, community composition, and contribution to N_2O production in agricultural soils.

Fungi carrying the denitrification marker gene *nirK* were rare and cosmopolitan and compared to prokaryotic denitrifiers, they were most abundant in forests and croplands, although prokaryotes dominated in all biomes. Agricultural management practices affected the abundance and community composition of fungal denitrifiers through changes in the availability of carbon and nitrogen. Long-term fertilization increased their abundance, irrespective of soil type or climate, but did not affect fungal contributions to potential N_2O production. Instead, the genetic potential of bacterial denitrifiers was more important. In unfertilized soils, biotic and abiotic controls of N_2O production rates were important, but in fertilized soils only abiotic soil properties were involved. Inversion tillage, compared with other types of tillage, led to a reduction of the genetic potential for fungal denitrification relative to that of bacterial denitrifiers and selected for fungi with opportunistic lifestyles. These results highlight that fungal denitrifiers are found across all terrestrial ecosystems, are significantly influenced by soil management but contribute less to N_2O emissions than their prokaryotic counterparts.

Keywords: fungal denitrification, genetic potential, nitrous oxide, soil

Author's address: Yvonne Bösch, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology , Uppsala, Sweden.

Denitrifierande svampars ekologi i terrestra ekosystem

Sammanfattning

Den mikrobiella processen för denitrifikation är den dominerande källan till lustgas (N_2O) från terrestra ekosystem, en långlivad växthusgas som också bryter ned ozonskiktet. Denitrifikation studerades nästan uteslutande i bakterier tills det upptäcktes att svampar kan bidra till processen, vilket gör dem till en potentiellt viktig N_2O källa. Syftet med denna doktorsavhandling är att bredda vår förståelse av ekologin hos denna mindre studerade grupp i kvävets kretslopp genom att undersöka deras globala förekomst och utbredning i terrestra ekosystem och effekterna av brukningsåtgärder på deras antal, samhällssammansättning och eventuella bidrag till N_2O-produktion i jordbruksmark.

Denitrifierande svampar som har genen *nirK,* en markör för denitrifikation, var sällsynta och kosmopolitiska i den globala utbredningstudien. Jämfört med prokaryota denitrifierare var de vanligast i skogsjordar och åkermark, men prokaryoter dominerade i alla biom. Vissa brukningsåtgärder påverkade förekomst och sammansättning av de denitrifierande svampsamhällena genom att förändraa tillgång till kol och kväve. Kvävegödsling under lång tid ökade deras förekomst oberoende av jordtyp eller klimat, men påverkade inte svamparnas potentiella bidrag till N_2O-produktion. Det var istället den genetiska potentialen hos denitrifierande bakterierna som förklarade N_2O-produktionen. I ogödslade jordar kontrollerade både biotiska och abiotiska faktorer N_2O-produktionen, men i gödslade jordar var endast abiotiska markegenskaper inblandade. Plöjning med vändplog jämfört med andra typer av jordbearbetning ledde till en minskning av svamparnas genetiska potential för denitrifikation i förhållande till den hos bakterier och selekterade för specifika, opportunistiska svampsläkten. Resultaten understryker att denitrifierande svampar finns i alla terrestra ekosystem, att de påverkas väsentligt av vissa brukningsåtgärder, men bidrar i mindre utsträckning till N_2O-utsläpp jämfört med deras prokaryota motsvarigheter.

Keywords: genetisk potential, jord, lustgas, svampdenitrifikation

Dedication

To my family –
Für meine Familie

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Bösch, Y., Pold, G., Saghaï, A., Karlsson, M., Jones, C.M. and Hallin, S. Global distribution patterns of fungal denitrifiers across major biomes. (manuscript).

II. Bösch, Y. Jones, C.M., Putz, T. Finlay, R., Karlsson, M. and Hallin, S. Impact of long-term fertilization on fungal denitrifiers across Swedish soils. (manuscript)

III. Bösch, Y., Jones, C.M., Finlay, R., Karlsson, M., Larsbo, M., Keller, T., Hallin, S. (2022). Minimizing tillage modifies fungal denitrifier communities, increases denitrification rates and enhances the genetic potential for fungal, relative to bacterial, denitrification. *Soil Biology and Biochemistry* (170) 108718.

The contribution of Yvonne Bösch to the papers included in this thesis was as follows:

I. Contributed to conceptualization and participated in the phylogenetic analysis, performed the statistical analyses and was the lead author in writing of the manuscript.

II. Performed lab work on fungal communities and performed all data analysis and was the lead author in writing of the manuscript.

III. Performed most of the lab work, analyzed the data and was the lead author in writing of the manuscript.

List of figures

Abbreviations

N_2O	Nitrous oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NH_4^+	Ammonium
nirK	Gene coding for the copper-binding nitrite reductase
nirS	Gene coding for the heme-binding nitrite reductase
p450nor	Gene coding for the nitric oxide reductase in fungi
*nos*Z	Gene coding for the nitrous oxide reductase
DNRA	Dissimilatory Nitrate Reduction to Ammonium

1. Introduction

Nitrogen is a major element in many biomolecules such as DNA, RNA and amino acids, the fundamental building blocks of life, and is thus an essential element for all living organisms. As such, the availability of nitrogen in soils is often the most limiting factor for plant growth and thereby primary production in terrestrial ecosystems. This makes nitrogen essential for food production, which is particularly important given the need to feed a growing world population.

In soils, nitrogen is present in organic (e.g., amino acids, amides) and inorganic forms such as nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+). Besides weathering of bedrocks (Houlton et al. 2018), nitrogen enters the soil by lightning and biological nitrogen-fixation, which is carried out by prokaryotic microbes that are free living or live in mutualistic relationships with other organisms, e.g. leguminous plants, alder trees and buckthorn. These organisms convert di-nitrogen (N_2) from the atmosphere into ammonium. Ammonium is either assimilated or converted to NO_3^- by the oxygen-dependent microbial process of nitrification. Nitrate can also be assimilated into biomass and then ultimately mineralized during the breakdown of dead material when an organism dies, and ammonium is released. However, as NO_3^- is highly soluble in water and can be easily be lost from soil through leaching or alternatively through the anaerobic microbial process of denitrification in which gaseous forms of nitrogen are returned to the atmosphere, thereby closing the nitrogen cycle (Figure 1). Nitrogen can also be retained by the microbial transformation of NO_3^- to NH_4^+ through the process of dissimilatory NO_3^- reduction to NH_4^+ (DNRA).

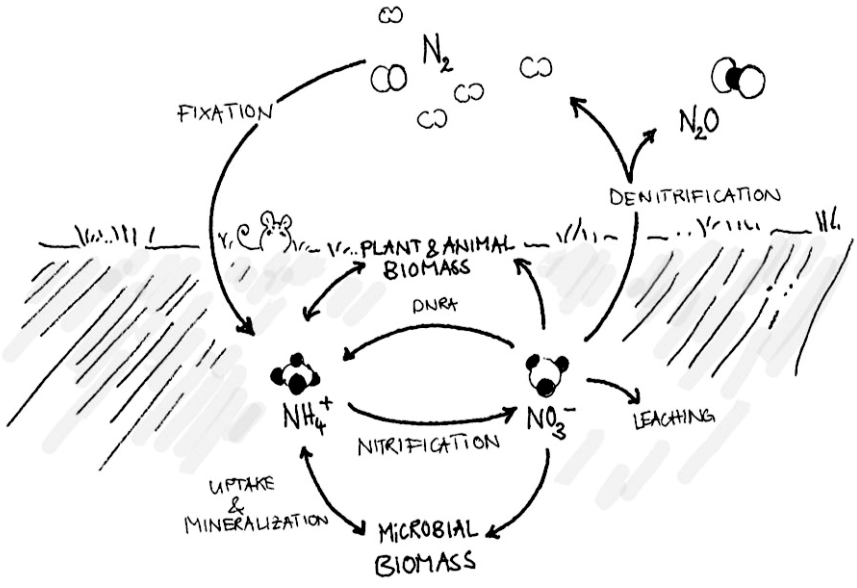


Figure 1. Terrestrial nitrogen cycle with the major nitrogen transformation processes indicated. DNRA, dissimilatory nitrate reduction to ammonium.

Denitrification is the dominating process that closes the nitrogen cycle by converting reactive nitrogen compounds back to the inert gas N_2. Terrestrial systems account for about 22% of the annual export of nitrogen via denitrification, whereas the continental shelf and oceanic minimum zones contribute 58% and freshwaters, including groundwater, 20% (Seitzinger et al. 2006).

Denitrification is a process that serves as an alternative to aerobic respiration and involves the stepwise reduction of NO_3^- to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and N_2 when oxygen is limited. Denitrification results in nitrogen loss from ecosystems, which limits primary production. However, this can alleviate eutrophication by removal of NO_3^- and NO_2^- from water, thereby improving water quality. In addition, denitrification may result in the production of N_2O, a strong greenhouse gas with a global warming potential almost 300 times that of carbon dioxide. Nitrous oxide is also the major contributor to the depletion of the stratospheric ozone layer in the 21st century (Ravishankara et al. 2009). Soils are the dominant sources of N_2O and contribute to 10.0 ± 2.0 Tg N_2O-

N/year, equal to more than 57% of total annual N_2O emissions (Tian et al. 2019, 2020). Therefore, terrestrial denitrification not only results in unwanted nitrogen losses from soils, but also in the emission of N_2O, that contributes to global warming. Understanding the underlying biological mechanisms of denitrification and their interaction with environmental factors in terrestrial ecosystems is thus vital for the development of strategies to mitigate N_2O emissions.

The ability to denitrify was formerly thought to be restricted to bacteria, but was later found in some Archaea and eukaryotes such as fungi, protozoa, *Foraminifera,* and *Gromiida* (Zumft 1997; Philippot et al. 2007; Kim et al. 2009; Piña-Ochoa et al. 2010). The process was almost exclusively studied in bacteria until Shoun and Tanimoto (1991) characterized the enzymatic pathways of dissimilatory reduction of nitrate in the fungus *Fusarium oxysporum*, revealing that some fungi possess similar sets of enzymes performing denitrification as bacteria. Since then, many more fungal species have been reported to be capable of denitrification (Maeda et al. 2015; Mothapo et al. 2015). However, denitrifying fungi do not carry the gene for nitrous oxide reductase, the last step of denitrification, and hence denitrification in fungi always terminates with N_2O. Consequently, fungal denitrification is considered a potential source of N_2O, particularly as some studies show that fungal denitrification might exceed bacterial denitrification under certain conditions (Laughlin & Stevens 2002; Long et al. 2013). However, recent advances indicate that fungal denitrifiers may not be as important for nitrogen losses in the form of N_2O as their prokaryotic counterparts (Yu et al. 2020; Wei et al. 2021). Yet knowledge of the ecology of these organisms and factors influencing their activity is limited and better understanding of how they interact with other organisms and respond to particular environmental conditions is necessary to improve understanding of nitrogen cycling in terrestrial ecosystems. This knowledge can contribute to the refinement of models, the development of good nitrogen management strategies to maintain soil fertility and forecast the contribution of soils to global warming.

1.1 Aims and objectives

The overarching aim of this thesis was to acquire a general understanding of the ecology of fungal denitrifiers and the importance of this understudied

group of microorganisms for production of N_2O in soils. More specifically, I aimed to gain a deeper understanding of the global distribution of fungal denitrifiers in different terrestrial environments, their diversity, community composition and abundance in soils, and soil factors that promote these organisms. As soil management practices are known to influence soil properties in multiple ways, I further aimed to understand how common agricultural practices like soil tillage and nitrogen fertilization affect these organisms. To achieve these aims, I combined a comparative metagenomic survey and analyses of fungal denitrifiers in agricultural long-term field experiments.

In **Paper I,** I and my co-authors performed comparative metagenomic analyses based on nearly 2000 samples to study the global distribution of fungal denitrifiers and the overall genetic potential for denitrification of fungi relative to bacteria in terrestrial biomes. For a subset of the metagenomes, associated edaphic factors that could explain the observed patterns were incorporated in the analyses. This study thereby sheds new light on soil factors that drive the abundance of fungal denitrifiers in terrestrial biomes.

In **Paper II,** we assessed the effects of long-term nitrogen fertilization, a major driver of increased N_2O emissions, on fungal denitrifiers and their possible contribution to N_2O production relative to bacterial denitrifiers. We investigated whether there are consistent effects of nitrogen fertilization on the abundance, diversity and community composition of fungal denitrifiers across Sweden using 14 field experiments (established 1956-1998) in which fertilized, and non-fertilized soils are compared. We hypothesized that fertilization modifies the fungal denitrifier communities and leads to an increase of fungal denitrifier abundance, i.e. their genetic denitrification potential and thus their contribution to N_2O emissions, as a result of increased denitrification.

Paper III focuses on the impact of inversion tillage on the abundance, diversity and genetic potential for denitrification of fungi relative to bacteria. We further evaluated potential relationships of soil chemical and physical factors with denitrification rates and fungal denitrifier abundance. We hypothesized that increasing soil disturbance by inversion tillage reduces

the genetic potential of the fungal community for denitrification. Consequently, reduced tillage would increase the potential for fungal denitrification and its contribution to N_2O production.

2. Fungal denitrifiers

2.1 Denitrification by fungi

Fungi are important contributors in the global biogeochemical cycle of nitrogen at several stages. Fungi make nitrogen available through decomposition of organic material, as well as, in the case of mutualistic species like mycorrhizal fungi, by delivery of soil-derived nitrogen to their plant hosts. Fungal activity can reduce nitrogen availability through immobilization of nitrogen in mycelial biomass or by denitrification resulting in the loss of nitrogen to the atmosphere. However, the knowledge of the fungi involved in denitrification, is still limited.

The denitrification pathway in fungi and bacteria is a dissimilatory respiratory process that uses oxidized nitrogen compounds as electron acceptors when oxygen is limited. Denitrification generates energy through the successive enzymatic reduction of nitrate and nitrite to nitric oxide, N_2O and finally N_2, contributing to the cellular proton-motive force, used to conserve energy in the form of ATP. In bacteria, these steps are catalyzed by four metalloenzymes: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) (Figure 2). Of the different steps, the reduction of nitrite is considered the defining step for denitrification and is performed by either the copper dependent NirK or the iron-heme dependent enzyme NirS, which are seldom found together in the same organism (Graft et al., 2014). Most denitrifying organisms are facultative anaerobes and oxygen respiration is favored as the energy yield is much higher than with NO_3^- respiration/denitrification (Chen & Strous 2013).

Denitrification in fungal cells involves similar enzymes as in bacteria, although unlike many bacterial denitrifiers, N_2O is the end-product since fungi lack the genetic capacity for N_2O reduction (Figure 2). Moreover, most fungi start denitrification with the reduction of NO_2^- rather than NO_3^- (Maeda et al. 2015; Mothapo et al. 2015). Fungal denitrification takes place in the mitochondria and is special in the sense that it requires small amounts of oxygen (Zhou et al. 2001). Ma et al. (2008) suggested a coupling of the fungal denitrification pathway with oxygen respiration by NADH, which delivers the electrons for the last reduction step in fungal denitrification.

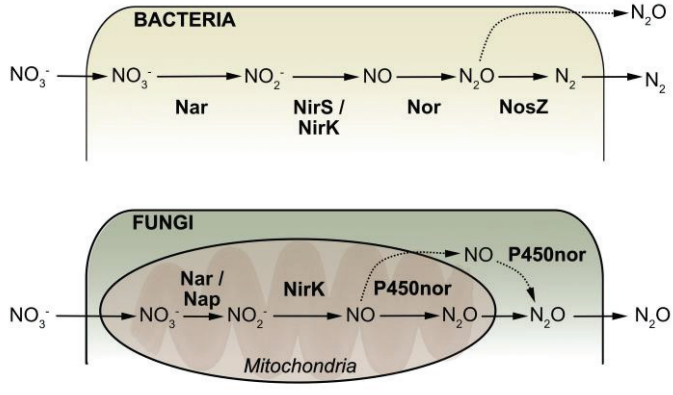


Figure 2. Denitrification pathway in bacterial and fungal cells, indicating the enzymes performing the reducing reactions (adapted from Mothapo et *al*. 2015). Fungal denitrification terminates with N_2O as fungi, in contrast to bacteria, lack the nitrous oxide reductase (NosZ). The fungal enzyme P450nor exists in two isoforms, one present in the mitochondria, whereas the other is found in the cytosol. Nar, nitrate reductase; Nap, periplasmic nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase.

Fungal denitrifiers possess the copper-containing NirK-type NO_2^- reductase, which is encoded by the *nirK* gene and is structurally and phylogenetically related to NirK of bacterial and archaeal denitrifiers (Figure 3). The high degree of sequence similarity of fungal and bacterial NirK, as well as the location of fungal NirK within the mitochondrial membrane, suggest that the fungal denitrification system has a common ancestor with bacteria (Kim et al. 2009).

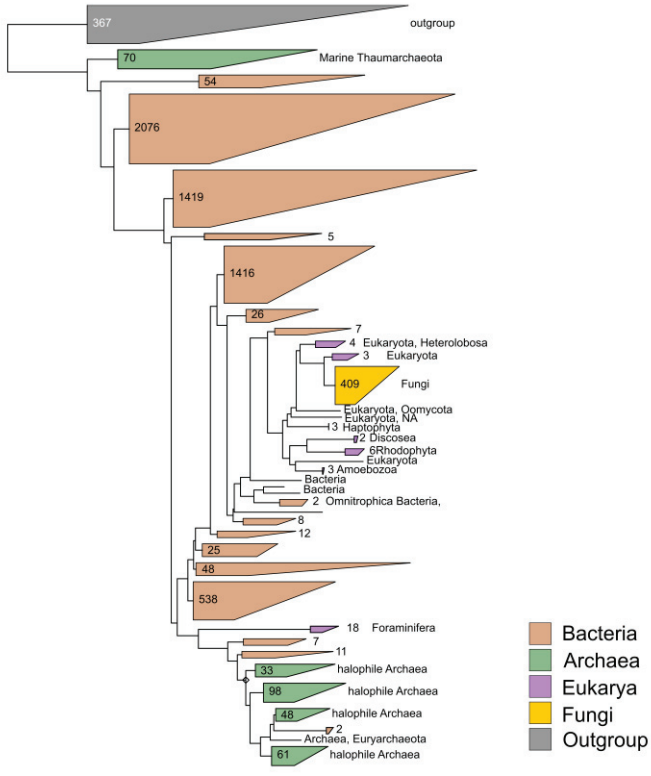


Figure 3. Phylogenetic maximum likelihood tree of 6789 NirK amino acid sequences obtained from genomes. Leaves were collapsed and leaf colors indicate the three taxonomic domains of life with the fungi within the Eukarya highlighted in yellow. The 409 fungal species are interleaved between other eukaryotes and bacterial taxa.

The third protein of the fungal denitrification pathway, Nor, belongs to the large superfamily of P450nor heme-based enzymes (Shoun et al. 2012). Two isoforms of the *p450nor* gene encoding this enzyme exist that differ at their translation initiation site, which results in P450nor being found in both the mitochondria and the cytosol (Takaya 2002; Shoun et al. 2012). This suggests that P450nor was acquired from a proto-mitochondrion, though the enzyme has undergone modifications over time (Shoun & Fushinobu 2016). P450nor receives the electrons for the reduction of NO directly from NAD(P)H (Nakahara et al. 1993) and is therefore disconnected from the respiratory electron transport chain (Takaya et al. 2003) and does not contribute to energy conservation. Consequently, the involvement of

P450nor in fungal denitrification is under debate and involvement in detoxification, fungal pathogenicity and secondary metabolism have been discussed (Higgins et al. 2018).

Genome analysis has shown that some fungal species possess only *nirK* or *p450nor* (Higgings et al., 2018) and fungi were shown to produce N_2O despite the lack of *nirK* or *p450nor* likely for NO detoxification (Keuschnig et al. 2020). This makes studying fungal denitrification even more complex. Furthermore, in strains lacking *p450nor*, the co-expression of flavohemoglobin NO dioxygenase (Fhb), that converts NO to NO_3^-, and fungal *nirK* was observed (Kim et al. 2010; Cánovas et al. 2016), demanding clarification of the role of Fhb in fungal denitrification. Hence, Aldossari and Ishii (2021) suggested calling fungi carrying both denitrification genes, *nirK* and *p450nor* putative denitrifiers.

Besides denitrification, a second pathway, called co-denitrification, exists. In this process, NO or NO_2^- is reduced together with amines and imines resulting in the formation of hybrid N_2O or N_2 with one N atom derived from the inorganic nitrogen source and the other from the co-substrate. Whether N_2O or N_2 is formed depends on the redox state of the co-substrate (Shoun et al. 2012). Co-denitrification can even occur simultaneously with denitrification and is suggested to also be mediated by P450nor (Shoun & Fushinobu 2016). Elevated levels of NO_2^- as well as anoxic conditions have been shown to promote this process in certain fungi (Tanimoto et al. 1992; Clough et al. 2017).

Ammonia fermentation (dissimilatory nitrate reduction to ammonium, DNRA) is, besides co-denitrification, one of the two additional metabolic pathways that exist in fungi involved in the reduction of NO_3^- and NO_2^- (Figure 4).

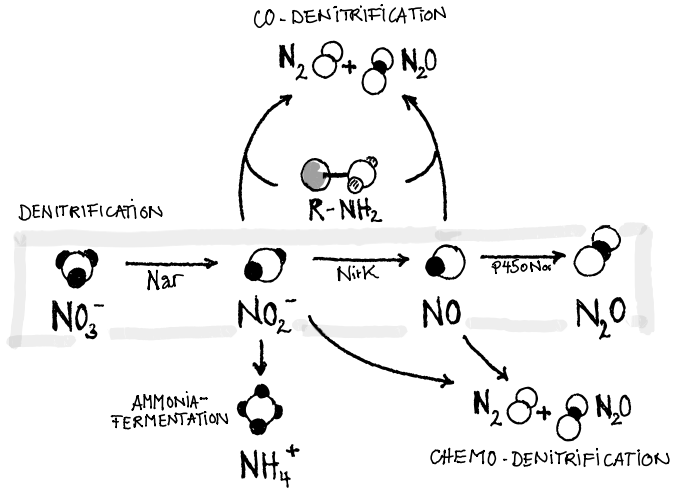


Figure 4. Selection of dissimilatory N-transforming processes performed by fungi starting with the reduction of NO_3^- (adapted from Aldossari and Ishii 2021). Nar, nitrate reductase; NirK, nitrite reductase; P450Nor, nitric oxide reductase; R-NH_2, co-substrate.

It has been reported as a novel fungal metabolic pathway to produce energy under anoxic conditions by reducing NO_3^- via NO_2^- to NH_4^+. Although similar to DNRA in bacteria, the fungal process involves the assimilatory enzymes, aNar and aNir, and is combined with ethanol or acetate oxidation (Takasaki et al. 2004). In addition, the abiotic process of nitrosation (chemo-denitrification) can convert metabolic by-products or intermediates to N_2O, but the importance of chemodenitrification to N_2O emissions from soil is not fully resolved.

2.1.1 Taxonomic and genetic diversity of fungal denitrifiers

The fungi *Fusarium oxysporum* and *Fusarium solani* were the first reported fungal species shown to produce N_2O from NO_2^- (Bollag & Tung 1972) but it took nearly 20 years until the enzymatic pathway of denitrification in fungi was verified by Shoun and Tanimoto (1991). Testing for N_2O production in pure culture has long been the most common method to identify fungal denitrifiers. For example, Maeda et al. (2015) screened over 200 fungal strains, confirming the ability to produce N_2O in various species within the orders Hypocreales, Eurotiales, Sordariales and Chaetosphaeriales in pure culture. However, recently developed molecular tools have enabled

identification of many more potential denitrifying fungal species, specifically via sequencing of fungal denitrification marker genes *nirK* and *p450nor*. More than 167 species from 60 different genera are currently known, with Ascomycetes being the dominant phylum. Representative denitrifiers from the main genera *Fusarium*, *Aspergillus*, *Trichoderma* and *Penicillium* have been isolated from agricultural soils multiple times. Furthermore, ectomycorrhizal species have been reported to produce N_2O (Prendergast-Miller et al. 2011), although the contribution to denitrification of this functional group of fungi is not clear and recently it was even suggested that ectomycorrhizal fungi reduce N_2O production (Okiobe et al. 2022). However, N_2O production by denitrification seems to be an evolutionarily conserved trait in the fungal subphylum Pezizomycotina. Nitrous oxide production from NO_3^- and NO_2^- reduction in pure culture is more common in the fungal orders of Eurotiales and Hypocreales (Mothapo et al. 2015) which include the known filamentous saprotrophs *Aspergillus*, *Penicillium*, *Trichoderma* and *Fusarium* (Figure 5). Furthermore, the presence of the denitrification genes *nirK* and *p450nor* in many of these species has been confirmed (Long et al. 2015; Maeda et al. 2015; Chen et al. 2016; Higgins et al. 2018). Notably, many fungal species carrying denitrification genes are known pathogens.

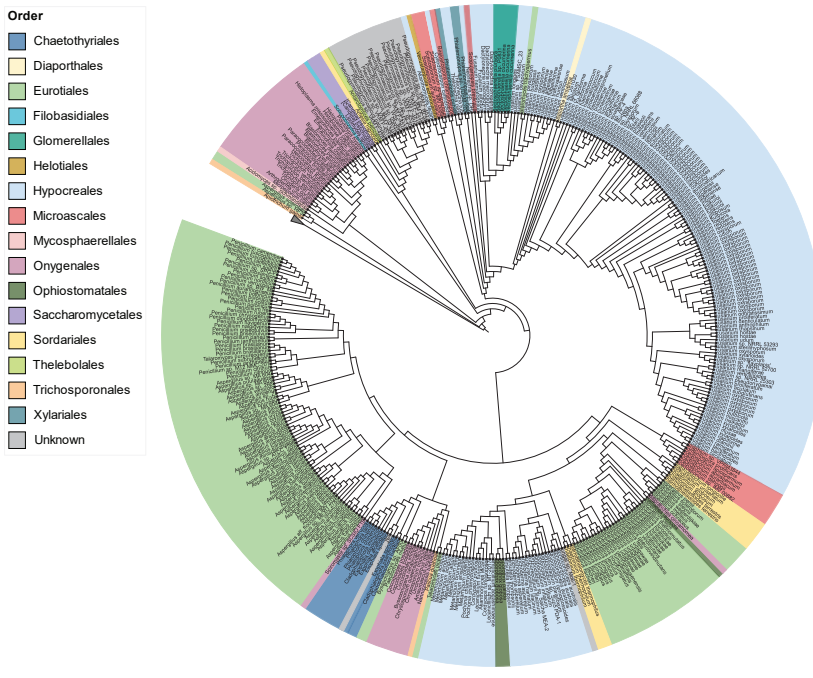


Figure 5. Cladogram derived from a phylogenetic maximum likelihood tree based on 409 full-length sequences of the fungal denitrification marker gene *nirK* (based on figure S1 in **paper III)**. The tree leaves with species names are colored by taxonomic order.

2.2 Measuring fungal denitrifier abundance, diversity and activity in soil

Tools used to study fungal denitrifiers and fungal denitrification have been developed recently and are still under evaluation. A set of culture- and DNA-based methods combined with selective respiration inhibition measurements, and stable isotope analysis, exists to assess the relevance of fungal denitrifiers in various ecosystems (Aldossari & Ishii 2021). Culture-based approaches were initially used to identify fungal species with the ability to reduce NO_3^- to N_2O (Bollag & Tung 1972) and are still relevant to verify denitrification activity in newly discovered fungi carrying fungal marker genes for denitrification (Aldossari & Ishii 2021). DNA-based, molecular tools, such as primers targeting protein coding genes specific to fungal denitrifiers, are used to measure fungal denitrifier abundance and diversity.

To measure (potential) denitrification activity of fungi, several different assays have been developed, of which the most prominent are substrate-induced respiration inhibition assays, followed by stable isotope labelling approaches. The following section will briefly introduce these methods and highlight challenges and perspectives of each.

2.2.1 Culture-based identification of denitrifying fungi

Culture based approaches, where denitrifying fungi are isolated from environmental samples by dilution of soil samples and cultivation on media plates, have the advantage that they enable detailed studies of physiology as well as excluding bacterial contamination or endosymbionts as possible sources of N_2O from denitrification (Sato et al. 2010). It is further possible to test the denitrification activity and its regulation of an individual species in liquid culture amended with either NO_3^- or NO_2^-. Subsequent analysis of biomass increase can then confirm that the reduction of the electron donors was used for energy conservation and biomass growth (Maeda et al. 2015). However, cultivation approaches suffer from a lack of consensus on the cultivation protocols (Mothapo et al. 2015) and the persistent limitations of cultivating certain species with currently available tools, leading to a cultivation bias (Prakash et al. 2021).

2.2.2 DNA-based community composition, diversity and abundance analysis

A continuing challenge in microbial ecology is the detection of taxonomic and functional groups of organisms of interest with as few false positives (non-target organisms) as possible, while still covering the entire spectrum of those within the target group. Thus, any method applied needs careful evaluation of the balance of specificity versus sensitivity.

Amplicon sequencing of fungal denitrifier marker genes

As with prokaryotic denitrifiers, the relatively large taxonomic diversity of fungal denitrifiers prevents the use of taxonomic marker genes to study fungal denitrifiers. The development of primers targeting fungal denitrifiers has therefore largely focused on the fungal denitrification marker genes *nirK* and *p450nor*. However, there are challenges in designing PCR primers for these genes. First, protein coding genes tend to have greater variation in DNA sequences due to wobble in the third position of codons. Second, primer

design is always limited by the availability of high-quality sequences in databases. This is particularly the case for fungal denitrifiers, where protein sequences of representative species are lacking. Careful evaluation of which primers to use is therefore crucial.

A promising approach to investigate fungal denitrifiers is to target the gene of the fungal NO-reductase P450nor, as this enzyme is unique to the fungal denitrification system. However, P450nor comes with the drawback of belonging to a large protein superfamily, resulting in considerable sequence similarity overlap between P450nor and other proteins of different functions. Consequently, Chen & Shi (2017) reported the existence of only two conserved regions in the protein sequence of P450nor, which restricts primer design. Several studies have since published primers targeting fungal *p450nor* in order to amplify and sequence the gene from environmental samples (Higgins et al. 2016; Novinscak et al. 2016; Rohe et al. 2020; Chen & Shi 2017). However, no study is yet published where fungal *p450nor* has been directly amplified and sequenced from soil without using a nested-PCR approach, which does not allow unbiased comparison of community structure and diversity (Yu et al. 2015). Further, when examining the evolution of fungal P450nor it has been proposed that this enzyme may have a role in secondary metabolism (Higgins et al. 2018), and its role in denitrification has been questioned. Consequently, analysis of more fungal genomes and experiments examining active gene transcription under field conditions are needed to clarify the involvement of *p450nor* in denitrification (Aldossari & Ishii 2021).

Several primer sets exist to amplify the fungal marker gene *nirK* from soil (Long et al. 2015; Maeda et al. 2015; Chen et al. 2016; Novinscak et al. 2016). The primers targeting fungal *nirK* have been recently compared *in-silico* and differed in coverage and specificity (Bonilla-Rosso et al. 2016; Ma et al. 2019). In particular, primers with good coverage would also amplify bacterial *nirK*, although to varying degrees (Chen et al. 2016). Thus, deep sequencing is necessary to capture the comparably rare fungal sequences. The results from **paper II** and **III**, with an average of 0.5 -1 % fungal *nirK* amplicons in the obtained sequences, underpin the *in silico* findings. The updated phylogenetic tree of *nirK* offers a visual explanation for these findings, showing that eukaryotic and fungal *nirK* are embedded in between *nirK* of bacterial species (Figure 3), depicting the close evolutionary relationship due to bacterial origin of this gene in eukaryotes.

Quantitative PCR

The abundance of fungal denitrifiers can be assessed by quantitative PCR (qPCR) of the fungal denitrification genes. Most reported assays have used the primer sets of Long and Chen (Long et al. 2015; Chen et al. 2016) targeting fungal *nirK*. However due to co-amplification of bacterial *nirK*, fungal *nirK* abundance will be overestimated by 2-3 orders of magnitude, unless the abundance data is corrected based on the ratio of fungal *nirK* sequences in the sample. Thus, correct quantification of absolute fungal *nirK* abundance can only be obtained by combining qPCR and sequencing data with identical primers, as demonstrated in Lourenço et al. (2022) and in **papers II** and **III**.

Metagenomics

A third option to detect fungal denitrifiers that is independent of PCR amplification bias is the screening of metagenomes for the protein-coding fungal denitrification genes, as performed in **paper I**. Higgins et al. (2016, 2018) revealed that, in most screened, putative, denitrifying fungal species, denitrification genes are present as single copies. In combination with the generally low abundance of fungal DNA in soil extracts, sequencing depth of metagenomes is expected to limit the detection of fungal denitrification genes. In **paper I,** we used a phylogenetically informed approach to search for fungal *nirK* sequences in nearly 2000 terrestrial metagenomes of similar sequencing depth and technology. The observed frequency of 0.01-0.06 fungal *nirK* sequences per fungal ITS2 sequence, underpinned a generally low abundance of fungal denitrification genes in metagenomes, but also showed that metagenomic approaches are able to report the most abundant fungal taxa harboring *nirK*.

2.2.3 Denitrification and N_2O production activity in soil

Stable isotope analysis

Tracing and quantifying the sources of N_2O from soil is challenging, particularly since both biotic, and abiotic sources, such as chemodenitrification can contribute to N_2O production (Wei et al. 2019). Furthermore, N_2O can be an intermediate or final product of denitrification but also a by-product of nitrification, further complicating the analysis. Nevertheless, analysis of the dual-isotopic signature of N_2O (^{15}N, ^{18}O) has been found to be a suitable method to discriminate between different biotic

processes producing N_2O (Yoshida & Toyoda 2000; Ostrom et al. 2007; Lewicka-Szczebak et al. 2015). Due to different site preferences of bacterial and fungal denitrifiers for ^{15}N in N_2O, likely caused by the differing Nor enzymes (Toyoda et al. 2002), it is possible to estimate the contribution of each group individually (Toyoda et al. 2005; Sutka et al. 2008). However, Yu et al. (2020) showed that the observed site preference values used to identify N_2O of fungal origin overlap with those for the process of nitrification. Furthermore, it is not possible to evaluate the fraction of N_2O produced by co-denitrification and chemodenitrification, and the site preference values for fungal denitrification can vary with differing pH and redox conditions (Otte et al. 2019; Wei et al. 2019; Yu et al. 2020).

Measuring potential denitrification and N_2O production

To estimate the denitrification activity of microbial communities, measurements of potential denitrification and N_2O production rates are frequently performed. In brief, soils are incubated anaerobically as soil slurries to allow equal diffusion of substrates, and carbon sources as electron donors and NO_3^- or NO_2^- as electron acceptors for denitrification are added in non-limiting amounts. After the addition of the substrate, gas samples are taken and the amount of N_2O produced is measured with gas chromatography. Since the end-product of denitrification can be both N_2O and N_2, and commonly N_2O alone is measured, the assays are usually conducted with addition of acetylene (C_2H_2) that inhibits the reduction of N_2O to N_2 to assess the total activity. These assays, however, do not represent the N_2O production in the field but rather what the active part of the community is producing under optimal conditions. Furthermore, chemodenitrification can occur and contribute to N_2O production, influencing the results. Hence, controlling of the assay's pH is suggested (Keuschnig et al. 2020). Nevertheless, these types of assays can be used to estimate the possible contribution of fungal and prokaryotic denitrifiers to denitrification and/or N_2O production rates. This approach was used in **paper II,** combined with structural equation modelling to determine the relative contribution of fungi and bacteria to N_2O production in fertilized and non-fertilized soils. In **paper III**, potential denitrification and N_2O production rates were determined and correlation analyses with the genetic potential of fungi and bacteria were performed.

Substrate induced selective respiration inhibition

Substrate induced selective respiration inhibition assays are used to directly determine the individual contributions of fungal and bacterial denitrification to potential N_2O production rates. The principle is similar to the potential denitrification activity measurements; soils are incubated anaerobically and a carbon source and NO_3^- or NO_2^- are added, but in combination with either a bacterial or fungal inhibitor. Controls are also performed in which both or no inhibitors are added. As in potential denitrification / N_2O production assays, acetylene is added to ensure that N_2O is not further reduced and thereby total denitrification activity can be measured.

Central to the selective inhibition method is a careful evaluation of the inhibitors. It has been shown that differences in soil type can affect the efficacy of inhibitors (Ladan & Jacinthe 2016). Furthermore, some inhibitors may not be effective in inhibiting the target organisms; for example, Streptomycin has been shown to inhibit only 60 % of bacterial respiration (Rousk et al. 2009b) or show off-target effects, like cycloheximide which besides fungi, can also inhibit bacteria (Castaldi & Smith 1998; Swallow & Quideau 2020). The duration of incubation must be carefully evaluated, as many inhibitors have a comparably short half-life (Moore & Stretton 1981; Badalucco et al. 1994; European Food Safety Authority 2014). Hence, measuring potential activity of a soil sample for a longer period than the half-life of the added inhibitors leads to unreliable results, particularly when measured over a time span of several days. Furthermore, the choice of the added carbon source might select for a specific fungal group (Hanson et al. 2008), thus a mixture of carbon sources with different degrees of complexity is suggested. Finally, considerations about anaerobic versus microaerobic incubations might have to be made with respect to the demand of small amounts of O_2 for fungal denitrification (Zhou et al. 2001). Recently, selective respiration inhibition was compared with stable isotope assays and large discrepancies have been found between the two methods (Rohe et al. 2021a). Due to the persistent difficulties with selective and efficient inhibitors, it is likely that stable isotope analysis will become more relevant in the future to discriminate the sources of N_2O from soil.

3. Ecological significance of fungal denitrification in terrestrial ecosystems

3.1 Presence of fungal denitrifiers in terrestrial biomes

Fungal denitrifiers have been isolated multiple times from soils but the majority of isolates originate from managed soils (cropland, plantation forestry, pasture) (Chen et al. 2014; Maeda et al. 2015; Mothapo et al. 2015). Consequently, our knowledge of the abundance and ecology of fungal denitrifiers across terrestrial habitats, and in particular unmanaged soils is limited (Mothapo et al. 2015). In **paper I**, I addressed this knowledge gap using a metagenomic approach to screen almost 2000 soil metagenomes from 608 locations, representing six terrestrial biomes (forests, grasslands, deserts, tundra, croplands and the rhizosphere of 10 different plants), for the fungal denitrifier marker gene *nirK*, taking advantage of the rapidly growing number of publicly available metagenomes. Metagenomic approaches enable evaluation of entire microbial communities present in a sample without any PCR amplification bias (Brooks et al. 2015; Krehenwinkel et al. 2017) which is central to estimating the proportion of fungal denitrifiers relative to the total fungal as well as to bacterial *nirK* abundance. This assessment of the genetic potential of fungal, relative to bacterial, *nirK* in global terrestrial ecosystems provides a first estimation of the possible fungal contribution to N_2O production through denitrification in major soil biomes. Furthermore, the global survey allowed us to examine the diversity and distribution of this functional group of fungi and assess whether certain fungal denitrifiers are coupled to biome-related patterns thereby expanding our knowledge of the ecology of these organisms.

Based on the abundance of the marker gene *nirK* normalized to the number of total reads to account for variation in sequencing depth of the metagenomes, we show that fungal denitrifiers were abundant in all terrestrial biomes. The highest abundance of fungal *nirK* was found in forests, driven by tropical and subtropical dry broadleaf forests, and croplands whereas it was the lowest in tundra and rhizosphere. Relative to overall fungi, quantified by 18S rRNA gene abundance, the proportion of fungal denitrifiers was the lowest in forest and the rhizosphere. The highest ratio of fungal *nirK* to prokaryotic abundance was found in forest and tundra biomes, indicating that they may play a more important role for denitrification in these biomes (Fig. 1, Table 1, **paper I**). However, compared to their prokaryotic counterparts, fungal *nirK* sequences were rare, totaling an average of 1% of the prokaryotic *nirK* sequences across biomes. The general low abundance of fungal denitrifiers, as found by Lourenço et al. (2022) and in **paper II and III** when abundance data is combined with sequencing data**,** supported our findings. Using metagenomics for environmental surveys also introduces some limitations. In particular, the search for rare gene sequences entails the risk of undersampling in metagenomes (Zhou et al. 2015; Zaheer et al. 2018). This means that if no fungal *nirK* is detected in a metagenome, this could be because these organisms are absent or, more likely, that the sequencing depth of the metagenome is insufficient to capture these rare sequences. However, we were able to detect fungal *nirK* in 76% of the screened metagenomes although with varying frequency (**paper I**, Fig S2).

The most prevalent species of putative fungal denitrifiers in terrestrial biomes were identified using a phylogenetically informed approach to classify the obtained, metagenomic *nirK* sequences based on a reference phylogeny of *nirK*. We used the method of phylogenetic placement that determined the branches in the *nirK* reference tree to which the retrieved metagenomic *nirK* sequences were most closely evolutionarily related (Czech et al. 2022). The phylogenetic placements of the obtained sequences were then visualized on a *nirK* reference tree to compare aggregations of placements between biomes (Fig. 2, **paper I**). Most sequences were placed in regions of the tree associated with the classes Eurotiomycetes, Dothideomycetes, Leotiomycetes and Sordarioycetes. Many of them were related to well-known cosmopolitan species, such as *Aspergillus westerdijkiae*, *A. sydowii*, *Penicillium solitum,* and *Fusarium*

neocosmosporiellum. *Aspergillus* and *Penicillium* species were discovered in a range of biomes, including Antarctica, tropical forests, and deserts (Abdel-Hafez 1981; Sterflinger et al. 2012; Cox et al. 2016) and are known for their stress tolerance (Lamb et al. 2008; Gostinčar et al. 2009) and efficient dispersal strategies (Golan & Pringle 2017). The capacity to inhabit many ecosystems requires metabolic flexibility, which could be provided through denitrification and hence the option for facultative anaerobic growth.

Some taxa also exhibited biome related abundance patterns, when comparing the placements across biomes visually. For example, placements associated with the hardwood pathogen *Thelonectria* aggregated in forest biomes, whereas placements of the entomopathogenic genera *Tolypocladium* and *Metarhizium* were aggregated in desert biomes. In addition, placements of the genus *Fusarium* were almost exclusively found in rhizosphere metagenomes of 10 plant species. This relationship of fungal taxa with potential hosts support that pathogenic traits, including secondary metabolism may play an important role for these organisms, as was suggested by Higgins et al. (2018).

3.2 Drivers of fungal denitrifiers

Soil fungi play a central role in nutrient cycling and their prevalence, often compared to bacteria, is influenced by altitude and climatic factors (precipitation, temperature), soil nutrients (soil organic carbon (SOC), total nitrogen, C:N), soil pH and texture (Fierer et al. 2009; Tedersoo et al. 2014; He et al. 2020) as well as biotic factors, particularly interactions with soil bacteria (Bahram et al. 2018) and plants (Tedersoo et al. 2014). Assessment of the richness of fungal functional groups such as saprotrophs, ectomycorrhizal fungi and plant pathogens in relation to biomes and associated soil factors suggests that functional traits are connected to soil factors. For example, the richness of saprotrophic fungi decreases with increasing carbon to nitrogen ratio (C:N) (Tedersoo et al. 2014). Similarly, fungal denitrifiers can also be driven by biotic and abiotic factors, although little is known about how environmental factors influence fungal denitrifiers.

Only a few studies have compared fungal denitrification activities in different ecosystems, revealing higher fungal denitrification activity in forest plantations and arid grasslands (Crenshaw et al. 2008; Chen et al. 2014). However, the underlying factors remain largely unknown. Microbial

denitrification in soil is controlled by several factors, of which the most important are carbon and nitrogen availability as electron donors and acceptors combined with the absence of oxygen. Chen et al. (2015a; b) reported that fungal denitrification was stimulated by complex C-compounds and exhibited a higher denitrification potential compared to bacteria under sub-anoxic and more acidic conditions. Combined with other studies, this suggests that fungal denitrification activity, compared to that of bacteria, increases with organic C supply in soil (Senbayram et al. 2018; Zhong et al. 2018). The ratio of C:N has further been shown to control the denitrification end-product ratio (Senbayram et al. 2012; Rummel et al. 2021), but the effect on fungal denitrifiers is not clear. Amongst others, fungal denitrifiers include saprotrophs and endophytes, representing differing functional traits (Treseder & Lennon 2015) and possibly differing carbon and nitrogen demands.

As fungal denitrification requires small amounts of oxygen (Zou et al. 2001; Ma et al. 2008), soil texture likely has a profound effect on fungal denitrifiers as it controls soil porosity and thus soil water content, as well as soil aeration. Significant effects of soil texture on community structure have recently been reported (Xu et al. 2019) and were also observed in **papers II** and **III**. We also noted increasing fungal *nirK* abundance with elevated soil clay content, both in forest and cropland biomes, which aligns with the observation that clay has been the dominant factor controlling the anoxic volume in soil (Keiluweit et al. 2018), which is obviously of high relevance for denitrification (Rohe et al. 2021b). Clay soils are also known to be rich in nutrients due to their reactive properties, which also favor fungal denitrifiers. Small pores in clay soil were shown to be favored by fungi, as they offer protection against predation (Elliott et al. 1980; Six et al. 2006) but might also become anaerobic more rapidly, favoring fungi with the capacity for facultative anaerobic respiration, for example through denitrification.

Soil pH has been shown to be a strong driver of bacterial and fungal abundance and community composition (Enwall et al. 2005; Fierer & Jackson 2006; Baggs et al. 2010), as well as denitrification activity (ŠImek & Cooper 2002; Wallenstein et al. 2006). The response of microbes to pH in general is well documented and caused by changes to their biochemical environment and indirect effects on nutrient availability, for example iron (Fe) and copper (Cu) (Lammel et al. 2018). A lack of Fe and Cu availability

can limit denitrification as these metals are central components of bacterial and fungal denitrification enzymes (Glass & Orphan 2012). Fungi have been shown to have a less restricted pH range for growth than bacteria, tolerating also lower soil pH (Rousk et al. 2010), and it has been demonstrated that fungal denitrification can exceed bacterial denitrification in highly acidic soils (Huang et al. 2017) although there are contrasting reports (Herold et al. 2012). The observed negative relation with soil pH and fungal *nirK* abundance, as well as fungal *nirK* relative to prokaryotic *nirK* in **paper I**, was in line with previous reports of increasing fungal denitrifier abundance with lower soil pH (Huang et al. 2017; Xu et al. 2019). However, when compared to fungi in general, we showed denitrifying fungi are favored by a higher soil pH (**paper I**).

The observation that a large proportion of known fungal denitrifiers exhibit a pathogenic lifestyle suggests that biotic factors could be involved in regulating fungal denitrifier abundance and activity. Plant roots have been shown to affect denitrification by stimulation of microbial activity through nutrient rich exudates. This, combined with root respiration in turn leads to oxygen consumption causing anaerobic hotspots in the rhizosphere, promoting denitrification. Plant exudates have also been shown to regulate the nitrogen cycle by recruiting nitrogen fixing microorganisms and repressing nitrifying and denitrifying microorganisms (Bardon et al. 2014; Coskun et al. 2017), and Ma et al (2008) found that formate from plant exudates controlled fungal denitrification. Furthermore, interactions with other microorganisms, particularly arbuscular mycorrhizal fungi (AMF) might play a role. It has been shown that AMF, which are closely associated with plant roots, affect the availability of labile carbon and nitrogen (Hodge 2003) but also compete for nitrate with other microorganisms (Storer et al. 2018), thereby also possibly affecting fungal denitrifiers.

In **paper I** we found a positive correlation of fungal *nirK* abundance across all terrestrial biomes with SOC, ammonium and clay content as well as soil moisture and negative relationships with C:N and pH (Fig. 3, **paper I**) . These findings are consistent with the hypothesis, that the abundance of carbon- and nitrogen resources, which are essential for denitrification, are drivers of fungal denitrifier abundance. In **paper II**, we found that the C:N ratio was a driving factor for fungal *nirK* communities amongst others, whereas in **paper III** it became the sole significant edaphic factor associated with fungal *nirK* community structure. This agrees with Bahram et al. (2018)

who identified C:N as the best predictor of the richness of fungal functional genes and affecting the relative abundance of filamentous Ascomycota fungi involved in biosynthesis of antibiotic and reactive oxygen species, including the major groups holding fungal denitrifiers, linking back to our findings in **paper I.**

The total abundance of fungal denitrifiers, but also relative to the total fungal community, was promoted by increasing soil nitrogen contents (total nitrogen, nitrate, ammonium), although differing patterns between biomes were noted (**paper I**). For example, NO_3^- was positively associated with the fungal *nirK* to ITS ratio in forest biomes, whereas the opposite was found in croplands. These observations were most likely explained by the inherent differences in nitrogen levels in these biomes, as described in several studies, (Batjes 1996; Butterbach-Bahl et al. 2011; Scharlemann et al. 2014) with generally low NO_3^- levels in forests (Ambus & Zechmeister-Boltenstern 2007). The negative relationship in NO_3^- rich croplands on the other hand indicated a potential nitrate threshold causing restructuring of fungal communities, with negative effects on denitrifiers.

When comparing fungal abundance in rhizosphere metagenomes of different plant species we found that fungal *nirK* relative to total fungal abundance was lowest in the rhizosphere of plant species belonging to the family of Poaceae (**paper I**), which are often associated with AMF (Endresz et al. 2013; Emery et al. 2022). However, since there was no additional information on environmental conditions in the rhizosphere, it was impossible to distinguish whether the observed differences were caused by changes in abiotic soil factors which could also partly be plant induced. Nevertheless, these findings align well with previous reports of AMF being associated with decreasing N_2O emissions from soil through regulation of denitrifier abundance (Gui et al. 2021).

The combined effects of the various soil properties most likely resulted in the observed biome-related frequency patterns of fungal species with *nirK* (Fig. 2, **paper I**). In addition to the factors described above, there remain anthropogenic factors that might affect fungal denitrifiers. Globalization allows fungal pathogens to spread more easily, which is also supported by our finding of many cosmopolitan denitrifying species, belonging to the genera of *Aspergillus*, *Penicillium* and *Fusarium* in **paper I**. Furthermore, modifications of soil properties such as pH, fertility, moisture and plant cover through soil management practices, has profound effects on soil fungal

communities (Ellouze et al. 2014), and necessitates fungal metabolic flexibility and stress tolerance as observed in these species (Lamb et al. 2008; Gostinčar et al. 2009).

4. Effects of agricultural soil management practices on fungal denitrification

4.1 Fungal denitrification and agriculture

Agricultural soil management practices, such as tillage, crop rotations and irrigation as well as fertilization can have a tremendous effect on soil fungi (Srour et al. 2020; Orrù et al. 2021; Deng et al. 2022). Tillage, for example, has been shown to disrupt hyphal networks thereby hampering filamentous fungi, including beneficial AMF (Schalamuk & Cabello 2010). Soil management practices, especially nitrogen fertilization, are also known to have profound effects on denitrification through changes in the availability of carbon and nitrogen resources, and soil texture, which influence soil aeration (Munch & Velthof 2007). Consequently, agricultural soils have become a major source of N_2O due to enhanced denitrification (Tian et al. 2020). Besides the contribution to global warming, this poses great challenges in the efforts of minimizing nutrient losses and maintaining soil fertility for crop production.

Fungal denitrification has been reported from many different managed soils, including arable soils, grasslands and pastures, but its contribution to N2O production is not yet clear as conflicting results have been reported (Wei et al. 2014; Yamamoto et al. 2017). Moreover, a better understanding of fungal denitrification in agricultural soils, besides gaseous nitrogen losses, might also be relevant for plant health, as many fungi with denitrification genes are known pathogens.

4.2 Effects of nitrogen fertilization

4.2.1 Impact of nitrogen fertilization on soil microbial communities

Primary production in terrestrial ecosystems is mainly limited by nitrogen availability, which in turn is regulated by biotic and abiotic factors controlling nitrogen cycling (Vitousek et al. 2002). In agriculture, nitrogen is removed from the ecosystem with the harvested crop. Hence, nitrogen fertilization is essential to maintain fertility and support crop yields. Fertilizers are applied in different forms, either as mineral fertilizers including different forms of nitrogen as ammonia, nitrate, and urea, or in organic forms such as manure or compost (Figure 6). However, excessive nitrogen-application can lead to nitrogen losses from soil of up to 50% to the environment, both, as gaseous losses due to microbial processes of denitrification and ammonia oxidation, and ammonia volatilization, as well as through leaching of NO_3^- to waterbodies (Lassaletta et al. 2014, Figure 6).

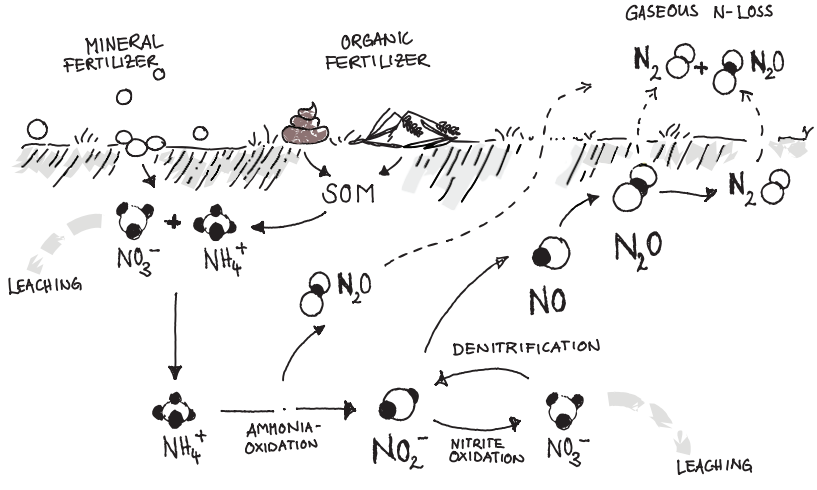


Figure 6. Fate of mineral and organic nitrogen fertilizer in the soil nitrogen cycle. Losses of soil nitrogen occur as gaseous nitrogen species via denitrification and leaching of nitrate to water bodies.

Fertilization is known to affect soil properties; particularly the decrease in soil pH has been observed in soils receiving regular input of certain types of nitrogen fertilizers (Vašák et al. 2016). Furthermore, profound effects of

fertilization on soil microbial communities have been detected, reflected in increased microbial biomass (Rousk et al. 2009a; Geisseler & Scow 2014) and changes in the community composition and structure (Enwall et al. 2007; Geisseler & Scow 2014).. The observed shifts in microbial communities towards increased denitrifier abundance appear to be associated with higher N_2O production rates (Ding et al. 2010; Jäger et al. 2011; Jones et al. 2022), and are attributed to higher levels of biological and chemical denitrification, as well as nitrification (Wang et al. 2021)

4.2.2 Effects of fertilization on fungal denitrifiers

Fertilization effects have been observed on fungal communities, leading to an overall increased fungal abundance but also increased relative abundance of certain fungal groups, particularly Ascomycota (Sun et al. 2016; Wen et al. 2020). Previous research has shown that the fungal phylum Ascomycota, to which many fungal denitrifiers belong (Mothapo et al., 2015), predominates in fungal communities in agro-ecosystems (Lienhard et al., 2014). Combined with increased N_2O emissions from fertilized soils, observed shifts in fungal communities could point towards increased abundance of denitrifying fungi. Increased fungal N_2O production in fertilized soils was later reported in microcosm experiments using selective inhibition. Nevertheless, the contribution of fungi relative to bacteria remains inconclusive due to differing results (Wei et al. 2014; Yamamoto et al. 2017).

Data on fertilization effects on fungal denitrifiers is scarce. Furthermore, most studies have been performed in soils with a comparatively short history of fertilization and at single sites, whereas long-term experiments and multi-site comparisons are lacking. Hence, it is unknown whether there are general trends regarding effects on fungal denitrifiers in relation to nitrogen fertilization or if they are site-specific and mainly influenced by edaphic factors as suggested by Xu et al. (2019). In **paper II,** we addressed if there are predictive trends of N-fertilization on the abundance and community composition of fungal denitrifiers across Sweden by using multiple long-term experiments. We further aimed to determine the relative importance of fungal and bacterial denitrifiers for potential N_2O production activity based on the statistical approach of structural equation modelling (SEM) that enabled evaluation of direct and indirect effects of biotic and abiotic factors on N_2O production.

Our study includes soils from 14 long-term fertilization experiments, established between 1956-1998. Each site included mineral-fertilized soils (ammonium-nitrate [50/50] at 12 sites and calcium nitrate at two sites) and an unfertilized control. The analysis of soil properties revealed an increase of total and organic carbon, total and reactive nitrogen species as well as Cu, Fe, Ca, phosphorus (P) and potassium (K) in fertilized soils (Table S2, **paper II**). However, soil pH did not decrease under long-term nitrogen-fertilization since only mildly or non-acidifying fertilizers were used.

Increased potential N_2O emissions were observed in fertilized soils (Jones et al., 2022) combined with an overall higher fungal abundance quantified by qPCR targeting ITS2. Fertilization also had a significant effect on the abundance of denitrifiers: both, fungal *nirK* and bacterial *nirK* were significantly more abundant in fertilized plots (Table 1, **paper II**). Conversely, the abundance of *nirS*-type denitrifiers did not change in response to fertilization and was six times more abundant than *nirK*, most likely explained by previously reported, differing niche preferences (Enwall et al. 2010; Azziz et al. 2017; Wang et al. 2020) and primer coverage (Bonilla-Rosso et al., 2016). The relative genetic potential of fungal denitrifiers however, specified as fungal *nirK* abundance relative to the sum of bacterial *nir* (fungal nirK/*nirK*+*nirS*), did not change in response to fertilization. We further noted a significant correlation of fungal *nirK* abundance with potential N_2O production rates, but only in unfertilized soils (Fig. 1, **paper II**). However, the community structure of fungal *nirK* was significantly affected by fertilization as well as site, and was driven by a number of soil factors, the strongest being clay and sand content, certain minerals and total organic carbon (TOC) content (Fig. 2C, **paper II**). TOC, and total nitrogen and carbon differed between fertilized and unfertilized sites, indicating a soil resource-related shift in the fungal denitrifying community.

Unlike prior studies (Wei et al. 2014; Xu et al. 2019), the results from our modeling approach did not show any significant fungal contribution to N_2O production rates, neither in fertilized nor unfertilized soils. The observed positive co-variance of fungal *nirK* and bacterial *nir* abundance in unfertilized soils suggests that fungal denitrifiers may indirectly, through a positive interaction with prokaryotic denitrifiers, contribute to N_2O production (Fig. 3A, **paper II**). In fertilized soils, however, N_2O production was solely driven by a combination of abiotic factors (Fig. 3B, **paper II**).

The missing aspect of N_2O reducers in the model could limit the conclusions, particularly with respect to biotic control. The findings of Jones et al. (2022), however, suggest that the increasing resource availability could lead to a threshold above which the ratio of N_2O consumers to producers has no effect on the net N_2O production, and support the observed loss of biological control of N_2O production in fertilized soils in the present work.

4.3 Soil tillage

4.3.1 Tillage effects on soil properties and N_2O emissions

Tillage is an integral part of seedbed preparation in many areas of the world, with strong implications for soil structure and nutrient homogenization, as well as plant pathogen control. Several studies have found that conventional tillage practices such as mouldboard ploughing have a significant impact on microbial abundance and community structure (Sommermann et al. 2018; Sun et al. 2018). Soil inversion by plowing brings nutrients and plant residues into the deeper layers of the soil, but soil structure, in particular the presence of macropores, deteriorates, which, along with uncovered soil surfaces, promotes soil erosion, increases water evaporation, and accelerates the loss of organic matter through higher decomposition and respiration rates (Ben-Noah & Friedman 2018). As a result, conservation or minimum tillage has been widely promoted to improve soil health through enhanced microbial activity and increased soil organic matter on the surface, which also retains soil humidity (Six et al. 2002; Chen et al. 2020; Krauss et al. 2020).

For reduced tillage increased N_2O emissions have been observed (Six et al. 2002; Lognoul et al. 2017), accompanied with a general enhancement of microbial activity (Doran, 1980; Melero et al., 2011). These emissions were found to be associated with cover crop residues and originate near the soil surface (Petersen et al. 2011). Since fungal denitrification is also related to crop residues (Yamamoto et al. 2017), it has been hypothesized that fungal denitrifiers could be the source of these N_2O emissions. In **paper III** we tested this hypothesis by examining how inversion tillage regimes influenced fungal denitrifiers, using a long-term tillage experiment located in Ultuna, Sweden. At this site, different tillage systems have been monitored since 1974. The denitrifier community (bacteria and fungi) as well as the overall fungal and bacterial communities were investigated, and potential

denitrification activity was assessed in relation to tillage regime, including inversion tillage, inversion frequency, and non-inversion tillage, and soil depth.

4.3.2 Effects of tillage on denitrification and denitrifying fungi

Inversion tillage has been shown to have profound effects on soil properties (Haddaway et al. 2017) in agreement with our findings of significant effects on total carbon, nitrogen as well as on bound P, K and available K. Untilled soils typically accumulate organic matter in the topsoil, which in tilled soils is transferred to deeper soil levels. This was reflected in the significantly higher amount of total carbon and nitrogen in the upper soil layer in the non-inverted treatment (Table 1, **paper III**). The different tillage regimes and associated changes in soil properties led to structural changes in the microbial communities. Differences in both bacterial and overall fungal communities followed a gradient of increasing tillage intensity (no inversion to occasional inversion (every 4-5 years) to conventional annual inversion), and by soil sampling depth. Similar effects were also observed by Sun et al. (2018) and explained by changes in the vertical distribution of soil carbon and carbon sequestration caused by different tillage regimes.

Our results further highlighted a gradient in the C:N ratio, which was significantly associated with the structure of the fungal *nirK* community (Fig. 3C, **paper III**), a relationship which was also found in **paper II**. The sample similarities based on fungal denitrifier community composition clustered in a similar way as the overall communities with respect to tillage treatment and soil depth, with most pronounced difference between soil layers in the non-inverted soils. This showed profound differences between the fungal denitrifier communities of the upper, nutrient rich, and nutrient poor lower non-inverted soils. These differences were driven by members of Tremellomycetes and *Trichoderma* in the upper levels, and Tremellomycetes and *Fusarium* in the lower soil levels. Also, in line with previous studies, the relative abundance of *Trichoderma* and *Penicillium* increased in the upper non-inverted soils (Bockus & Shroyer 1998; Nesci et al. 2006; Meng et al. 2010; Degrune et al. 2017), whereas *Fusarium* became more abundant in the lower soil layer, suggesting competition with other decomposers in the upper soil layer (Leplat et al. 2013). The observed patterns indicated the existence of depth-related niche differences that could be associated with differences

in labile carbon availability caused by differing stages of crop-residue decomposition (Rummel et al. 2021).

We found that the genetic potential for denitrification of fungi relative to that of bacteria was highest in non-inverted upper soil layers, based on the ratio of fungal *nirK* to the sum of bacterial *nirK*+*nirS* abundance. This coincided with significantly higher potential denitrification rates compared to the conventionally ploughed soils, when NO_2^- was used as electron acceptor. Furthermore, a significant positive correlation between fungal *nirK* abundance and denitrification activity with NO_2^- was observed. Combined with the report that fungi often start denitrification with NO_2^- rather than NO_3^- (Maeda et al. 2015), these results provide evidence of the fungal contribution to denitrification in non-inverted upper soil layers. However, compared to bacteria, their denitrification potential was orders of magnitudes lower (Fig. 2C, **paper III**), which, in line with the findings of **papers I** and **II**, suggests that bacteria are more significant contributors to N_2O emissions.

5. Conclusions and perspectives

Given that denitrification in terrestrial ecosystems is the major source of N_2O, a thorough understanding of the ecology of the microorganisms involved in producing this greenhouse gas is crucial. Moreover, the loss of nitrogen from soil has a severe impact on agricultural productivity. Research on denitrifying fungi is important since it helps to set priorities in the development of N_2O mitigation strategies and improved nitrogen usage efficiency in agriculture.

This thesis shed light onto the abundance, diversity and relevance of the understudied microbial group of fungal denitrifiers in soil and how soil management practices affect these organisms. These findings provide a valuable contribution to a better understanding of the ecology of this not well understood component of the denitrifying community. The findings in **paper I –III** highlight that:

- Fungal denitrifiers are found in all terrestrial biomes across the globe and are associated with soil factors known to promote denitrification, i.e. availability of resources such as organic carbon and reactive nitrogen, as well as elevated soil moisture.
- The genetic potential for denitrification of fungal denitrifiers is orders of magnitude lower than their prokaryotic counterparts, suggesting a minor role of fungi in global terrestrial denitrification.
- Many fungal denitrifiers are cosmopolitan, stress-tolerant species that may benefit from global warming. Particularly the opportunistic pathogens among denitrifying fungi could pose an increasing threat to food production and at the same time provide unwanted positive feedback to global warming by N_2O production.

- Soil management practices that affect the distribution and availability of soil carbon and nitrogen have a profound effect on denitrifying organisms, including fungi. Improved management strategies increasing N-use efficiency are promising tools to mitigate N_2O emissions from agricultural soils.
- Quantification of fungal *nirK* with qPCR results in an overestimation of fungal denitrifiers due to co-amplification of bacterial *nirK*. Therefore, correct quantification of absolute fungal *nirK* abundance can only be obtained by combining qPCR and sequencing data with identical primers and subsequent correction of the abundance data by considering the proportion of fungal *nirK* in the sequence data.

These results suggest a novel perspective on fungal denitrifiers should be adopted, focusing more on their pathogenic traits and how these are related to the acquisition of the denitrification genes. The central question of why these organisms maintain denitrification capacity although its contribution to energy conservation is low, remains unsolved. Is it an alternative to other NO detoxification pathways or an enhancement to survive suboxic conditions? Also, in light of the finding of the prevalence of *Fusarium* in rhizosphere metagenomes of different plants, according to Keuschnig et al. (2020) hitherto the sole genus hosting true denitrifiers, host-interactions should be considered in future research. The study of fungal isolates and their hosts in combination with gene expression assays could help improve our understanding of the role of denitrification in fungi.

In **paper III** it was shown that there is a potential interaction between fungi harboring *nirK* and prokaryotes with *nirK* and *nirS* in soils with lower resource availability. Fungal interactions with N_2O reducing microorganisms, either complete denitrifiers or non-denitrifying N_2O-reducers, would be relevant to investigate in future studies. A first step could be to perform co-occurrence analyses to search for putative interactions and shared niches between denitrifying fungi and N_2O reducers.

This thesis has highlighted the limitations of the molecular methods available to detect and quantify fungal denitrifiers. Efforts are needed to improve these methods as they offer powerful tools to study the ecology of fungal denitrifiers. Quantitative data is necessary and should be combined with novel and improved stable isotope analysis (Rohe et al. 2020). The results of this thesis illustrate that we still do not fully comprehend the complexity of processes that lead to N_2O emissions from soils. Future work

should therefore aim to provide a more comprehensive picture of how fungal denitrifiers contribute to global warming.

References

Abdel-Hafez, S.I.I. (1981). Halophilic fungi of desert soils in Saudi Arabia. *Mycopathologia*, 75 (2), 75–80. https://doi.org/10.1007/BF00505781

Aldossari, N. & Ishii, S. (2021). Fungal denitrification revisited – Recent advancements and future opportunities. *Soil Biology and Biochemistry*, 157, 108250. https://doi.org/10.1016/j.soilbio.2021.108250

Ambus, P. & Zechmeister-Boltenstern, S. (2007). Chapter 22 - Denitrification and N-Cycling in Forest Ecosystems. In: Bothe, H., Ferguson, S.J., & Newton, W.E. (eds.) *Biology of the Nitrogen Cycle*. Amsterdam: Elsevier, 343–358. https://doi.org/10.1016/B978-044452857-5.50023-0

Azziz, G., Monza, J., Etchebehere, C. & Irisarri, P. (2017). nirS- and nirK-type denitrifier communities are differentially affected by soil type, rice cultivar and water management. *European Journal of Soil Biology*, 78, 20–28. https://doi.org/10.1016/j.ejsobi.2016.11.003

Badalucco, L., Pomaré, F., Grego, S., Landi, L. & Nannipieri, P. (1994). Activity and degradation of streptomycin and cycloheximide in soil. *Biology and Fertility of Soils*, 18 (4), 334–340. https://doi.org/10.1007/BF00570637

Baggs, E.M., Smales, C.L. & Bateman, E.J. (2010). Changing pH shifts the microbial sourceas well as the magnitude of N2O emission from soil. *Biology and Fertility of Soils*, 46 (8), 793–805. https://doi.org/10.1007/s00374-010-0484-6

Bahram, M., Hildebrand, F., Forslund, S.K., Anderson, J.L., Soudzilovskaia, N.A., Bodegom, P.M., Bengtsson-Palme, J., Anslan, S., Coelho, L.P., Harend, H., Huerta-Cepas, J., Medema, M.H., Maltz, M.R., Mundra, S., Olsson, P.A., Pent, M., Põlme, S., Sunagawa, S., Ryberg, M., Tedersoo, L. & Bork, P. (2018). Structure and function of the global topsoil microbiome. *Nature*, 560 (7717), 233–237. https://doi.org/10.1038/s41586-018-0386-6

Bardon, C., Piola, F., Bellvert, F., el Zahar Haichar, F., Comte, G., Meiffren, G., Pommier, T., Puijalon, S., Tsafack, N. & Poly, F. (2014). Evidence for biological denitrification inhibition (BDI) by plant secondary metabolites. *New Phytologist*, 204 (3), 620–630. https://doi.org/10.1111/nph.12944

Batjes, N. H. (1996). Total carbon and nitrogen in the soils of the world. *European Journal of Soil Science*, 47 (2), 151–163. https://doi.org/10.1111/j.1365-2389.1996.tb01386.x

Ben-Noah, I. & Friedman, S.P. (2018). Review and Evaluation of Root Respiration and of Natural and Agricultural Processes of Soil Aeration. *Vadose Zone Journal*, 17 (1), 170119. https://doi.org/10.2136/vzj2017.06.0119

Bockus, W.W. & Shroyer, J.P. (1998). The impact of reduced tillage on soilborne plant pathogens. *Annual Review of Phytopathology*, 36 (1), 485–500. https://doi.org/10.1146/annurev.phyto.36.1.485

Bollag, J.-M. & Tung, G. (1972). Nitrous oxide release by soil fungi. *Soil Biology and Biochemistry*, 4 (3), 271–276. https://doi.org/10.1016/0038-0717(72)90021-1

Bonilla-Rosso, G., Wittorf, L., Jones, C.M. & Hallin, S. (2016). Design and evaluation of primers targeting genes encoding NO-forming nitrite reductases: implications for ecological inference of denitrifying communities. *Scientific Reports*, 6 (1). https://doi.org/10.1038/srep39208

Brooks, J.P., Edwards, D.J., Harwich, M.D., Rivera, M.C., Fettweis, J.M., Serrano, M.G., Reris, R.A., Sheth, N.U., Huang, B., Girerd, P., Strauss, J.F., Jefferson, K.K., Buck, G.A., & Vaginal Microbiome Consortium (additional members) (2015). The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiology*, 15 (1), 66. https://doi.org/10.1186/s12866-015-0351-6

Butterbach-Bahl, K., Gundersen, P., Ambus, P., Augustin, J., Beier, C., Boeckx, P., Dannenmann, M., Sanchez Gimeno, B., Ibrom, A., Kiese, R., Kitzler, B., Rees, R.M., Smith, K.A., Stevens, C., Vesala, T. & Zechmeister-Boltenstern, S. (2011). Nitrogen processes in terrestrial ecosystems. *The European Nitrogen Assessment: Sources, Effects and Policy Perspectives*. Cambridge University Press, 99–125. https://doi.org/10.1017/CBO9780511976988.009

Cánovas, D., Marcos, J.F., Marcos, A.T. & Strauss, J. (2016). Nitric oxide in fungi: is there NO light at the end of the tunnel? *Current Genetics*, 62 (3), 513–518. https://doi.org/10.1007/s00294-016-0574-6

Castaldi, S. & Smith, K.A. (1998). Effect of cycloheximide on N2O and NO3/- production in a forest and an agricultural soil. *Biology and Fertility of Soils*, 27 (1), 27–34. https://doi.org/10.1007/s003740050395

Charles Munch, J. & Velthof, G.L. (2007). Chapter 21 - Denitrification and Agriculture. In: Bothe, H., Ferguson, S.J., & Newton, W.E. (eds.)

Biology of the Nitrogen Cycle. Amsterdam: Elsevier, 331–341. https://doi.org/10.1016/B978-044452857-5.50022-9

Chen, H., Dai, Z., Veach, A.M., Zheng, J., Xu, J. & Schadt, C.W. (2020). Global meta-analyses show that conservation tillage practices promote soil fungal and bacterial biomass. *Agriculture, Ecosystems & Environment*, 293, 106841. https://doi.org/10.1016/j.agee.2020.106841

Chen, H., Mothapo, N.V. & Shi, W. (2014). The significant contribution of fungi to soil N2O production across diverse ecosystems. *Applied Soil Ecology*, 73, 70–77. https://doi.org/10.1016/j.apsoil.2013.08.011

Chen, H., Mothapo, N.V. & Shi, W. (2015a). Fungal and bacterial N2O production regulated by soil amendments of simple and complex substrates. *Soil Biology and Biochemistry*, 84, 116–126. https://doi.org/10.1016/j.soilbio.2015.02.018

Chen, H., Mothapo, N.V. & Shi, W. (2015b). Soil Moisture and pH Control Relative Contributions of Fungi and Bacteria to N2O Production. *Microbial Ecology*, 69 (1), 180–191. https://doi.org/10.1007/s00248-014-0488-0

Chen, H. & Shi, W. (2017). Opening up the N2O-producing fungal community in an agricultural soil with a cytochrome p450nor-based primer tool. *Applied Soil Ecology*, 119, 392–395. https://doi.org/10.1016/j.apsoil.2017.07.022

Chen, H., Yu, F. & Shi, W. (2016). Detection of N2O-producing fungi in environment using nitrite reductase gene (*nirK*)-targeting primers. *Fungal Biology*, 120 (12), 1479–1492. https://doi.org/10.1016/j.funbio.2016.07.012

Chen, J. & Strous, M. (2013). Denitrification and aerobic respiration, hybrid electron transport chains and co-evolution. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1827 (2), 136–144. https://doi.org/10.1016/j.bbabio.2012.10.002

Clough, T.J., Lanigan, G.J., de Klein, C.A.M., Samad, M.S., Morales, S.E., Rex, D., Bakken, L.R., Johns, C., Condron, L.M., Grant, J. & Richards, K.G. (2017). Influence of soil moisture on codenitrification fluxes from a urea-affected pasture soil. *Scientific Reports*, 7 (1), 2185. https://doi.org/10.1038/s41598-017-02278-y

Coskun, D., Britto, D.T., Shi, W. & Kronzucker, H.J. (2017). How Plant Root Exudates Shape the Nitrogen Cycle. *Trends in Plant Science*, 22 (8), 661–673. https://doi.org/10.1016/j.tplants.2017.05.004

Cox, F., Newsham, K.K., Bol, R., Dungait, J.A.J. & Robinson, C.H. (2016). Not poles apart: Antarctic soil fungal communities show similarities

to those of the distant Arctic. *Ecology Letters*, 19 (5), 528–536. https://doi.org/10.1111/ele.12587

Crenshaw, C.L., Lauber, C., Sinsabaugh, R.L. & Stavely, L.K. (2008). Fungal Control of Nitrous Oxide Production in Semiarid Grassland. *Biogeochemistry*, 87 (1), 17–27

Czech, L., Stamatakis, A., Dunthorn, M. & Barbera, P. (2022). Metagenomic Analysis Using Phylogenetic Placement—A Review of the First Decade. *Frontiers in Bioinformatics*, 2. https://www.frontiersin.org/article/10.3389/fbinf.2022.871393

Degrune, F., Theodorakopoulos, N., Colinet, G., Hiel, M.-P., Bodson, B., Taminiau, B., Daube, G., Vandenbol, M. & Hartmann, M. (2017). Temporal Dynamics of Soil Microbial Communities below the Seedbed under Two Contrasting Tillage Regimes. *Frontiers in Microbiology*, 8, 1127. https://doi.org/10.3389/fmicb.2017.01127

Deng, Q., Wu, Y., Zhao, X., Qiu, C., Xia, S., Feng, Y. & Liu, H. (2022). Influence of different irrigation methods on the alfalfa rhizosphere soil fungal communities in an arid region. *PLOS ONE*, 17 (6), e0268175. https://doi.org/10.1371/journal.pone.0268175

Ding, W., Yagi, K., Cai, Z. & Han, F. (2010). Impact of Long-Term Application of Fertilizers on N2O and NO Production Potential in an Intensively Cultivated Sandy Loam Soil. *Water, Air, & Soil Pollution*, 212 (1), 141–153. https://doi.org/10.1007/s11270-010-0328-x

Elliott, E.T., Anderson, R.V., Coleman, D.C. & Cole, C.V. (1980). Habitable Pore Space and Microbial Trophic Interactions. *Oikos*, 35 (3), 327–335. https://doi.org/10.2307/3544648

Ellouze, W., Esmaeili Taheri, A., Bainard, L.D., Yang, C., Bazghaleh, N., Navarro-Borrell, A., Hanson, K. & Hamel, C. (2014). Soil Fungal Resources in Annual Cropping Systems and Their Potential for Management. *BioMed Research International*, 2014, e531824. https://doi.org/10.1155/2014/531824

Emery, S.M., Bell-Dereske, L., Stahlheber, K.A. & Gross, K.L. (2022). Arbuscular mycorrhizal fungal community responses to drought and nitrogen fertilization in switchgrass stands. *Applied Soil Ecology*, 169, 104218. https://doi.org/10.1016/j.apsoil.2021.104218

Endresz, G., Somodi, I. & Kalapos, T. (2013). Arbuscular mycorrhizal colonisation of roots of grass species differing in invasiveness. *Community Ecology*, 14 (1), 67–76. https://doi.org/10.1556/comec.14.2013.1.8

Enwall, K., Throbäck, I.N., Stenberg, M., Söderström, M. & Hallin, S. (2010). Soil Resources Influence Spatial Patterns of Denitrifying

Communities at Scales Compatible with Land Management. *Applied and environmental microbiology*, 76, 2243–50. https://doi.org/10.1128/AEM.02197-09

Enwall, K., Nyberg, K., Bertilsson, S., Cederlund, H., Stenström, J. & Hallin, S. (2007). Long-term impact of fertilization on activity and composition of bacterial communities and metabolic guilds in agricultural soil. *Soil Biology and Biochemistry*, 39 (1), 106–115. https://doi.org/10.1016/j.soilbio.2006.06.015

Enwall, K., Philippot, L. & Hallin, S. (2005). Activity and Composition of the Denitrifying Bacterial Community Respond Differently to Long-Term Fertilization. *Applied and Environmental Microbiology*, 71 (12), 8335–8343. https://doi.org/10.1128/AEM.71.12.8335-8343.2005

European Food Safety Authority (2014). Reasoned opinion on the review of the existing maximum residue levels (MRLs) for captan according to Article 12 of Regulation (EC) No 396/2005. *EFSA Journal*, 12 (4). https://doi.org/10.2903/j.efsa.2014.3663

Fierer, N. & Jackson, R.B. (2006). The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences*, 103 (3), 626–631. https://doi.org/10.1073/pnas.0507535103

Fierer, N., Strickland, M.S., Liptzin, D., Bradford, M.A. & Cleveland, C.C. (2009). Global patterns in belowground communities. *Ecology Letters*, 12 (11), 1238–1249. https://doi.org/10.1111/j.1461-0248.2009.01360.x

Geisseler, D. & Scow, K.M. (2014). Long-term effects of mineral fertilizers on soil microorganisms – A review. *Soil Biology and Biochemistry*, 75, 54–63. https://doi.org/10.1016/j.soilbio.2014.03.023

Glass, J. & Orphan, V.J. (2012). Trace Metal Requirements for Microbial Enzymes Involved in the Production and Consumption of Methane and Nitrous Oxide. *Frontiers in Microbiology*, 0. https://doi.org/10.3389/fmicb.2012.00061

Golan, J.J. & Pringle, A. (2017). Long-Distance Dispersal of Fungi. *Microbiology Spectrum,* 5(4), https://doi.org/10.1128/microbiolspec.FUNK-0047-2016

Gostinčar, C., Grube, M., De Hoog, S., Zalar, P. & Gunde-Cimerman, N. (2009). Extremotolerance in fungi: evolution on the edge. *FEMS Microbiology Ecology*, 71 (1), 2–11. https://doi.org/10.1111/j.1574-6941.2009.00794.x

Gui, H., Gao, Y., Wang, Z., Shi, L., Yan, K. & Xu, J. (2021). Arbuscular mycorrhizal fungi potentially regulate N2O emissions from

agricultural soils via altered expression of denitrification genes. *The Science of the Total Environment*, 774, 145133. https://doi.org/10.1016/j.scitotenv.2021.145133

Haddaway, N.R., Hedlund, K., Jackson, L.E., Kätterer, T., Lugato, E., Thomsen, I.K., Jørgensen, H.B. & Isberg, P.-E. (2017). How does tillage intensity affect soil organic carbon? A systematic review. *Environmental Evidence*, 6 (1), 30. https://doi.org/10.1186/s13750-017-0108-9

Hanson, C.A., Allison, S.D., Bradford, M.A., Wallenstein, M.D. & Treseder, K.K. (2008). Fungal Taxa Target Different Carbon Sources in Forest Soil. *Ecosystems*, 11 (7), 1157–1167. https://doi.org/10.1007/s10021-008-9186-4

He, L., Mazza Rodrigues, J.L., Soudzilovskaia, N.A., Barceló, M., Olsson, P.A., Song, C., Tedersoo, L., Yuan, F., Yuan, F., Lipson, D.A. & Xu, X. (2020). Global biogeography of fungal and bacterial biomass carbon in topsoil. *Soil Biology and Biochemistry*, 151, 108024. https://doi.org/10.1016/j.soilbio.2020.108024

Heinze, S., Raupp, J. & Joergensen, R.G. (2010). Effects of fertilizer and spatial heterogeneity in soil pH on microbial biomass indices in a long-term field trial of organic agriculture. *Plant and Soil*, 328 (1), 203–215. https://doi.org/10.1007/s11104-009-0102-2

Herold, M.B., Baggs, E.M. & Daniell, T.J. (2012). Fungal and bacterial denitrification are differently affected by long-term pH amendment and cultivation of arable soil. *Soil Biology and Biochemistry*, 54, 25–35. https://doi.org/10.1016/j.soilbio.2012.04.031

Higgins, S.A., Schadt, C.W., Matheny, P.B. & Löffler, F.E. (2018). Phylogenomics Reveal the Dynamic Evolution of Fungal Nitric Oxide Reductases and Their Relationship to Secondary Metabolism. *Genome Biology and Evolution*, 10 (9), 2474–2489. https://doi.org/10.1093/gbe/evy187

Higgins, S.A., Welsh, A., Orellana, L.H., Konstantinidis, K.T., Chee-Sanford, J.C., Sanford, R.A., Schadt, C.W. & Löffler, F.E. (2016). Detection and Diversity of Fungal Nitric Oxide Reductase Genes (p450nor) in Agricultural Soils. *Applied and Environmental Microbiology*, 82 (10), 2919–2928. https://doi.org/10.1128/AEM.00243-16

Hodge, A. (2003). Plant nitrogen capture from organic matter as affected by spatial dispersion, interspecific competition and mycorrhizal colonization. *New Phytologist*, 157 (2), 303–314. https://doi.org/10.1046/j.1469-8137.2003.00662.x

Houlton, B.Z., Morford, S.L. & Dahlgren, R.A. (2018). Convergent evidence for widespread rock nitrogen sources in Earth's surface environment. *Science*, 360 (6384), 58–62. https://doi.org/10.1126/science.aan4399

Huang, Y., Xiao, X. & Long, X. (2017). Fungal denitrification contributes significantly to N2O production in a highly acidic tea soil. *Journal of Soils and Sediments*, 17 (6), 1599–1606. https://doi.org/10.1007/s11368-017-1655-y

Jäger, N., Stange, C.F., Ludwig, B. & Flessa, H. (2011). Emission rates of N2O and CO2 from soils with different organic matter content from three long-term fertilization experiments—a laboratory study. *Biology and Fertility of Soils*, 47 (5), 483–494. https://doi.org/10.1007/s00374-011-0553-5

Jones, C.M., Putz, M., Tiemann, M. & Hallin, S. (2022). Reactive nitrogen restructures and weakens microbial controls of soil N2O emissions. *Communications Biology*, 5 (1), 1–12. https://doi.org/10.1038/s42003-022-03211-4

Keiluweit, M., Gee, K., Denney, A. & Fendorf, S. (2018). Anoxic microsites in upland soils dominantly controlled by clay content. *Soil Biology and Biochemistry*, 118, 42–50. https://doi.org/10.1016/j.soilbio.2017.12.002

Keuschnig, C., Gorfer, M., Li, G., Mania, D., Frostegård, Å., Bakken, L. & Larose, C. (2020). NO and N_2O transformations of diverse fungi in hypoxia: evidence for anaerobic respiration only in *Fusarium* strains. *Environmental Microbiology*, 22 (6), 2182–2195. https://doi.org/10.1111/1462-2920.14980

Kim, S.-W., Fushinobu, S., Zhou, S., Wakagi, T. & Shoun, H. (2009). Eukaryotic nirK Genes Encoding Copper-Containing Nitrite Reductase: Originating from the Protomitochondrion? *Applied and Environmental Microbiology*, 75 (9), 2652–2658. https://doi.org/10.1128/AEM.02536-08

Kim, S.-W., Fushinobu, S., Zhou, S., Wakagi, T. & Shoun, H. (2010). The Possible Involvement of Copper-Containing Nitrite Reductase (NirK) and Flavohemoglobin in Denitrification by the Fungus *Cylindrocarpon tonkinense*. *Bioscience, Biotechnology, and Biochemistry*, 74 (7), 1403–1407. https://doi.org/10.1271/bbb.100071

Krauss, M., Berner, A., Perrochet, F., Frei, R., Niggli, U. & Mäder, P. (2020). Enhanced soil quality with reduced tillage and solid manures in organic farming – a synthesis of 15 years. *Scientific Reports*, 10 (1), 4403. https://doi.org/10.1038/s41598-020-61320-8

Krehenwinkel, H., Wolf, M., Lim, J.Y., Rominger, A.J., Simison, W.B. & Gillespie, R.G. (2017). Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. *Scientific Reports*, 7 (1), 17668. https://doi.org/10.1038/s41598-017-17333-x

Ladan, S. & Jacinthe, P.-A. (2016). Evaluation of antibacterial and antifungal compounds for selective inhibition of denitrification in soils. *Environmental Science: Processes & Impacts*, 18 (12), 1519–1529. https://doi.org/10.1039/C6EM00456C

Lamb, B.C., Mandaokar, S., Bahsoun, B., Grishkan, I. & Nevo, E. (2008). Differences in spontaneous mutation frequencies as a function of environmental stress in soil fungi at "Evolution Canyon," Israel. *Proceedings of the National Academy of Sciences*, 105 (15), 5792–5796. https://doi.org/10.1073/pnas.0801995105

Lammel, D.R., Barth, G., Ovaskainen, O., Cruz, L.M., Zanatta, J.A., Ryo, M., de Souza, E.M. & Pedrosa, F.O. (2018). Direct and indirect effects of a pH gradient bring insights into the mechanisms driving prokaryotic community structures. *Microbiome*, 6 (1), 106. https://doi.org/10.1186/s40168-018-0482-8

Lassaletta, L., Billen, G., Grizzetti, B., Anglade, J. & Garnier, J. (2014). 50 year trends in nitrogen use efficiency of world cropping systems: the relationship between yield and nitrogen input to cropland. *Environmental Research Letters*, 9 (10), 105011. https://doi.org/10.1088/1748-9326/9/10/105011

Laughlin, R.J. & Stevens, R.J. (2002). Evidence for Fungal Dominance of Denitrification and Codenitrification in a Grassland Soil. *Soil Science Society of America Journal*, 66 (5), 1540–1548. https://doi.org/10.2136/sssaj2002.1540

Leplat, J., Friberg, H., Abid, M. & Steinberg, C. (2013). Survival of Fusarium graminearum, the causal agent of Fusarium head blight. A review. *Agronomy for Sustainable Development*, 33 (1), 97–111. https://doi.org/10.1007/s13593-012-0098-5

Lewicka-Szczebak, D., Well, R., Bol, R., Gregory, A.S., Matthews, G.P., Misselbrook, T.H., Whalley, W.R. & Cardenas, L.M. (2015). Isotope fractionation factors controlling isotopocule signatures of soil-emitted N2O produced by denitrification processes of various rates. *Rapid Communications in Mass Spectrometry*, 29 (3), 269–282. https://doi.org/10.1002/rcm.7102

Lognoul, M., Theodorakopoulos, N., Hiel, M.-P., Regaert, D., Broux, F., Heinesch, B., Bodson, B., Vandenbol, M. & Aubinet, M. (2017). Impact of tillage on greenhouse gas emissions by an agricultural crop and dynamics of N2O fluxes: Insights from automated closed

chamber measurements. *Soil and Tillage Research*, 167, 80–89. https://doi.org/10.1016/j.still.2016.11.008

Long, A., Heitman, J., Tobias, C., Philips, R. & Song, B. (2013). Co-Occurring Anammox, Denitrification, and Codenitrification in Agricultural Soils. *Applied and Environmental Microbiology*, 79 (1), 168–176. https://doi.org/10.1128/AEM.02520-12

Long, A., Song, B., Fridey, K. & Silva, A. (2015). Detection and diversity of copper containing nitrite reductase genes (nirK) in prokaryotic and fungal communities of agricultural soils. *FEMS Microbiology Ecology*, 91 (2), 1–9. https://doi.org/10.1093/femsec/fiu004

Lourenço, K.S., Costa, O.Y. de A., Cantarella, H. & Kuramae, E.E. (2022). Ammonia-oxidizing bacteria and fungal denitrifier diversity are associated with N2O production in tropical soils. *Soil Biology and Biochemistry*, 166, 108563. https://doi.org/10.1016/j.soilbio.2022.108563

Ma, W.K., Farrell, R.E. & Siciliano, S.D. (2008). Soil Formate Regulates the Fungal Nitrous Oxide Emission Pathway. *Applied and Environmental Microbiology*, 74 (21), 6690–6696. https://doi.org/10.1128/AEM.00797-08

Ma, Y., Zilles, J.L. & Kent, A.D. (2019). An evaluation of primers for detecting denitrifiers via their functional genes. *Environmental Microbiology*, 21 (4), 1196–1210. https://doi.org/10.1111/1462-2920.14555

Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.-C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C. & Philippot, L. (2015). N2O production, a widespread trait in fungi. *Scientific Reports*, 5 (1). https://doi.org/10.1038/srep09697

Meng, J., Xu, Y., Li, S., Li, C., Zhang, X., Dong, D. & Chen, P. (2010). Soybean Growth and Soil Microbial Populations under Conventional and Conservational Tillage Systems. *Journal of Crop Improvement*, 24 (4), 337–348. https://doi.org/10.1080/15427528.2010.499864

Moore, K.E. & Stretton, R.J. (1981). The Effect of pH, Temperature and Certain Media Constituents on the Stability and Activity of the Preservative, Bronopol. *Journal of Applied Bacteriology*, 51 (3), 483–494. https://doi.org/10.1111/j.1365-2672.1981.tb01267.x

Mothapo, N., Chen, H., Cubeta, M.A., Grossman, J.M., Fuller, F. & Shi, W. (2015). Phylogenetic, taxonomic and functional diversity of fungal denitrifiers and associated N2O production efficacy. *Soil Biology and Biochemistry*, 83, 160–175. https://doi.org/10.1016/j.soilbio.2015.02.001

Nakahara, K., Tanimoto, T., Hatano, K., Usuda, K. & Shoun, H. (1993). Cytochrome P-450 55A1 (P-450dNIR) acts as nitric oxide reductase employing NADH as the direct electron donor. *The Journal of biological chemistry*, 268, 8350–5

Nesci, A., Barros, G., Castillo, C. & Etcheverry, M. (2006). Soil fungal population in preharvest maize ecosystem in different tillage practices in Argentina. *Soil and Tillage Research*, 91 (1), 143–149. https://doi.org/10.1016/j.still.2005.11.014

Novinscak, A., Goyer, C., Zebarth, B.J., Burton, D.L., Chantigny, M.H. & Filion, M. (2016). Novel P450nor Gene Detection Assay Used To Characterize the Prevalence and Diversity of Soil Fungal Denitrifiers. *Applied and Environmental Microbiology*, 82 (15), 4560–4569. https://doi.org/10.1128/AEM.00231-16

Okiobe, S.T., Pirhofer-Walzl, K., Leifheit, Eva.F., Rillig, M.C. & Veresoglou, S.D. (2022). Proximal and distal mechanisms through which arbuscular mycorrhizal associations alter terrestrial denitrification. *Plant and Soil*,. https://doi.org/10.1007/s11104-022-05534-x

Orrù, L., Canfora, L., Trinchera, A., Migliore, M., Pennelli, B., Marcucci, A., Farina, R. & Pinzari, F. (2021). How Tillage and Crop Rotation Change the Distribution Pattern of Fungi. *Frontiers in Microbiology*, 0. https://doi.org/10.3389/fmicb.2021.634325

Ostrom, N.E., Pitt, A., Sutka, R., Ostrom, P.H., Grandy, A.S., Huizinga, K.M. & Robertson, G.P. (2007). Isotopologue effects during N2O reduction in soils and in pure cultures of denitrifiers. *Journal of Geophysical Research: Biogeosciences*, 112 (G2). https://doi.org/10.1029/2006JG000287

Otte, J.M., Blackwell, N., Ruser, R., Kappler, A., Kleindienst, S. & Schmidt, C. (2019). N2O formation by nitrite-induced (chemo)denitrification in coastal marine sediment. *Scientific Reports*, 9 (1), 10691. https://doi.org/10.1038/s41598-019-47172-x

Petersen, S.O., Mutegi, J.K., Hansen, E.M. & Munkholm, L.J. (2011). Tillage effects on N2O emissions as influenced by a winter cover crop. *Soil Biology and Biochemistry*, 43 (7), 1509–1517. https://doi.org/10.1016/j.soilbio.2011.03.028

Philippot, L., Hallin, S. & Schloter, M. (2007). Ecology of Denitrifying Prokaryotes in Agricultural Soil. *Advances in Agronomy*. Elsevier, 249–305. https://doi.org/10.1016/S0065-2113(07)96003-4

Piña-Ochoa, E., Høgslund, S., Geslin, E., Cedhagen, T., Revsbech, N.P., Nielsen, L.P., Schweizer, M., Jorissen, F., Rysgaard, S. & Risgaard-Petersen, N. (2010). Widespread occurrence of nitrate storage and

denitrification among Foraminifera and Gromiida. *Proceedings of the National Academy of Sciences*, 107 (3), 1148–1153. https://doi.org/10.1073/pnas.0908440107

Prakash, O., Parmar, M., Vaijanapurkar, M., Rale, V. & Shouche, Y.S. (2021). Recent trend, biases and limitations of cultivation-based diversity studies of microbes. *FEMS Microbiology Letters*, 368 (17), fnab118. https://doi.org/10.1093/femsle/fnab118

Prendergast-Miller, M.T., Baggs, E.M. & Johnson, D. (2011). Nitrous oxide production by the ectomycorrhizal fungi Paxillus involutus and Tylospora fibrillosa. *FEMS Microbiology Letters*, 316 (1), 31–35. https://doi.org/10.1111/j.1574-6968.2010.02187.x

Ravishankara, A.R., Daniel, J.S. & Portmann, R.W. (2009). Nitrous oxide (N2O): the dominant ozone-depleting substance emitted in the 21st century. *Science (New York, N.Y.)*, 326 (5949), 123–125. https://doi.org/10.1126/science.1176985

Rohe, L., Anderson, T.-H., Flessa, H., Goeske, A., Lewicka-Szczebak, D., Wrage-Mönnig, N. & Well, R. (2021a). Comparing modified substrate-induced respiration with selective inhibition (SIRIN) and N₂O isotope approaches to estimate fungal contribution to denitrification in three arable soils under anoxic conditions. *Biogeosciences*, 18 (15), 4629–4650. https://doi.org/10.5194/bg-18-4629-2021

Rohe, L., Apelt, B., Vogel, H.-J., Well, R., Wu, G.-M. & Schlüter, S. (2021b). Denitrification in soil as a function of oxygen availability at the microscale. *Biogeosciences*, 18 (3), 1185–1201. https://doi.org/10.5194/bg-18-1185-2021

Rohe, L., Oppermann, T., Well, R. & Horn, M.A. (2020). Nitrite induced transcription of p450nor during denitrification by Fusarium oxysporum correlates with the production of N2O with a high 15N site preference. *Soil Biology and Biochemistry*, 151, 108043. https://doi.org/10.1016/j.soilbio.2020.108043

Rousk, J., Bååth, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R. & Fierer, N. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *The ISME Journal*, 4 (10), 1340–1351. https://doi.org/10.1038/ismej.2010.58

Rousk, J., Brookes, P.C. & Bååth, E. (2009a). Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization. *Applied and Environmental Microbiology*, 75 (6), 1589–1596. https://doi.org/10.1128/AEM.02775-08

Rousk, J., Demoling, L.A. & Bååth, E. (2009b). Contrasting Short-Term Antibiotic Effects on Respiration and Bacterial Growth

Compromises the Validity of the Selective Respiratory Inhibition Technique to Distinguish Fungi and Bacteria. *Microbial Ecology*, 58 (1), 75–85. https://doi.org/10.1007/s00248-008-9444-1

Rummel, P.S., Well, R., Pausch, J., Pfeiffer, B. & Dittert, K. (2021). Carbon Availability and Nitrogen Mineralization Control Denitrification Rates and Product Stoichiometry during Initial Maize Litter Decomposition. *Applied Sciences*, 11 (11), 5309. https://doi.org/10.3390/app11115309

Sato, Y., Narisawa, K., Tsuruta, K., Umezu, M., Nishizawa, T., Tanaka, K., Yamaguchi, K., Komatsuzaki, M. & Ohta, H. (2010). Detection of betaproteobacteria inside the mycelium of the fungus mortierella elongata. *Microbes and Environments*, 25 (4), 321–324. https://doi.org/10.1264/jsme2.ME10134

Schalamuk, S. & Cabello, M. (2010). Chapter 13: Effect of tillage systems on the arbuscular mycorrhizal fungi (AMF) propagule bank in soils. *Management of Fugal Plant Pathogens: Current Trends and Progress*. CAB International

Scharlemann, J.P., Tanner, E.V., Hiederer, R. & Kapos, V. (2014). Global soil carbon: understanding and managing the largest terrestrial carbon pool. *Carbon Management*, 5 (1), 81–91. https://doi.org/10.4155/cmt.13.77

Seitzinger, S., Harrison, J.A., Böhlke, J.K., Bouwman, A.F., Lowrance, R., Peterson, B., Tobias, C. & Drecht, G.V. (2006). Denitrification Across Landscapes and Waterscapes: A Synthesis. *Ecological Applications*, 16 (6), 2064–2090. https://doi.org/10.1890/1051-0761(2006)016[2064:DALAWA]2.0.CO;2

Senbayram, M., Chen, R., Budai, A., Bakken, L. & Dittert, K. (2012). N2O emission and the N2O/(N2O+N2) product ratio of denitrification as controlled by available carbon substrates and nitrate concentrations. *Agriculture, Ecosystems & Environment*, 147, 4–12. https://doi.org/10.1016/j.agee.2011.06.022

Senbayram, M., Well, R., Bol, R., Chadwick, D.R., Jones, D.L. & Wu, D. (2018). Interaction of straw amendment and soil NO3− content controls fungal denitrification and denitrification product stoichiometry in a sandy soil. *Soil Biology and Biochemistry*, 126, 204–212. https://doi.org/10.1016/j.soilbio.2018.09.005

Shoun, H. & Fushinobu, S. (2016). CHAPTER 14:Denitrification in Fungi. *Metalloenzymes in Denitrification*. 331–348. https://doi.org/10.1039/9781782623762-00331

Shoun, H., Fushinobu, S., Jiang, L., Kim, S.-W. & Wakagi, T. (2012). Fungal denitrification and nitric oxide reductase cytochrome P450nor.

Philosophical Transactions of the Royal Society B: Biological Sciences, 367 (1593), 1186–1194. https://doi.org/10.1098/rstb.2011.0335

Shoun, H. & Tanimoto, T. (1991). Denitrification by the fungus Fusarium oxysporum and involvement of cytochrome P-450 in the respiratory nitrite reduction. *The Journal of Biological Chemistry*, 266 (17), 11078–11082

ŠImek, M. & Cooper, J.E. (2002). The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. *European Journal of Soil Science*, 53 (3), 345–354. https://doi.org/10.1046/j.1365-2389.2002.00461.x

Six, J., Feller, C., Denef, K., Ogle, S.M., de Moraes, J.C. & Albrecht, A. (2002). Soil organic matter, biota and aggregation in temperate and tropical soils - Effects of no-tillage. *Agronomie*, 22 (7–8), 755–775. https://doi.org/10.1051/agro:2002043

Six, J., Frey, S.D., Thiet, R.K. & Batten, K.M. (2006). Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. *Soil Science Society of America Journal*, 70 (2), 555–569. https://doi.org/10.2136/sssaj2004.0347

Sommermann, L., Geistlinger, J., Wibberg, D., Deubel, A., Zwanzig, J., Babin, D., Schlüter, A. & Schellenberg, I. (2018). Fungal community profiles in agricultural soils of a long-term field trial under different tillage, fertilization and crop rotation conditions analyzed by high-throughput ITS-amplicon sequencing. *PLOS ONE*, 13 (4), e0195345. https://doi.org/10.1371/journal.pone.0195345

Srour, A.Y., Ammar, H.A., Subedi, A., Pimentel, M., Cook, R.L., Bond, J. & Fakhoury, A.M. (2020). Microbial Communities Associated With Long-Term Tillage and Fertility Treatments in a Corn-Soybean Cropping System. *Frontiers in Microbiology*, 11, 1363. https://doi.org/10.3389/fmicb.2020.01363

Sterflinger, K., Tesei, D. & Zakharova, K. (2012). Fungi in hot and cold deserts with particular reference to microcolonial fungi. *Fungal Ecology*, 5 (4), 453–462. https://doi.org/10.1016/j.funeco.2011.12.007

Storer, K., Coggan, A., Ineson, P. & Hodge, A. (2018). Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N2O hotspots. *New Phytologist*, 220 (4), 1285–1295. https://doi.org/10.1111/nph.14931

Sun, R., Dsouza, M., Gilbert, J.A., Guo, X., Wang, D., Guo, Z., Ni, Y. & Chu, H. (2016). Fungal community composition in soils subjected to long-term chemical fertilization is most influenced by the type of

organic matter. *Environmental Microbiology*, 18 (12), 5137–5150. https://doi.org/10.1111/1462-2920.13512

Sun, R., Li, W., Dong, W., Tian, Y., Hu, C. & Liu, B. (2018). Tillage Changes Vertical Distribution of Soil Bacterial and Fungal Communities. *Frontiers in Microbiology*, 9. https://doi.org/10.3389/fmicb.2018.00699

Sutka, R.L., Adams, G.C., Ostrom, N.E. & Ostrom, P.H. (2008). Isotopologue fractionation during N2O production by fungal denitrification. *Rapid Communications in Mass Spectrometry*, 22 (24), 3989–3996. https://doi.org/10.1002/rcm.3820

Swallow, M.J.B. & Quideau, S.A. (2020). Evidence of rapid non-targeted effects of cycloheximide on soil bacteria using 13C-PLFA analysis. *Canadian Journal of Soil Science*, 100 (4), 356–362. https://doi.org/10.1139/cjss-2019-0152

Takasaki, K., Shoun, H., Yamaguchi, M., Takeo, K., Nakamura, A., Hoshino, T. & Takaya, N. (2004). Fungal Ammonia Fermentation, a Novel Metabolic Mechanism That Couples the Dissimilatory and Assimilatory Pathways of Both Nitrate and Ethanol: ROLE OF ACETYL CoA SYNTHETASE IN ANAEROBIC ATP SYNTHESIS*. *Journal of Biological Chemistry*, 279 (13), 12414–12420. https://doi.org/10.1074/jbc.M313761200

Takaya, N. (2002). Dissimilatory nitrate reduction metabolisms and their control in fungi. *Journal of Bioscience and Bioengineering*, 94 (6), 506–510. https://doi.org/10.1016/S1389-1723(02)80187-6

Takaya, N., Kuwazaki, S., Adachi, Y., Suzuki, S., Kikuchi, T., Nakamura, H., Shiro, Y. & Shoun, H. (2003). Hybrid Respiration in the Denitrifying Mitochondria of Fusarium oxysporum. *The Journal of Biochemistry*, 133 (4), 461–465. https://doi.org/10.1093/jb/mvg060

Tanimoto, T., Hatano, K., Kim, D., Uchiyama, H. & Shoun, H. (1992). Co-denitrification by the denitrifying system of the fungus Fusarium oxysporum. *FEMS Microbiology Letters*, 93 (2), 177–180. https://doi.org/10.1111/j.1574-6968.1992.tb05086.x

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., Smith, M.E., Sharp, C., Saluveer, E., Saitta, A., Rosas, M., Riit, T., Ratkowsky, D., Pritsch, K., Põldmaa, K., Piepenbring, M., Phosri, C., Peterson, M., Parts, K., Pärtel, K., Otsing, E., Nouhra, E., Njouonkou, A.L., Nilsson, R.H., Morgado, L.N., Mayor, J., May, T.W., Majuakim, L., Lodge, D.J., Lee, S.S., Larsson, K.-H., Kohout, P., Hosaka, K., Hiiesalu, I., Henkel, T.W., Harend, H., Guo, L., Greslebin, A., Grelet, G., Geml, J., Gates, G., Dunstan, W., Dunk,

C., Drenkhan, R., Dearnaley, J., Kesel, A.D., Dang, T., Chen, X., Buegger, F., Brearley, F.Q., Bonito, G., Anslan, S., Abell, S. & Abarenkov, K. (2014). Global diversity and geography of soil fungi. *Science*, 346 (6213). https://doi.org/10.1126/science.1256688

Tian, H., Xu, R., Canadell, J.G., Thompson, R.L., Winiwarter, W., Suntharalingam, P., Davidson, E.A., Ciais, P., Jackson, R.B., Janssens-Maenhout, G., Prather, M.J., Regnier, P., Pan, N., Pan, S., Peters, G.P., Shi, H., Tubiello, F.N., Zaehle, S., Zhou, F., Arneth, A., Battaglia, G., Berthet, S., Bopp, L., Bouwman, A.F., Buitenhuis, E.T., Chang, J., Chipperfield, M.P., Dangal, S.R.S., Dlugokencky, E., Elkins, J.W., Eyre, B.D., Fu, B., Hall, B., Ito, A., Joos, F., Krummel, P.B., Landolfi, A., Laruelle, G.G., Lauerwald, R., Li, W., Lienert, S., Maavara, T., MacLeod, M., Millet, D.B., Olin, S., Patra, P.K., Prinn, R.G., Raymond, P.A., Ruiz, D.J., van der Werf, G.R., Vuichard, N., Wang, J., Weiss, R.F., Wells, K.C., Wilson, C., Yang, J. & Yao, Y. (2020). A comprehensive quantification of global nitrous oxide sources and sinks. *Nature*, 586 (7828), 248–256. https://doi.org/10.1038/s41586-020-2780-0

Tian, H., Yang, J., Xu, R., Lu, C., Canadell, J.G., Davidson, E.A., Jackson, R.B., Arneth, A., Chang, J., Ciais, P., Gerber, S., Ito, A., Joos, F., Lienert, S., Messina, P., Olin, S., Pan, S., Peng, C., Saikawa, E., Thompson, R.L., Vuichard, N., Winiwarter, W., Zaehle, S. & Zhang, B. (2019). Global soil nitrous oxide emissions since the preindustrial era estimated by an ensemble of terrestrial biosphere models: Magnitude, attribution, and uncertainty. *Global Change Biology*, 25 (2), 640–659. https://doi.org/10.1111/gcb.14514

Toyoda, S., Mutobe, H., Yamagishi, H., Yoshida, N. & Tanji, Y. (2005). Fractionation of N2O isotopomers during production by denitrifier. *Soil Biology and Biochemistry*, 37 (8), 1535–1545. https://doi.org/10.1016/j.soilbio.2005.01.009

Toyoda, S., Yoshida, N., Miwa, T., Matsui, Y., Yamagishi, H., Tsunogai, U., Nojiri, Y. & Tsurushima, N. (2002). Production mechanism and global budget of N2O inferred from its isotopomers in the western North Pacific. *Geophys. Res. Lett*, 29 (3)

Treseder, K.K. & Lennon, J.T. (2015). Fungal Traits That Drive Ecosystem Dynamics on Land. *Microbiology and Molecular Biology Reviews*, 79 (2), 243–262. https://doi.org/10.1128/MMBR.00001-15

Vašák, F., Černý, J., Buráňová, Š., Kulhánek, M. & Balík, J. (2016). Soil pH changes in long-term field experiments with different fertilizing systems. *Soil and Water Research*, 10 (No. 1), 19–23. https://doi.org/10.17221/7/2014-SWR

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H. & Tilman, D.G. (1997). Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. *Ecological Applications*, 7 (3), 737–750. https://doi.org/10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2

Vitousek, P.M., Hättenschwiler, S., Olander, L. & Allison, S. (2002). Nitrogen and Nature. *AMBIO: A Journal of the Human Environment*, 31 (2), 97–101. https://doi.org/10.1579/0044-7447-31.2.97

Wallenstein, M.D., Myrold, D.D., Firestone, M. & Voytek, M. (2006). Environmental Controls on Denitrifying Communities and Denitrification Rates: Insights from Molecular Methods. *Ecological Applications*, 16 (6), 2143–2152. https://doi.org/10.1890/1051-0761(2006)016[2143:ECODCA]2.0.CO;2

Wang, F., Liang, X., Ma, S., Liu, L. & Wang, J. (2021). Ammonia-oxidizing archaea are dominant over comammox in soil nitrification under long-term nitrogen fertilization. *Journal of Soils and Sediments*, 21 (4), 1800–1814. https://doi.org/10.1007/s11368-021-02897-z

Wang, Y., Guo, J., Vogt, R.D., Mulder, J., Wang, J. & Zhang, X. (2018). Soil pH as the chief modifier for regional nitrous oxide emissions: New evidence and implications for global estimates and mitigation. *Global Change Biology*, 24 (2), e617–e626. https://doi.org/10.1111/gcb.13966

Wang, Y., Qi, L., Huang, R., Wang, F., Wang, Z. & Gao, M. (2020). Characterization of Denitrifying Community for Application in Reducing Nitrogen: a Comparison of nirK and nirS Gene Diversity and Abundance. *Applied Biochemistry and Biotechnology*, 192 (1), 22–41. https://doi.org/10.1007/s12010-020-03250-9

Wei, J., Ibraim, E., Brüggemann, N., Vereecken, H. & Mohn, J. (2019). First real-time isotopic characterisation of N2O from chemodenitrification. *Geochimica et Cosmochimica Acta*, 267, 17–32. https://doi.org/10.1016/j.gca.2019.09.018

Wei, W., Isobe, K., Shiratori, Y., Nishizawa, T., Ohte, N., Otsuka, S. & Senoo, K. (2014). N2O emission from cropland field soil through fungal denitrification after surface applications of organic fertilizer. *Soil Biology and Biochemistry*, 69, 157–167. https://doi.org/10.1016/j.soilbio.2013.10.044

Wei, W., Isobe, K., Shiratori, Y., Yano, M., Toyoda, S., Koba, K., Yoshida, N., Shen, H. & Senoo, K. (2021). Revisiting the involvement of ammonia oxidizers and denitrifiers in nitrous oxide emission from

cropland soils. *Environmental Pollution*, 287, 117494. https://doi.org/10.1016/j.envpol.2021.117494

Wen, Y.-C., Li, H.-Y., Lin, Z.-A., Zhao, B.-Q., Sun, Z.-B., Yuan, L., Xu, J.-K. & Li, Y.-Q. (2020). Long-term fertilization alters soil properties and fungal community composition in fluvo-aquic soil of the North China Plain. *Scientific Reports*, 10 (1), 7198. https://doi.org/10.1038/s41598-020-64227-6

Wu, D., Well, R., Cárdenas, L.M., Fuß, R., Lewicka-Szczebak, D., Köster, J.R., Brüggemann, N. & Bol, R. (2019). Quantifying N2O reduction to N2 during denitrification in soils via isotopic mapping approach: Model evaluation and uncertainty analysis. *Environmental Research*, 179, 108806. https://doi.org/10.1016/j.envres.2019.108806

Xu, H., Sheng, R., Xing, X., Zhang, W., Hou, H., Liu, Y., Qin, H., Chen, C. & Wei, W. (2019). Characterization of Fungal nirK-Containing Communities and N2O Emission From Fungal Denitrification in Arable Soils. *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.00117

Yamamoto, A., Akiyama, H., Nakajima, Y. & Hoshino, Y.T. (2017). Estimate of bacterial and fungal N2O production processes after crop residue input and fertilizer application to an agricultural field by 15N isotopomer analysis. *Soil Biology and Biochemistry*, 108, 9–16. https://doi.org/10.1016/j.soilbio.2017.01.015

Yoshida, N. & Toyoda, S. (2000). Constraining the atmospheric N2O budget from intramolecular site preference in N2O isotopomers. *Nature*, 405 (6784), 330–334. https://doi.org/10.1038/35012558

Yu, G., Fadrosh, D., Goedert, J.J., Ravel, J. & Goldstein, A.M. (2015). Nested PCR Biases in Interpreting Microbial Community Structure in 16S rRNA Gene Sequence Datasets. *PLoS ONE*, 10 (7), e0132253. https://doi.org/10.1371/journal.pone.0132253

Yu, L., Harris, E., Lewicka-Szczebak, D., Barthel, M., Blomberg, M.R.A., Harris, S.J., Johnson, M.S., Lehmann, M.F., Liisberg, J., Müller, C., Ostrom, N.E., Six, J., Toyoda, S., Yoshida, N. & Mohn, J. (2020). What can we learn from N2O isotope data? – Analytics, processes and modelling. *Rapid Communications in Mass Spectrometry*, 34 (20). https://doi.org/10.1002/rcm.8858

Zaheer, R., Noyes, N., Ortega Polo, R., Cook, S.R., Marinier, E., Van Domselaar, G., Belk, K.E., Morley, P.S. & McAllister, T.A. (2018). Impact of sequencing depth on the characterization of the microbiome and resistome. *Scientific Reports*, 8 (1), 5890. https://doi.org/10.1038/s41598-018-24280-8

Zhong, L., Bowatte, S., Newton, P.C.D., Hoogendoorn, C.J. & Luo, D. (2018). An increased ratio of fungi to bacteria indicates greater potential for N2O production in a grazed grassland exposed to elevated CO2. *Agriculture, Ecosystems & Environment*, 254, 111–116. https://doi.org/10.1016/j.agee.2017.11.027

Zhou, J., He, Z., Yang, Y., Deng, Y., Tringe, S.G. & Alvarez-Cohen, L. (2015). High-Throughput Metagenomic Technologies for Complex Microbial Community Analysis: Open and Closed Formats. *mBio*, 6 (1), e02288-14. https://doi.org/10.1128/mBio.02288-14

Zhou, Z., Takaya, N., Sakairi, M.A.C. & Shoun, H. (2001). Oxygen requirement for denitrification by the fungus Fusarium oxysporum. *Archives of Microbiology*, 175 (1), 19–25. https://doi.org/10.1007/s002030000231

Zou, C., Penfold, C., Sands, R., Misra, R.K. & Hudson, I. (2001). Effects of soil air-filled porosity, soil matric potential and soil strength on primary root growth of radiata pine seedlings. *Plant and Soil*, 236 (1), 105–115. https://doi.org/10.1023/A:1011994615014

Zumft, W.G. (1997). Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*, 61 (4), 533–616. https://doi.org/10.1128/mmbr.61.4.533-616.1997

Popular science summary

Nitrous oxide (N_2O), also known as 'laughing gas', is a strong greenhouse gas that is nearly 300 times more powerful than carbon dioxide. Besides contributing to global warming, N_2O is currently also the single most prominent ozone depleting chemical. Primary sources of N_2O include terrestrial soils, particularly agricultural soils. They receive large inputs of nitrogen in the form of fertilizers to promote plant growth and thereby crop yield. Nitrous oxide from soils is produced by microbial processes that convert excess bioavailable nitrogen into N_2O. As much as 50% of the added nitrogen as fertilizer can be lost as gaseous nitrogen.

Denitrification is the most important N_2O producing process in soils. Denitrification is performed by bacteria, archaea and fungi and converts nitrate through successive steps to nitrogen gas, via several intermediates of which N_2O is one. Denitrification in fungi always ends with N_2O and fungi are therefore potentially important sources of this greenhouse gas. However, since denitrification has almost exclusively been studied in bacteria, we know little about denitrifying fungi in soil.

The studies in this thesis aimed to increase our understanding of the ecology of denitrifying fungi in soils by determining their global distribution, how they are affected by soil properties, their possible contribution to N_2O emissions, and how they are affected by common management practices in crop production systems.

The first study revealed that fungal denitrifiers are generally rare and about 200 times less abundant than other denitrifiers, predominantly bacteria. Fungal denitrifiers were present in all types of soil environments but the highest numbers were in forest soils, particularly in tropical and subtropical broadleaf forests. In relation to other denitrifiers, they were most abundant in forest and tundra soils. The amount of fungal denitrifiers correlated with

soil properties that are known to promote denitrification, i.e. moist soils with high content of carbon and nitrogen. Many of the ones identified in the soils studied in this thesis are cosmopolitan molds and plant pathogens such as *Aspergillus*, *Penicillium* and *Fusarium* species that can flourish under a broad range of environmental conditions. Since global warming promotes stress tolerant organisms, we might expect an increase in fungal denitrifiers.

The second and third studies examined how common management practices like fertilization and tillage affect denitrifying fungi. Fertilization increased nutrient availability along with the abundance of denitrifying fungi, but also changed their community composition, reducing the relative abundance of *Fusarium* species but increasing that of *Penicillium* species. Similarly, conventional tillage with soil inversion resulted in differing soil properties and composition and abundance of fungal denitrifier communities in comparison to less tilled soils in the topsoil. The accumulation of organic material in the upper soil layers of non-inverted soils could explain this. However, no significant contribution to N_2O production by fungi was observed in any of the studies, suggesting that fungi are less relevant for denitrification in agricultural soils than previously suggested.

Overall, this thesis shows that fungal denitrifiers constitute a low-abundant group within the nitrogen cycle and that the genetic potential for fungal denitrification is much lower than that for bacteria. However, the fungal denitrifier community can change in soils subject to management and thereby their contribution to N_2O production can change. To reduce N_2O emissions through soil management strategies, it is crucial to have a better knowledge of the ecology of denitrifying microorganisms, including fungi, and how they control N_2O emissions.

Populärvetenskaplig sammanfattning

Lustgas (N_2O) är en växthusgas som är 300 gånger starkare än koldioxid. Förutom att bidra till den globala uppvärmningen är N_2O numera också den enskilt viktigaste substansen som bryter ner ozonskiktet . Mark av olika slag, särskilt åkermark, är den största källan till N_2O eftersom åkermark gödslas med stora mängder kväve för att främja goda skördar. Lustgas produceras när mikroorganismer i jorden omvandlar olika former av kväve.

Den viktigaste processen för N_2O produktion är denitrifikation. Så mycket som 50% av tillfört kväve kan försvinna från marken som gasformigt kväve. Denitrifikation kan utföras av bakterier, arkéer och svampar och omvandlar nitrat till kvävgas via flera steg, varav N_2O är en mellanprodukt. Denitrifikation hos svampar slutar dock alltid med N_2O. Svampar är därför potentiellt viktiga källor till denna växthusgas. Eftersom denitrifikation nästan uteslutande har studerats i bakterier vet vi inte så mycket om denitrifierande svampar.

Denna avhandling syftade till att öka förståelsen om de denitrifierande svampars ekologi genom att bestämma deras globala utbredning, påverkan av markfaktorer, möjliga bidrag till N_2O emissioner och hur de påverkas av vanliga brukningsåtgärder i våra odlingssystem.

Den första studien visade att denitrifierande svampar är sällsynta och cirka 200 gånger mindre förekommande än övriga denitrifierande mikroorganismer. De påträffades i alla typer av markekosystem men fanns i störst antal i skogsmarker, särskilt i tropiska och subtropiska lövskogar. Deras andel inom det denitrifierande mikrobiella samhället var störst i skogs- och tundrajordar. Förekomsten av denitrifierande svampar korrelerade med markegenskaper kända för att främja denitrifikation, d.v.s. fuktiga jordar med hög halt av kol och kväve. Många av de denitrifierande svamparna som identifierades är kosmopolitiska mögelsvampar och växtpatogener som

Aspergillus, *Penicillium* och *Fusarium* som kan gynnas under många olika miljöförhållanden. Eftersom den pågående klimatförändringen förväntas gynna stresstoleranta organismer, kan vi eventuellt förvänta oss en ökning av denitrifierande svampar.

Den andra och tredje studien undersökte hur vanliga brukningsåtgärder inom jordbruket påverkar denitrifierande svampar. Kvävegödsling ökade markens näringsinnehåll samtidigt som antalet denitrifierande svampar ökade, men samhällssammansättning ändrades, t.ex. blev *Fusarium* blir relativt färre och *Penicillium* rikligare. På liknande sätt visades att jordar med konventionell plöjning jämfört med andra typer av jordbearbetning hade andramarkegenskaper, liksom fler och andra denitrifierande svampar i det övre jordlagret Det kan eventuellt förklaras av det ökade kolinnehållet i det övre jordskiktet med denna bearbetningsåtgärd.

Sammanfattningsvis visar avhandlingsarbetet att denitrifierande svampar är en liten grupp inom kvävets kretslopp, och att den genetiska potentialen för denitrifikation hos svampar är lägre än den för bakterier. För att minska N_2O -utsläppen genom ändrade brukningsåtgärder är ökad kunskap om ekologin hos denitrifierande mikroorganismer, inklusive svampar, och hur de påverkar emissioner av N_2O avgörande.

Acknowledgements

To my supervisors:

Sara, thank you so much for this privilege and opportunity to learn from you and this great team how to do great research. I appreciate a lot the energy and time you invested to teach, encourage and bring me back on track.

Chris, I'm very grateful to have had you as my co-supervisor. Thanks you so much for your patience, advice, support and time to explain, finding the error in the code and all the encouragements.

Roger, thanks a lot for all your encouragements, the input especially at the end of this PhD journey. I appreciate a lot.

Magnus, thank you for your support during the last years, checking on me every now and then and your help especially during the challenging last weeks.

Grace and Aurélien: Without your help, the last one and a half years would not have worked out as they did. Thank you so much for the time you took to explain and help me with bioinformatics, discussing and explaining.

Arpita, Carles, Dominik, Jaanis, Karina, Laura, Lea, Louis, Mathilde, Maria, Stephanie, Tina, Valerie and Monica: thank you for making our group such a welcoming place, to learn laugh and work together.

Thomas, Ararso, Mats, Tino: Thank you for your collaboration, your patience with a newbie in soil science and providing me with insight into soil physics.

Laurent, David: Thank you for your warm welcome in Dijon, the great time I could spend there. I appreciate a lot.

To my fellow PhD students: thank you for your friendship, encouragement, all the good talks and fun we had together.

To the members of the Mykopat department: Thank you every one of you for the great atmosphere you create. It was a pleasure to meet you all, work with you and learn from you.

To my family in Switzerland and Austria: You always inspired and challenged me in so many positive ways. Thank you for your endless support! Ihr Lieben, danke für eure Unterstützung, Inspiration und zu wissen, dass ihr immer für mich da seid. Danke.

To Samuel: Without you, this journey would never have come so far. Thank you for traveling with me and enduring me. I love you.

THANK YOU!

III

Soil Biology and Biochemistry 170 (2022) 108718

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

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

Yvonne Bösch [a], Christopher M. Jones [a], Roger Finlay [a], Magnus Karlsson [a], Mats Larsbo [b], Thomas Keller [b,c], Sara Hallin [a,*]

[a] Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology, Box 7026, 750 07, Uppsala, Sweden
[b] Swedish University of Agricultural Sciences, Department of Soil and Environment, Box 7014, 750 07, Uppsala, Sweden
[c] Agroscope, Department of Agroecology and Environment, Reckenholzstrasse 191, 8046, Zürich, Switzerland

ARTICLE INFO

Keywords:
Fungal denitrification
nirK
Nitrous oxide
Ploughing
Tillage

ABSTRACT

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

* Corresponding author.
E-mail address: Sara.Hallin@slu.se (S. Hallin).

https://doi.org/10.1016/j.soilbio.2022.108718
Received 30 December 2021; Received in revised form 12 May 2022; Accepted 14 May 2022
Available online 17 May 2022

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). Multicopper 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 ± 0.3% (mean ± SD) and 0.4 ± 0.2% (mean ± 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 ± 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^{a}	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.

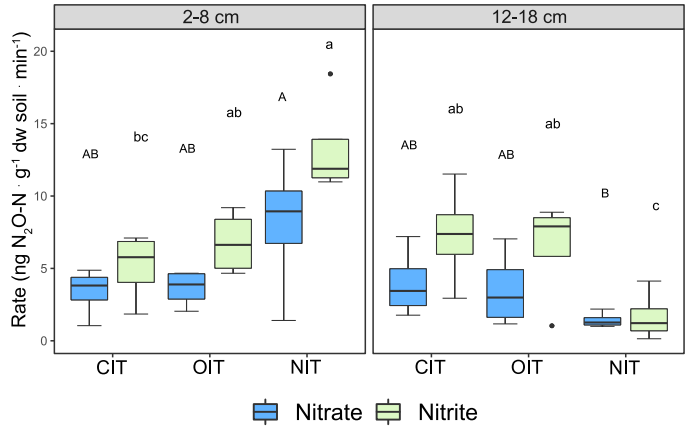


Fig. 1. Potential N_2O production with addition of NO_3^- or NO_2^- in the upper and lower layer of the topsoil subject to conventional inversion (CIT), occasional inversion (OIT) and non-inversion tillage (NIT). Letters above the boxes indicate statistically significant differences among tillage and 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

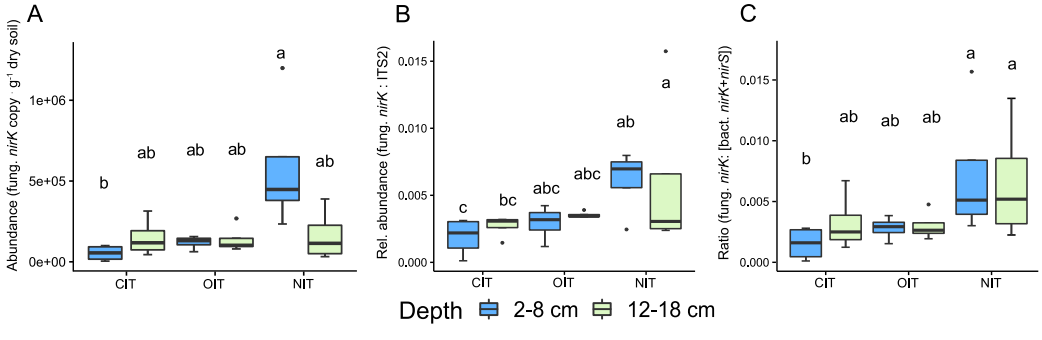


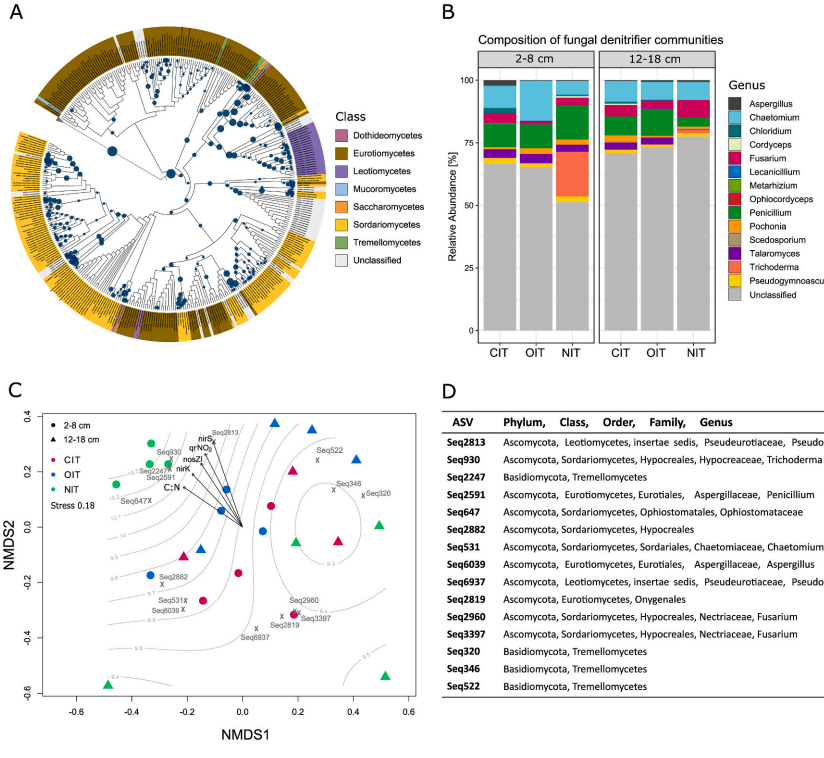
Fig. 2. Abundance of fungal denitrification genes in the upper and lower layer of the topsoil subject to conventional inversion (CIT), occasional inversion (OIT) and non-inversion tillage (NIT). **A)** Absolute abundance of fungal denitrifiers based on fungal *nirK* copy numbers. The abundances were 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 N_2O 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



ASV	Phylum, Class, Order, Family, Genus
Seq2813	Ascomycota, Leotiomycetes, insertae sedis, Pseudeurotiaceae, Pseudogymnoascus
Seq930	Ascomycota, Sordariomycetes, Hypocreales, Hypocreaceae, Trichoderma
Seq2247	Basidiomycota, Tremellomycetes
Seq2591	Ascomycota, Eurotiomycetes, Eurotiales, Aspergillaceae, Penicillium
Seq647	Ascomycota, Sordariomycetes, Ophiostomatales, Ophiostomataceae
Seq2882	Ascomycota, Sordariomycetes, Hypocreales
Seq531	Ascomycota, Sordariomycetes, Sordariales, Chaetomiaceae, Chaetomium
Seq6039	Ascomycota, Eurotiomycetes, Eurotiales, Aspergillaceae, Aspergillus
Seq6937	Ascomycota, Leotiomycetes, insertae sedis, Pseudeurotiaceae, Pseudogymnoascus
Seq2819	Ascomycota, Eurotiomycetes, Onygenales
Seq2960	Ascomycota, Sordariomycetes, Hypocreales, Nectriaceae, Fusarium
Seq3397	Ascomycota, Sordariomycetes, Hypocreales, Nectriaceae, Fusarium
Seq320	Basidiomycota, Tremellomycetes
Seq346	Basidiomycota, Tremellomycetes
Seq522	Basidiomycota, Tremellomycetes

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 NO_2^--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 N_2O 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.

Acknowledgements

This work was supported by The Swedish Research Council Formas (grant number 2016-00477). We thank Katarina Ihrmark for helping with preparations for the amplicon sequencing of fungal *nirK* and David Bru and Laurent Philippot for their help with establishing the quantitative PCR assay for fungal *nirK*. We also thank Tino Colombi and Ararso Etana for collecting the soil samples, Ana Maria Mingot Soriano for help with soil analyses, and the staff of Lövsta field station for performing all field operations. We further thank the Faculty of Natural Resources and Agricultural Science of the Swedish University of Agricultural Sciences, for the financial support of the field experiment. Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala. The facility is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory. The SNP&SEQ Platform is also supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. The authors would like to acknowledge the support of the National Genomics Infrastructure (NGI)/Uppsala Genome Center and UPPMAX with assistance in massively parallel sequencing and computational infrastructure. Work performed at NGI/Uppsala Genome Center was funded by RFI/VR and Science for Life Laboratory, Sweden.

Appendix A. Supplementary data

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

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Arvidsson, J., Etana, A., Rydberg, T., 2014. Crop yield in Swedish experiments with shallow tillage and no-tillage 1983–2012. European Journal of Agronomy 52, 307–315. https://doi.org/10.1016/j.eja.2013.08.002.

Baggs, E.M., Stevenson, M., Pihlatie, M., Regar, A., Cook, H., Cadisch, G., 2003. Nitrous oxide emissions following application of residues and fertiliser under zero and conventional tillage. Plant and Soil 254, 361–370. https://doi.org/10.1023/A:1025593121839.

Bartossek, R., Spang, A., Weidler, G., Lanzen, A., Schleper, C., 2012. Metagenomic analysis of ammonia-oxidizing archaea affiliated with the soil group. Frontiers in Microbiology 3. https://doi.org/10.3389/fmicb.2012.00208.

Blanco-Canqui, H., Wortmann, C.S., 2020. Does occasional tillage undo the ecosystem services gained with no-till? A review. Soil and Tillage Research 198, 104534. https://doi.org/10.1016/j.still.2019.104534.

Bockus, W.W., Shroyer, J.P., 1998. The impact of reduced tillage on soilborne plant pathogens. Annual Review of Phytopathology 36, 485–500. https://doi.org/10.1146/annurev.phyto.36.1.485.

Bongiorno, G., Bünemann, E.K., Oguejiofor, C.U., Meier, J., Gort, G., Comans, R., Mäder, P., Brussaard, L., de Goede, R., 2019. Sensitivity of labile carbon fractions to tillage and organic matter management and their potential as comprehensive soil quality indicators across pedoclimatic conditions in Europe. Ecological Indicators 99, 38–50. https://doi.org/10.1016/j.ecolind.2018.12.008.

Bonilla-Rosso, G., Wittorf, L., Jones, C.M., Hallin, S., 2016. Design and evaluation of primers targeting genes encoding NO-forming nitrite reductases: implications for ecological inference of denitrifying communities. Scientific Reports 6. https://doi.org/10.1038/srep39208.

Boyd, J.A., Woodcroft, B.J., Tyson, G.W., 2018. GraftM: a tool for scalable, phylogenetically informed classification of genes within metagenomes. Nucleic Acids Research 46, e59. https://doi.org/10.1093/nar/gky174.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Castaño, C., Berlin, A., Durling, M.B., Ihrmark, K., Lindahl, B.D., Stenlid, J., Clemmensen, K.E., Olson, Å., 2020. Optimized metabarcoding with Pacific biosciences enables semi-quantitative analysis of fungal communities. New Phytologist 228. https://doi.org/10.1111/nph.16731.

Chen, H., Mothapo, N.V., Shi, W., et al., 2015a. Soil Moisture and pH Control Relative Contributions of Fungi and Bacteria to N_2O Production. Microbial Ecology 69 (1), 180–191. https://doi.org/10.1007/s00248-014-0488-0.

Chen, H., Mothapo, N.V., Shi, W., et al., 2015b. Fungal and bacterial N_2O production regulated by soil amendments of simple and complex substrates. Soil Biology and Biochemistry 84, 116–126. https://doi.org/10.1016/j.soilbio.2015.02.018.

Chen, H., Yu, F., Shi, W., 2016. Detection of N2O-producing fungi in environment using nitrite reductase gene (nirK)-targeting primers. Fungal Biology 120, 1479–1492. https://doi.org/10.1016/j.funbio.2016.07.012.

Clemmensen, K.E., Durling, M.B., Michelsen, A., Hallin, S., Finlay, R.D., Lindahl, B.D., 2021. A tipping point in carbon storage when forest expands into tundra is related to mycorrhizal recycling of nitrogen. Ecology Letters 24, 1193–1204. https://doi.org/10.1111/ele.13735.

Degrune, F., Theodorakopoulos, N., Colinet, G., Hiel, M.-P., Bodson, B., Taminiau, B., Daube, G., Vandenbol, M., Hartmann, M., 2017. Temporal dynamics of soil microbial communities below the seedbed under two contrasting tillage regimes. Frontiers in Microbiology 8, 1127. https://doi.org/10.3389/fmicb.2017.01127.

Doran, J.W., 1980. Soil microbial and biochemical changes associated with reduced tillage 1. Soil Science Society of America Journal 44, 765–771. https://doi.org/10.2136/sssaj1980.03615995004400040022x.

Eddy, S.R., 2008. A probabilistic model of local sequence alignment that simplifies statistical significance estimation. PLoS Computational Biology 4, e1000069. https://doi.org/10.1371/journal.pcbi.1000069.

Etana, A., Rydberg, T., Arvidsson, J., 2009. Readily dispersible clay and particle transport in five Swedish soils under long-term shallow tillage and mouldboard ploughing. Soil and Tillage Research 106, 79–84. https://doi.org/10.1016/j.still.2009.09.016.

Graf, D.R.H., Jones, C.M., Hallin, S., 2014. Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N2O emissions. PLoS One 9. https://doi.org/10.1371/journal.pone.0114118.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. Trichoderma species — opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2, 43–56. https://doi.org/10.1038/nrmicro797.

Heil, J., Vereecken, H., Brüggemann, N., 2016. A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. European Journal of Soil Science 67, 23–39. https://doi.org/10.1111/ejss.12306.

Hicks Pries, C.E., Sulman, B.N., West, C., O'Neill, C., Poppleton, E., Porras, R.C., Castanha, C., Zhu, B., Wiedemeier, D.B., Torn, M.S., 2018. Root litter decomposition slows with soil depth. Soil Biology and Biochemistry 125, 103–114. https://doi.org/10.1016/j.soilbio.2018.07.002.

Higgins, S.A., Schadt, C.W., Matheny, P.B., Löffler, F.E., 2018. Phylogenomics reveal the dynamic evolution of fungal nitric oxide reductases and their relationship to secondary metabolism. Genome Biology and Evolution 10, 2474–2489. https://doi.org/10.1093/gbe/evy187.

Higgins, S.A., Welsh, A., Orellana, L.H., Konstantinidis, K.T., Chee-Sanford, J.C., Sanford, R.A., Schadt, C.W., Löffler, F.E., 2016. Detection and diversity of fungal nitric oxide reductase genes (p450nor) in agricultural soils. Applied and Environmental Microbiology 82, 2919–2928. https://doi.org/10.1128/AEM.00243-16.

Hui, N., Liu, X., Jumpponen, A., Setälä, H., Kotze, D.J., Biktasheva, L., Romantschuk, M., 2018. Over twenty years farmland reforestation decreases fungal diversity of soils, but stimulates the return of ectomycorrhizal fungal communities. Plant and Soil 427, 231–244. https://doi.org/10.1007/s11104-018-3647-0.

Hydbom, S., Enfors, M., Birgander, J., Hollander, J., Jensen, E.S., Olsson, P.A., et al., 2017. Reduced tillage stimulated symbiotic fungi and microbial saprotrophs, but did

not lead to a shift in the saprotrophic microorganism community structure. Applied Soil Ecology 119, 104–114. https://doi.org/10.1016/j.apsoil.2017.05.032.
Ibraim, E., Wolf, B., Harris, E., Gasche, R., Wei, J., Yu, L., Kiese, R., Eggleston, S., Butterbach-Bahl, K., Zeeman, M., Tuzson, B., Emmenegger, L., Six, J., Henne, S., Mohn, J., 2019. Attribution of N_2O sources in a grassland soil with laser spectroscopy based isotopocule analysis. Biogeosciences 16, 3247–3266. https://doi.org/10.5194/bg-16-3247-2019.
Ihrmark, K., Bödeker T.M., I., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K.E., Lindahl, B.D., et al., 2012. New primers to amplify the fungal ITS2 region – evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiology Ecology 82 (3), 666–677. https://doi.org/10.1111/j.1574-6941.2012.01437.x.
Jones, C.M., Stres, B., Rosenquist, M., Hallin, S., 2008. Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. Molecular Biology and Evolution 25, 1955–1966. https://doi.org/10.1093/molbev/msn146.
Kaurin, A., Mihelič, R., Kastelec, D., Grčman, H., Bru, D., Philippot, L., Suhadolc, M., 2018. Resilience of bacteria, archaea, fungi and N-cycling microbial guilds under plough and conservation tillage, to agricultural drought. Soil Biology and Biochemistry 120, 233–245. https://doi.org/10.1016/j.soilbio.2018.02.007.
Kautz, T., 2015. Research on subsoil biopores and their functions in organically managed soils: a review. Renewable Agriculture and Food Systems 30, 318–327. https://doi.org/10.1017/S1742170513000549.
Kim, S.-W., Fushinobu, S., Zhou, S., Wakagi, T., Shoun, H., 2009. Eukaryotic nirK genes encoding copper-containing nitrite reductase: originating from the protomitochondrion? Applied and Environmental Microbiology 75, 2652–2658. https://doi.org/10.1128/AEM.02536-08.
Krauss, M., Berner, A., Perrochet, F., Frei, R., Niggli, U., Mäder, P., 2020. Enhanced soil quality with reduced tillage and solid manures in organic farming – a synthesis of 15 years. Scientific Reports 10, 4403. https://doi.org/10.1038/s41598-020-61320-8.
Kravchenko, A.N., Guber, A.K., Quigley, M.Y., Koestel, J., Gandhi, H., Ostrom, N.E., 2018. X-ray computed tomography to predict soil N2O production via bacterial denitrification and N2O emission in contrasting bioenergy cropping systems. GCB Bioenergy 10, 894–909. https://doi.org/10.1111/gcbb.12552.
Ladan, S., Jacinthe, P.-A., 2016. Evaluation of antibacterial and antifungal compounds for selective inhibition of denitrification in soils. Environmental Sciences: Processes & Impacts 18, 1519–1529. https://doi.org/10.1039/C6EM00456C.
Lahti, L., Shetty, S., 2017. In: Microbiome R Package. https://doi.org/10.18129/B9.bioc.microbiome.
Legrand, F., Picot, A., Cobo-Díaz, J.F., Carof, M., Chen, W., Le Floch, G., 2018. Effect of tillage and static abiotic soil properties on microbial diversity. Applied Soil Ecology 132, 135–145. https://doi.org/10.1016/j.apsoil.2018.08.016.
Leplat, J., Friberg, H., Abid, M., Steinberg, C., 2013. Survival of Fusarium graminearum, the causal agent of Fusarium head blight. A review. Agronomy for Sustainable Development 33, 97–111. https://doi.org/10.1007/s13593-012-0098-5.
Letunic, I., Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Research 49 (W1), W293–W296. https://doi.org/10.1093/nar/gkab301.
Liu, S., Schloter, M., Hu, R., Vereecken, H., Brüggemann, N., 2019. Hydroxylamine contributes more to abiotic N2O production in soils than nitrite. Frontiers in Environmental Science 7, 47. https://doi.org/10.3389/fenvs.2019.00047.
Lognoul, M., Theodorakopoulos, N., Hiel, M.-P., Regaert, D., Broux, F., Heinesch, B., Bodson, B., Vandenbol, M., Aubinet, M., 2017. Impact of tillage on greenhouse gas emissions by an agricultural crop and dynamics of N2O fluxes: insights from automated closed chamber measurements. Soil and Tillage Research 167, 80–89. https://doi.org/10.1016/j.still.2016.11.008.
Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, null, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüssmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.-H., 2004. ARB: a software environment for sequence data. Nucleic Acids Research 32, 1363–1371. https://doi.org/10.1093/nar/gkh293.
Ma, Y., Zilles, J.L., Kent, A.D., 2019. An evaluation of primers for detecting denitrifiers via their functional genes. Environmental Microbiology 21, 1196–1210. https://doi.org/10.1111/1462-2920.14555.
Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.-C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C., Philippot, L., 2015. N2O production, a widespread trait in fungi. Scientific Reports 5. https://doi.org/10.1038/srep09697.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.
Meng, J., Xu, Y., Li, S., Li, C., Zhang, X., Dong, D., Chen, P., 2010. Soybean growth and soil microbial populations under conventional and conservational tillage systems. Journal of Crop Improvement 24, 337–348. https://doi.org/10.1080/15427528.2010.499864.
Mothapo, N., Chen, H., Cubeta, M.A., Grossman, J.M., Fuller, F., Shi, W., 2015. Phylogenetic, taxonomic and functional diversity of fungal denitrifiers and associated N2O production efficacy. Soil Biology and Biochemistry 83, 160–175. https://doi.org/10.1016/j.soilbio.2015.02.001.
Mothapo, N.V., Chen, H., Cubeta, M.A., Shi, W., 2013. Nitrous oxide producing activity of diverse fungi from distinct agroecosystems. Soil Biology and Biochemistry 66, 94–101. https://doi.org/10.1016/j.soilbio.2013.07.004.
Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular Biology and Evolution 32, 268–274. https://doi.org/10.1093/molbev/msu300.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2020. In: Vegan: Community Ecology Package.
Parvin, N., Parvage, M.M., Etana, A., 2014. Effect of mouldboard ploughing and shallow tillage on sub-soil physical properties and crop performance. Soil Science & Plant Nutrition 60, 38–44. https://doi.org/10.1080/00380768.2013.847779.
Pell, M., Stenberg, B., Stenström, J., Torstensson, L., 1996. Potential denitrification activity assay in soil—with or without chloramphenicol? Soil Biology and Biochemistry 28, 393–398. https://doi.org/10.1016/0038-0717(95)00149-2.
Philippot, L., 2002. Denitrifying genes in bacterial and Archaeal genomes. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression 1577, 355–376. https://doi.org/10.1016/S0167-4781(02)00420-7.
Philippot, L., Hallin, S., 2005. Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. In: Peter J Peters and Joel Swanson, vol. 8, pp. 234–239. https://doi.org/10.1016/j.mib.2005.04.003. Current Opinion in Microbiology, Ecology and industrial microbiology/Edited by Sergio Sánchez and Betty Olson · Techniques/.
Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of denitrifying prokaryotes in agricultural soil. In: Advances in Agronomy, Advances in Agronomy. Academic Press, pp. 249–305. https://doi.org/10.1016/S0065-2113(07)96003-4.
Prendergast-Miller, M.T., Baggs, E.M., Johnson, D., 2011. Nitrous oxide production by the ectomycorrhizal fungi Paxillus involutus and Tylospora fibrillosa. FEMS Microbiology Letters 316, 31–35. https://doi.org/10.1111/j.1574-6968.2010.02187.x.
Ramesh, T., Bolan, N.S., Kirkham, M.B., Wijesekara, H., Kanchikerimath, M., Srinivasa Rao, C., Sandeep, S., Rinklebe, J., Ok, Y.S., Choudhury, B.U., Wang, H., Tang, C., Wang, X., Song, Z., Freeman II, O.W., 2019. Chapter One - soil organic carbon dynamics: impact of land use changes and management practices: a review. In: Sparks, D.L. (Ed.), Advances in Agronomy. Academic Press, pp. 1–107. https://doi.org/10.1016/bs.agron.2019.02.001.
Shan, J., Sanford, R.A., Chee-Sanford, J., Ooi, S.K., Löffler, F.E., Konstantinidis, K.T., Yang, W.H., 2021. Beyond denitrification: the role of microbial diversity in controlling nitrous oxide reduction and soil nitrous oxide emissions. Global Change Biology 27, 2669–2683. https://doi.org/10.1111/gcb.15545.
Shoun, H., Fushinobu, S., Jiang, L., Kim, S.-W., Wakagi, T., 2012. Fungal denitrification and nitric oxide reductase cytochrome P450nor. Philosophical Transactions of the Royal Society B: Biological Sciences 367, 1186–1194. https://doi.org/10.1098/rstb.2011.0335.
Shoun, H., Kim, D.-H., Uchiyama, H., Sugiyama, J., 1992. Denitrification by fungi. FEMS Microbiology Letters 94, 277–281. https://doi.org/10.1111/j.1574-6968.1992.tb05331.x.
Shoun, H., Takaya, N., 2002. Cytochromes P450nor and P450foxy of the fungus Fusarium oxysporum. International Congress Series, Oxygen and life: Oxygenases, oxidase and lipid mediators 1233, 89–97. https://doi.org/10.1016/S0531-5131(02)00378-3.
Six, J., Elliott, E.T., Paustian, K., 2000. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. Soil Biology and Biochemistry 32, 2099–2103. https://doi.org/10.1016/S0038-0717(00)00179-6.
Six, J., Feller, C., Denef, K., Ogle, S.M., de Moraes, J.C., Albrecht, A., 2002. Soil organic matter, biota and aggregation in temperate and tropical soils - effects of no-tillage. Agronomie 22, 755–775. https://doi.org/10.1051/agro:2002043.
Smith, C.R., Blair, P.L., Boyd, C., Cody, B., Hazel, A., Hedrick, A., Kathuria, H., Khurana, P., Kramer, B., Muterspaw, K., Peck, C., Sells, E., Skinner, J., Tegeler, C., Wolfe, Z., 2016. Microbial community responses to soil tillage and crop rotation in a corn/soybean agroecosystem. Ecology and Evolution 6, 8075–8084. https://doi.org/10.1002/ece3.2553.
Srour, A.Y., Ammar, H.A., Subedi, A., Pimentel, M., Cook, R.L., Bond, J., Fakhoury, A.M., 2020. Microbial communities associated with long-term tillage and fertility treatments in a corn-soybean cropping system. Frontiers in Microbiology 11, 1363. https://doi.org/10.3389/fmicb.2020.01363.
Sun, R., Li, W., Dong, W., Tian, Y., Hu, C., Liu, B., 2018. Tillage changes vertical distribution of soil bacterial and fungal communities. Frontiers in Microbiology 9. https://doi.org/10.3389/fmicb.2018.00699.
Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., Nishijima, M., 2014. Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. PLoS One 9, e105592. https://doi.org/10.1371/journal.pone.0105592.
Vujanovic, V., 2021. Tremellomycetes yeasts in kernel ecological niche: early indicators of enhanced competitiveness of endophytic and mycoparasitic symbionts against wheat pathobiota. Plants 10, 905. https://doi.org/10.3390/plants10050905.
Wagner-Riddle, C., Baggs, E.M., Clough, T.J., Fuchs, K., Petersen, S.O., 2020. Mitigation of nitrous oxide emissions in the context of nitrogen loss reduction from agroecosystems: managing hot spots and hot moments. Current Opinion in Environmental Sustainability, Climate Change, Reactive Nitrogen, Food Security and Sustainable Agriculture 47, 46–53. https://doi.org/10.1016/j.cosust.2020.08.002.
Waldrop, M.P., Balser, T.C., Firestone, M.K., 2000. Linking microbial community composition to function in a tropical soil. Soil Biology and Biochemistry 32, 1837–1846. https://doi.org/10.1016/S0038-0717(00)00157-7.
Wang, Z., Chen, Q., Liu, L., Wen, X., Liao, Y., et al., 2016. Responses of soil fungi to 5-year conservation tillage treatments in the drylands of northern China. Applied Soil Ecology 101, 132–140. https://doi.org/10.1016/j.apsoil.2016.02.002.
Wang, H., Marshall, C.W., Cheng, M., Xu, H., Li, H., Yang, X., Zheng, T., 2017. Changes in land use driven by urbanization impact nitrogen cycling and the microbial

community composition in soils. Scientific Reports 7, 44049. https://doi.org/10.1038/srep44049.
Wang, J., Zou, J., 2020. No-till increases soil denitrification via its positive effects on the activity and abundance of the denitrifying community. Soil Biology and Biochemistry 142, 107706. https://doi.org/10.1016/j.soilbio.2020.107706.
Ward, B.B., 2013. How nitrogen is lost. Science 341, 352–353. https://doi.org/10.1126/science.1240314.
Wei, W., Isobe, K., Shiratori, Y., Nishizawa, T., Ohte, N., Ise, Y., Otsuka, S., Senoo, K., 2015. Development of PCR primers targeting fungal nirK to study fungal denitrification in the environment. Soil Biology and Biochemistry 81, 282–286. https://doi.org/10.1016/j.soilbio.2014.11.026.
Wei, W., Isobe, K., Shiratori, Y., Nishizawa, T., Ohte, N., Otsuka, S., Senoo, K., 2014. N2O emission from cropland field soil through fungal denitrification after surface applications of organic fertilizer. Soil Biology and Biochemistry 69, 157–167. https://doi.org/10.1016/j.soilbio.2013.10.044.
Wortmann, C.S., Quincke, J.A., Drijber, R.A., Mamo, M., Franti, T., 2008. Soil microbial community change and recovery after one-time tillage of continuous No-till. Agronomy Journal 100, 1681–1686. https://doi.org/10.2134/agronj2007.0317.
Wuest, S.B., 2001. Soil biopore estimation: effects of tillage, nitrogen, and photographic resolution. Soil and Tillage Research 62, 111–116. https://doi.org/10.1016/S0167-1987(01)00218-5.
Yurkov, A.M., 2018. Yeasts of the soil – obscure but precious. Yeast 35, 369–378. https://doi.org/10.1002/yea.3310.
Zhang, D., Yang, X., Wang, Y., Zong, J., Ma, J., Li, C., 2020. Changes in soil organic carbon fractions and bacterial community composition under different tillage and organic fertiliser application in a maize–wheat rotation system. Acta Agriculturae Scandinavica Section B Soil and Plant Science 70, 457–466. https://doi.org/10.1080/09064710.2019.1700301.
Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Reviews 61, 533–616. https://doi.org/10.1128/mmbr.61.4.533-616.1997.

Table S1. Conditions and primers used in quantitative polymerase chain reaction (qPCR) assays for each marker gene.

Target	Primer Name	Sequence (5' > 3')	Reference	Cycling conditions	Efficiency
16S rRNA	341F	CCTACGGGAGGCAGCAG	Lopez-Gutierrez *et al.*, 2004	(95°C 7 min)x 1; (95°C 15 s, 60°C 30 s, 72°C 30 s, 80°C 30 s)x 35 (95°C 15 s;(60 - 95°C, 10 s, increment 0.5°C))x 1	104.02%
	543R	ATTACCGCGGCTGCTGGCA			
nirK	876F	ATYGGCGGVCAYGGCGA	Henry *et al.*, 2004	(95°C 7 min)x 1; (95°C 15 s, (63°C-58°C, -1°C/cycle) 30 s, 72°C 30 s)x 6; (95°C 15 s, 58°C 30 s, 80°C 30 s)x 35; (95°C 15 s;(60 - 95°C, 10 s, increment 0.5°C))x 1	93.49%
	1040R	GCCTCGATCAGRTTRTGGTT			
nirS	cd3aFm	AACGYSAAGGARACSGG	Throbäck *et al.*, 2004	(95°C 7 min)x 1; (95°C 15 s,(65°C-60°C, -1°C/cycle), 30s, 72°C 30 s)x 6; (95°C 15 s, 60°C 30 s, 72°C 30 s, 80°C 30 s)x 35 (95°C 15 s;(60 - 95°C, 10 s, increment 0.5°C))x 1	70.78%
	R3cdm	GASTTCGGRTGSGTCTTSAYGAA			
*nos*ZI	1840F	CGCRACGGCAASAAGGTSMSSGT	Henry *et al.*, 2004	(95°C 7 min)x 1; (95°C 15 s, 65°C 30s, 72°C 30s)x 5; (95°C 15 s, 60°C 30s, 72°C 30s, 80°C 10 s)x 34; (95°C 15 s; (70 - 95°C, 15 s, increment 0.5°C))x 1	81.56%
	2090R	CAKRTGCAKSGCRTGGCAGAA			
*nos*ZII	nosZII-F	CTIGGICCIYTKCAYAC	Jones *et al.*, 2013	(95°C 5 min)x 1; (95°C 15 s, 54°C 30 s, 72°C 45 s, 80°C 5 s)x 39 (95°C 15 s;(75 - 95°C, 10 s, increment 0.5°C))x 1	81.47%
	nosZII-R	GCIGARCARAAITCBGTRC			
ITS2	ITS3F	GCATCGATGAAGAACGCAGC	White *et al.*, 1990	(95°C 7 min)x 1; (95°C 15 s, 60°C 30s, 72°C 30 s, 80°C 30 s)x 35 (95°C 15 s;(60 - 90°C, 10 s, increment 0.5°C))x 1	88.67%
	ITS4R	TCCTCCGCTTATTGATATGC			
fungal *nirK*	EunirK-F1	GGBAAYCCICAYAAYATCGA	Maeda *et al.*, 2015	(95°C 7 min)x 1; (95°C 15 s, 60°C 30s, 72°C 30 s, 80°C 30 s)x 35 (95°C 15 s;(60 - 90°C, 10 s, increment 0.5°C))x 1	76.81%
	EunirK-R2	GGICCIGCRTTSCCRAAGAA			

Table S2. Primers and cycling conditions for PCR amplification of marker genes used for amplicon sequencing.

Target	Primer Name	Sequence (5´> 3´)	Reference	Cycling conditions
16S rRNA	Pro341F	CCTACGGGNBGCASCAG	Takahashi *et al.*, 2014	(98°C 3min) x 1; (98°C 15 s, 55°C 30 s, 72°C 40 s) x 25; (72°C 10 min) x1
	Pro805R	GACTACNVGGGTATCTAATCC		
ITS2	gITS7	GTGARTCATCGAATCTTTG	Ihrmark *et al*., 2012	(95 °C 5min) x 1; (95 °C 30s, 56°C 30s, 72 °C 30s) x 23-29
	ITS4	TCCTCCGCTTATTGATATGC		
fungal *nirK*	EunirK-F1	GGBAAYCCICAYAAYATCGA	Maeda *et al*., 2015	(98°C 5min) x1; (98°C 1 min, 53°C 30 s, 68°C 1 min) x 30; (68°C 10 min) x 1
	EunirK-R2	GGICCIGCRTTSCCRAAGAA		

Table S3. Effect of tillage regime and soil depth on edaphic factors based on two-way ANOVA. WFPS: water-filled pore space, AFP: air-filled porosity. Numerator and denominator degrees of freedom are indicated for each *F*-ratio as subscripts, and significant main and interaction effects are shown in bold (*0.01 < P < 0.05; **0.001 < P < 0.01; *** P < 0.001).

	ANOVA results		
Edaphic factor	**Tillage**	**Depth**	**Tillage × Depth**
HCl-K ($mg \cdot 100g^{-1}$)	**$F_{2,21}$= 23.35*** **	$F_{1,20}$=0.004	$F_{2,18}$=0.87
HCl-P ($mg \cdot 100g^{-1}$)	**$F_{2,21}$=11.80*** **	$F_{1,20}$=0.07	$F_{2,18}$=3.40
AL-K ($mg \cdot 100g^{-1}$)	**$F_{2,21}$=5.84***	$F_{1,21}$=3.93	$F_{2,18}$=2.74
AL-P ($mg \cdot 100g^{-1}$)	$F_{2,21}$=0.42	$F_{1,20}$=0.12	$F_{2,18}$=1.41
Total C (%)	**$F_{2,21}$=21.03*** **	**$F_{1,20}$=9.16****	**$F_{2,18}$=17.68*** **
Total N (%)	**$F_{2,21}$=12.39*** **	$F_{1,20}$=2.55	**$F_{2,18}$=7.21****
C:N	$F_{2,21}$=3.12	**$F_{1,20}$=8.29****	**$F_{2,18}$=8.89****
pH	$F_{2,21}$=1.04	$F_{1,20}$=0.06	$F_{2,18}$=1.95
Water content at sampling (%)	$F_{2,21}$=1.20	**$F_{1,20}$=7.59***	**$F_{2,18}$=4.12***
Bulk density ($g \cdot cm^{-3}$)	$F_{2,21}$= 0.45	$F_{1,20}$= 1.10	**$F_{2,18}$= 3.92***
Total Porosity ($cm^{-3} \cdot cm^{-3}$)	$F_{2,21}$= 0.57	$F_{1,20}$= 0.71	**$F_{2,18}$= 3.79***
WFPS -50 hPa (%)	$F_{2,21}$=0.84	$F_{1,20}$=1.50	$F_{2,18}$=2.60
WFPS -300 hPa (%)	$F_{2,21}$=0.54	$F_{1,20}$=0.002	$F_{2,18}$=2.84
WFPS -600 hPa (%)	$F_{2,21}$=0.84	$F_{1,20}$=1.41	$F_{2,18}$=2.55
AFP -50 hPa ($m^3\ m^{-3}$)	$F_{2,21}$=0.77	$F_{1,20}$=1.32	$F_{2,18}$=3.51
AFP -300 hPa ($m^3\ m^{-3}$)	$F_{2,21}$=1.15	$F_{1,20}$=0.02	**$F_{2,18}$=4.76***
AFP -600 hPa ($m^3\ m^{-3}$)	$F_{2,21}$=0.97	$F_{1,20}$=1.64	**$F_{2,18}$=4.07***

Table S4: Potential denitrification rates (mean ± SD, n=4, expressed as ng N_2O-N g^{-1} soil dry weight min^{-1}) in soils under conventional inversion (CIT), occasional inversion (OIT) or non-inversion tillage (NIT) at two different depths using NO_3^- or NO_2^- as the terminal electron acceptor. Generalized linear modelling followed by two-way ANOVA was used to test for tillage, depth, and interaction effects. Subscript numbers indicate the numerator and denominator degrees of freedom. Significant effects are in bold, and different letters indicate significant differences based on Šidák post hoc testing within each electron acceptor (*0.01 < P < 0.05; **0.001 < P < 0.01; *** P < 0.001).

Electron acceptor	Tillage	Soil layer		ANOVA results	
		Upper	Lower	Factor	F
NO_3^-	CIT	3.39 ± 1.67^{ab}	3.96 ± 2.38^{ab}	Tillage	$F_{2,15} = 0.09$
	OIT	3.62 ± 1.26^{ab}	3.55 ± 2.67^{ab}	Depth	**$F_{1,15} = 8.39$***
	NIT	8.13 ± 4.93^{a}	1.43 ± 0.53^{b}	Tillage x Depth	**$F_{2,15} = 8.14$****
NO_2^-	CIT	5.12 ± 2.41^{bc}	7.30 ± 3.52^{ac}	Tillage	$F_{2,18} = 0.40$
	OIT	6.78 ± 2.22^{ac}	6.43 ± 3.65^{ac}	Depth	**$F_{1,18} = 7.43$***
	NIT	13.30 ± 3.48^{a}	1.68 ± 1.73^{b}	Tillage x Depth	**$F_{2,18} = 12.56$*****

Table S5. Spearman's ranked correlation coefficients of soil physico-chemical properties, gene abundance, potential activities and community diversities (Shannon's *H´*). Significant values (P < 0.05) are shown in bold, and blue or red indicate positive or negative associations, respectively (*0.01 < P < 0.05; **0.001 < P < 0.01; *** P < 0.001; ns: non-significant).

		Soil Properties																	**Gene Abundance**										**Denit.**		**Shannon's *H***		
		HCL.K	**HCL.P**	**AL.K**	**AL.P**	**Tot. C**	**Tot. N**	**C:N**	**pH**	**Water cont.**	**Bulk density**	**Porosity**	**WFPS 50**	**WFPS 300**	**WFPS 600**	**AFP 50**	**AFP 300**	**AFP 600**	**ITS2**	**16S rRNA**	***nirK***	***nirS***	***nosZI***	***nosZII***	**fung. *nirK***	**fung. *nir*: bact. *nir***	**rel. fung. *nir***	**rel. bact. *nir***	**denit. NO_2^-**	**denit. NO_3^-**	**ITS2**	**16S rRNA**	**fung. *nirK***
Soil Properties	**HCL.K**	NA	**0.54**	**0.52**	0.08	0.11	0.19	-0.32	0.32	0.01	-0.09	0.11	-0.10	0.02	-0.05	0.11	0.00	0.04	-0.04	-0.07	-0.36	-0.13	-0.35	-0.06	-0.14	-0.04	-0.27	-0.25	-0.15	0.06	-0.15	-0.18	-0.12
	HCL.P	**	NA	**0.48**	**0.49**	**0.56**	**0.49**	0.37	-0.31	0.25	-0.26	0.24	**-0.44**	-0.31	-0.38	**0.47**	0.33	0.36	0.06	0.04	-0.13	0.15	0.02	0.06	-0.03	-0.05	-0.17	-0.02	0.18	0.20	0.14	0.18	0.37
	AL.K	**	*	NA	0.38	**0.51**	**0.59**	0.03	-0.05	**0.66**	-0.56	**0.52**	-0.25	-0.06	-0.20	0.27	0.13	0.21	0.40	0.33	0.11	0.40	0.23	0.19	0.25	0.16	0.08	0.17	**0.49**	**0.63**	0.16	0.16	0.13
	AL.P	ns	*	ns	NA	**0.46**	**0.50**	0.29	**-0.56**	0.39	-0.28	0.28	-0.31	-0.19	-0.23	0.34	0.21	0.25	0.26	-0.02	-0.06	0.16	0.21	-0.01	0.33	0.34	0.13	0.18	0.13	0.35	-0.10	-0.10	0.11
	Tot. C	ns	**	*	*	NA	**0.95**	**0.51**	**-0.65**	**0.63**	**-0.49**	**0.46**	-0.32	-0.21	-0.23	0.39	0.26	0.24	0.36	0.29	0.33	**0.41**	**0.44**	0.35	0.34	0.26	0.18	0.08	**0.42**	**0.46**	-0.06	0.04	**0.51**
	Tot. N	ns	*	**	*	***	NA	0.32	**-0.61**	**0.65**	**-0.48**	**0.46**	-0.24	-0.12	-0.15	0.30	0.18	0.15	0.40	0.33	0.30	0.35	0.39	0.32	**0.42**	**0.41**	0.27	0.01	0.40	**0.51**	-0.14	-0.02	**0.42**
	C:N	ns	ns	ns	ns	*	ns	NA	**-0.48**	0.29	-0.18	0.11	-0.36	-0.37	-0.36	0.38	0.34	0.33	0.32	0.27	**0.45**	**0.50**	**0.49**	0.36	0.23	0.00	-0.04	0.26	0.37	0.09	0.37	0.39	**0.54**
	pH	ns	ns	ns	**	***	**	*	NA	**-0.41**	0.40	-0.38	0.07	0.15	0.04	-0.15	-0.18	-0.10	-0.36	-0.06	-0.15	-0.30	-0.39	-0.12	-0.26	-0.25	-0.13	-0.26	-0.39	-0.32	0.27	0.03	-0.26
	Water cont.	ns	ns	***	ns	***	***	ns	*	NA	**-0.73**	0.44	-0.10	-0.03	-0.06	0.15	0.10	0.10	**0.66**	**0.48**	**0.42**	**0.67**	**0.71**	0.24	**0.51**	0.33	0.23	0.39	**0.75**	**0.84**	0.15	0.28	0.39
	Bulk density	ns	ns	**	ns	*	*	ns	ns	***	NA	**-0.99**	**0.41**	**0.46**	**0.42**	**-0.50**	**-0.55**	**-0.52**	-0.36	-0.12	-0.17	**-0.52**	-0.37	-0.07	-0.17	-0.04	-0.15	**-0.51**	**-0.60**	**-0.69**	-0.04	-0.04	0.02
	Porosity	ns	ns	**	ns	*	*	ns	ns	ns	***	NA	-0.39	**-0.44**	-0.40	**0.48**	**0.52**	**0.50**	0.34	0.09	0.11	**0.50**	0.32	0.06	0.14	0.02	0.13	**0.50**	**0.57**	**0.68**	-0.01	-0.05	0
	WFPS 50	ns	*	ns	ns	ns	ns	ns	ns	ns	*	ns	NA	**0.91**	**0.98**	**-0.99**	**-0.91**	**-0.96**	0.26	0.19	-0.10	-0.01	0.09	0.04	0.12	0.18	-0.14	-0.21	0.02	-0.02	**-0.45**	-0.13	-0.13
	WFPS 300	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	***	NA	**0.95**	**-0.92**	**-0.99**	**-0.96**	0.18	0.18	-0.16	-0.05	0.03	0.08	0.08	0.12	-0.11	-0.27	0.01	0.00	**-0.42**	-0.18	-0.04
	WFPS 600	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	***	***	NA	**-0.97**	**-0.95**	**-0.98**	0.24	0.13	-0.15	-0.07	0.08	0.05	0.13	0.22	-0.11	-0.25	-0.01	-0.02	**-0.49**	-0.20	-0.11
	AFP 50	ns	*	ns	ns	ns	ns	ns	ns	ns	*	*	***	***	***	NA	**0.93**	**0.96**	-0.23	-0.19	0.10	0.07	-0.05	-0.04	-0.11	-0.18	0.14	0.28	0.04	0.08	0.37	0.08	0.09
	AFP 300	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	**	***	***	***	***	NA	**0.98**	-0.16	-0.18	0.16	0.09	-0.01	-0.09	-0.09	-0.15	0.11	0.33	0.06	0.09	0.40	0.16	0.01
	AFP 600	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	*	***	***	***	***	***	NA	-0.22	-0.19	0.12	0.07	-0.06	-0.11	-0.14	-0.23	0.10	0.32	0.05	0.09	0.44	0.14	0.01
Gene Abundances	**ITS2**	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	NA	**0.66**	**0.54**	**0.71**	**0.68**	**0.58**	**0.69**	**0.51**	0.00	0.31	**0.63**	**0.52**	0.06	0.39	0.23
	16S rRNA	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	***	NA	**0.69**	**0.66**	**0.67**	**0.59**	**0.61**	0.33	0.17	-0.05	**0.56**	0.32	0.24	**0.52**	**0.50**
	nirK	ns	ns	ns	ns	ns	ns	*	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	**	***	NA	**0.62**	**0.73**	**0.51**	**0.70**	**0.42**	**0.43**	0.32	**0.56**	0.17	0.31	**0.47**	**0.42**
	nirS	ns	ns	ns	ns	*	ns	*	ns	***	**	*	ns	ns	ns	ns	ns	ns	***	***	**	NA	**0.80**	**0.46**	**0.53**	0.14	0.12	**0.62**	**0.80**	**0.57**	0.22	**0.52**	0.35
	nosZI	ns	ns	ns	ns	*	ns	*	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	***	***	***	***	NA	0.31	**0.73**	**0.43**	0.34	**0.44**	**0.68**	**0.47**	0.17	**0.47**	**0.44**
	nosZII	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	**	*	*	ns	NA	0.40	0.20	-0.05	0.01	0.40	0.05	0.12	0.22	0.17
	fung. *nirK*	ns	ns	ns	ns	ns	*	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	***	**	***	**	***	ns	NA	**0.86**	**0.61**	0.21	**0.42**	0.29	-0.04	0.27	0.17
	fung. *nir*: bact. *nir*	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*	ns	*	ns	***	NA	**0.65**	-0.06	0.14	0.17	-0.20	0.04	0.12
	rel. fung. *nir*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	**	***	NA	0.13	0.16	0.09	-0.11	-0.01	0.10
	rel. bact. *nir*	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	*	ns	ns	ns	ns	NA	**0.56**	0.40	0.15	0.28	-0.15
Denit.	**denit. NO_2^-**	ns	ns	*	ns	*	ns	ns	ns	***	**	**	ns	ns	ns	ns	ns	ns	*	**	**	***	***	ns	*	ns	ns	**	NA	**0.60**	0.16	**0.45**	0.31
	denit. NO_3^-	ns	ns	**	ns	*	*	ns	ns	***	***	***	ns	ns	ns	ns	ns	ns	**	ns	ns	**	*	ns	ns	ns	ns	ns	**	NA	0.21	0.24	0.24
Shannon's *H*	**ITS2**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	NA	**0.75**	**0.42**
	16S rRNA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	*	**	*	ns	ns	ns	ns	ns	*	ns	***	NA	**0.55**
	fung. *nirK*	ns	ns	ns	ns	*	*	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	ns	*	ns	ns	ns	ns	ns	ns	ns	*	**	NA

Table S6. Effect of tillage regime and soil depth on abundance of different genes based on two-way ANOVA analyses. The numerator and denominator degrees of freedom are given for each *F*-ratio as subscripts, and significant main and interaction effects are shown in bold (*0.01 < P < 0.05; **0.001 < P < 0.01; *** P < 0.001).

	ANOVA results		
Target	**Tillage**	**Depth**	**Tillage x Depth**
16S rRNA	**$F_{2,18}$ =6.12****	**$F_{1,18}$ =13.98****	**$F_{2,18}$ =15.31*****
ITS2	$F_{2,21}$ =1.76	$F_{1,20}$ =0.63	**$F_{2,18}$ =4.09***
fungal *nir*K	**$F_{2,21}$ =3.92***	$F_{1,20}$ <0.01	**$F_{2,18}$ =4.50***
nirK	**$F_{2,21}$ =4.41***	**$F_{1,20}$ =12.34****	**$F_{2,18}$ =10.33****
nirS	$F_{2,18}$ =0.24	**$F_{1,18}$ =13.37****	**$F_{2,18}$ =30.82*****
*nos*ZI	$F_{2,18}$ =2.79	**$F_{1,18}$ =11.56****	**$F_{2,18}$ =10.35****
*nos*ZII	$F_{2,18}$ =2.03	$F_{1,18}$ =0.36	**$F_{2,18}$ =6.78****
(*nirK*+ *nirS*) /16S	**$F_{2,18}$ =3.88***	$F_{1,18}$ =1.82	**$F_{2,18}$ =6.20****
fungal *nirK* /ITS2	**$F_{2,21}$ =5.48***	$F_{1,20}$ =0.51	$F_{2,18}$ =0.17
fungal *nirK*/(*nirS*+*nirK*)	**$F_{2,21}$ =4.75***	$F_{1,20}$ =0.79	$F_{2,18}$ =0.98

Table S7. Effects of conventional inversion-, occasional inversion- and no-inversion tillage on absolute abundance of different marker genes (mean ± SD, n=4, expressed as copy number per gram dry soil) and abundance ratios in the upper and lower layer of the topsoil. Different letters indicate significant differences ($P < 0.05$) among treatment and depth utilizing Šidák post-hoc testing for generalized linear models and Tukey's HSD testing for linear models.

	Upper soil layer			Lower soil layer		
Target	**CIT**	**OIT**	**NIT**	**CIT**	**OIT**	**NIT**
16S rRNA	6.29E+08 ± 1.69E+08 ^{b}	7.08E+08 ± 1.40E+08 ^{b}	1.28E+09 ± 2.09E+08 ^{a}	7.35E+08 ± 1.11E+08 ^{b}	6.10E+08 ± 1.34E+08 ^{b}	5.43E+08 ± 1.70E+08 ^{b}
ITS2	2.86E+07 ± 9.74E+06 ^{b}	4.35E+07 ± 9.52E+06 ^{ab}	9.86E+07 ± 5.80E+07 ^{a}	5.13E+07 ± 3.29E+07 ^{ab}	3.97E+07 ± 2.65E+07 ^{ab}	3.20E+07 ± 2.76E+07 ^{ab}
fungal *nir*K	5.39E+04 ± 4.85E+04 ^{b}	1.20E+05 ± 4.12E+04 ^{ab}	5.82E+05 ± 4.24E+05 ^{a}	1.49E+05 ± 1.19E+05 ^{ab}	1.38E+05 ± 8.75E+04 ^{ab}	1.62E+05 ± 1.63E+05 ^{ab}
nirK	9.98E+06 ± 1.19E+06 ^{b}	1.22E+07 ± 2.46E+06 ^{b}	3.25E+07 ± 1.12E+07 ^{a}	1.00E+07 ± 2.76E+06 ^{b}	1.16E+07 ± 2.82E+06 ^{b}	8.84E+06 ± 3.61E+06 ^{b}
nirS	2.64E+07 ± 2.70E+06 ^{bc}	3.07E+07 ± 7.13E+06 ^{c}	5.08E+07 ± 5.18E+06 ^{a}	3.37E+07 ± 4.97E+06 ^{c}	3.29E+07 ± 9.85E+06 ^{c}	1.32E+07 ± 5.36E+06 ^{b}
*nos*ZI	2.34E+05 ± 6.22E+04 ^{b}	3.83E+05 ± 1.53E+05 ^{ab}	1.13E+06 ± 5.13E+05 ^{a}	2.37E+05 ± 1.30E+05 ^{b}	4.09E+05 ± 2.44E+05 ^{ab}	1.53E+05 ± 9.41E+04 ^{b}
*nos*ZII	1.23E+07 ± 1.42E+06 ^{b}	1.45E+07 ± 1.07E+06 ^{ab}	2.04E+07 ± 3.08E+06 ^{a}	1.74E+07 ± 3.35E+06 ^{ab}	1.39E+07 ± 3.69E+06 ^{ab}	1.27E+07 ± 6.88E+06 ^{ab}
(*nirK*+ *nirS*) /16S	0.05 ± 0.02 ^{ab}	0.06 ± 0.02 ^{ab}	0.08 ± 0.05 ^{a}	0.07 ± 0.05 ^{ab}	0.06 ± 0.04 ^{a}	0.05 ± 0.04 ^{b}
fungal *nirK* /ITS2	0.002 ± 0.001 ^{c}	0.003 ± 0.001 ^{abc}	0.006 ± 0.003 ^{ab}	0.003 ± 0.001 ^{bc}	0.004 ± 0.0002 ^{abc}	0.006 ± 0.006 ^{a}
fungal *nirK*/ (*nirS*+*nirK*)	0.002 ± 0.001 ^{b}	0.003 ± 0.001 ^{ab}	0.007 ± 0.006 ^{a}	0.003 ± 0.002 ^{ab}	0.003 ± 0.001 ^{ab}	0.007 ± 0.005 ^{a}

Table S8. PERMANOVA showing effects of tillage regime and soil depth on bacterial, fungal and fungal denitrifier communities. The Adonis function with 999 permutations was used, and significant effects ($P < 0.05$) are indicated in bold. Subscript numbers indicate the numerator and denominator degrees of freedom for different F-ratios. R^2 values indicate variance explained by each factor (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$0.0001 < P < 0.001$).

.

Community (marker gene)	PERMANOVA results		
	Factor	**R^2**	**F**
Fungal denitrifiers (Eu*nirK*)	Tillage	0.118	$F_{2,17}$=**1.46***
	Depth	0.077	$F_{1,17}$=**1.90****
	Tillage x Depth	0.117	$F_{2,17}$=**1.45***
	Residuals	0.688	
Bacterial (16S rRNA)	Tillage	0.130	$F_{2,18}$=**1.84****
	Depth	0.105	$F_{1,18}$=**2.97*****
	Tillage x Depth	0.126	$F_{2,18}$=**1.78***
	Residuals	0.638	
Fungal (ITS2)	Tillage	0.145	$F_{2,18}$=**2.12****
	Depth	0.108	$F_{1,18}$=**3.15*****
	Tillage x Depth	0.131	$F_{2,18}$=**1.91****
	Residuals	0.616	

Table S9. Alpha diversity of total fungal (ITS2), total bacterial (16S rRNA gene) and fungal *nirK* (EunirK) communities measured as Shannon Wiener index. Values are arranged by tillage treatment and soil depth (mean ± SD, n=4). Different letters indicate significant differences among within each community based on Šidák post-hoc testing, and the effects of tillage regime, depth and their interaction are given as F-values, with significant factors in bold and the numerator and denominator degrees of freedom in subscript (*0.01 < P < 0.05; **0.001 < P < 0.01; ***0.0001 < P < 0.001).

		Diversity (Shannon's *H*)		
Tillage	**Soil layer**	**16S rRNA community**	**ITS2 community**	**Fungal *nirK* community**
CIT	Upper	6.25 ± 0.16 ^{a}	4.24 ± 0.17 ^{a}	2.90 ± 0.13 ^{ab}
	Lower	6.25 ± 0.24 ^{a}	4.06 ± 0.22 ^{a}	2.82 ± 0.26 ^{ab}
OIT	Upper	6.31 ± 0.15 ^{a}	4.22 ± 0.14 ^{a}	2.94 ± 0.24 ^{ab}
	Lower	6.13 ± 0.12 ^{ab}	3.99 ± 0.15 ^{a}	2.65 ± 0.14 ^{b}
NIT	Upper	6.41 ± 0.15 ^{a}	4.24 ± 0.11 ^{a}	3.25 ± 0.18 ^{a}
	Lower	5.82 ± 0.16 ^{b}	3.59 ± 0.17 ^{b}	2.76 ± 0.29 ^{b}
ANOVA	Tillage	$F_{2,21}$ = 1.51	$F_{2,21}$ = 3.16	$F_{2,17}$ = 2.04
	Depth	$F_{1,20}$ = **14.24 ****	$F_{1,20}$ = **26.71 *****	$F_{1,17}$ = **11.04 ****
	Interaction	$F_{2,18}$ = **6.81 ****	$F_{2,18}$ = **4.10***	$F_{2,17}$ = 1.74

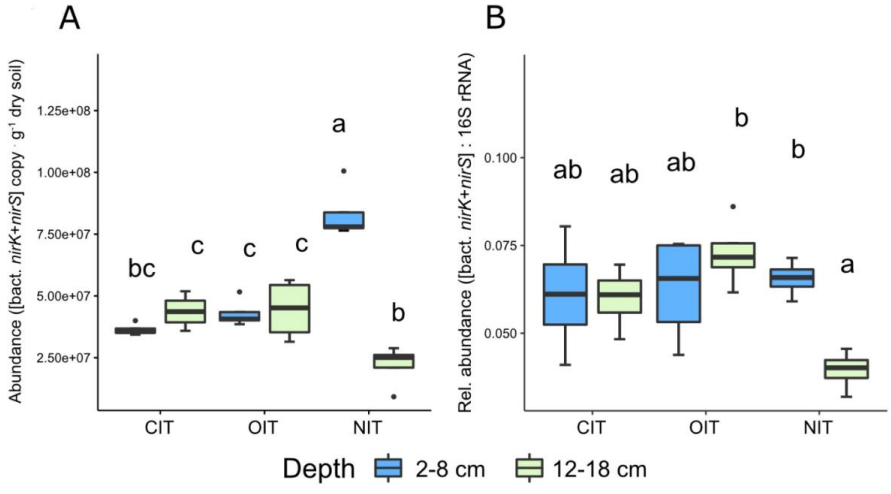


Figure S1. Abundance of bacterial denitrification genes in the upper and lower layer of the topsoil subjected to conventional inversion (CIT), occasional inversion (OIT) and non-inversion tillage (NIT). **A)** Abundance of bacterial denitrifiers determined as the sum of bacterial *nirK* and *nirS* copy numbers. **B)** Relative abundance of bacterial denitrifiers within the total bacterial community calculated as the ratio of the sum of bacterial *nirK* and *nirS* to the bacterial 16S rRNA gene copy numbers.

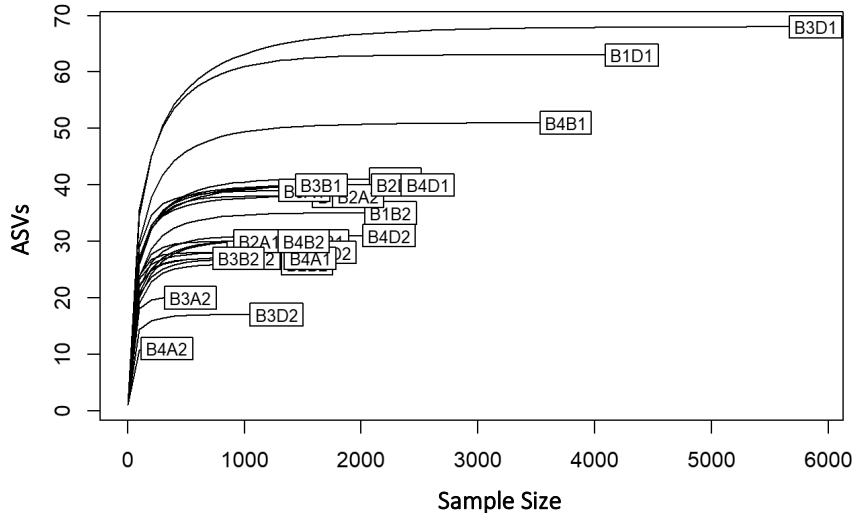


Figure S2. Rarefaction curve of fungal *nirK* communities. Due to the low observed sample size, sample B4A2 was excluded from the fungal *nirK* community analysis.

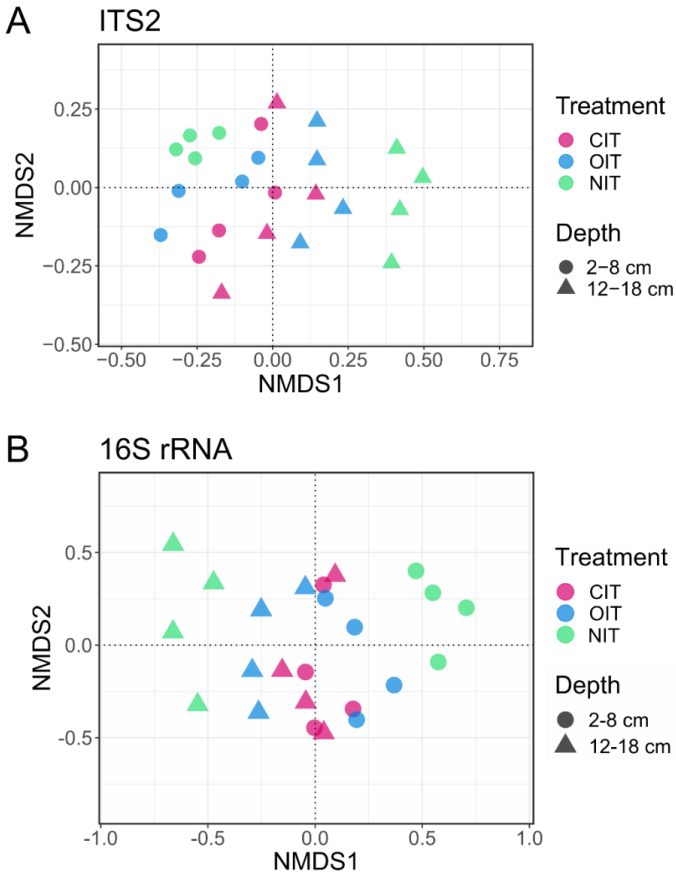


Figure S3. Total fungal (**A**) and bacterial (**B**) community structure visualized by non-metric multidimensional scaling plots. Samples are colored according to tillage regimes, and shapes represent soil sampling depth.

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2022:51

Fungi can produce nitrous oxide (N_2O), a strong greenhouse gas, through the process of denitrification. The ecology of this understudied group in the nitrogen cycle was investigated in terrestrial environments, with an emphasis on understanding their global distribution and the impact of agricultural management methods. The genetic potential for denitrification of these rare cosmopolitan fungi was primarily influenced by the availability of soil resources, which can be influenced by soil management methods, although it was orders of magnitude lower than that of their prokaryotic equivalents.

Yvonne Bösch received her graduate education at the Department of Forest Mycology and Plant Pathology, SLU, Uppsala. She received her M.Sc. in Cell Biology from the Swiss Federal Institute of Technology ETH Zurich, Switzerland.

Acta Universitatis Agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).

SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.

ISSN 1652-6880
ISBN (print version) 978-91-7760-977-3
ISBN (electronic version) 978-91-7760-978-0