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Nitrification Inhibitors Have Minimal Impact on Microbial and Collembola Communities in Danish Arable Soil

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Abstract

This study aimed to investigate the targeted and non-target impacts of nitrification inhibitors (NIs) on the soil microbial and collembola communities in arable soils under a spring barley cultivation system. By assessing soils where inhibitors were repeatedly applied alongside fertilizers, we evaluated the short-term effects of NI use on soil biodiversity and function composition using a combination of microscopic identification, quantitative PCR (qPCR) and amplicon sequencing. Our results revealed that neither ammonia-oxidizing microorganisms nor non-target bacterial and fungal communities, as well as collembola, were not significantly affected by short-term NI application, even at tenfold dosages than recommended. However, the choice of fertilizer—organic or chemical—had significant effects on microbial and collembola communities. The neutral community model (NCM) analysis indicated that bacterial communities under chemical fertilizer treatments and fungal communities under organic treatments were primarily governed by stochastic processes, highlighting the resilience and functional redundancy of these communities. Despite the limited direct impact of NIs, high concentrations of organic matter correlated significantly with microbial community structures under high NI conditions, underscoring the buffering role of organic matter. These findings suggest that while fertilizer type plays a crucial role in shaping soil ecology, NIs have minimal impact on both targeted and non-targeted groups in the short term. Future research should focus on the role of complete ammonia oxidizers (comammox) to gain a more comprehensive understanding of nitrogen cycling dynamics in these systems.

Acknowledgment

I had a very good time writing my thesis in Denmark and Sweden! Besides my wonderful supervisors, I appreciate Paul Henning Krogh for introducing me into Denmark and guided me through collembola classification, and Zdenek Gavor who gave me a very useful collembola identification lecture. A lot of thanks to soil microbe group at Aarhus university for designing the field experiment and assistance in sample collection & molecular labwork. Lab technicians Tina Thane and Tanya Begovic are very helpful organizing workplace in the wet lab and preparing necessary consumables.

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1. Introduction

Nitrogen is an essential element for soil fertility and is considered a limiting element for primary productivity in terrestrial ecosystems. Nitrogen fertilizers have been extensively used in agricultural soil to sustain a suitable N concentration for crop growth. However, excessive use of nitrogen fertilizers can accelerate the nitrification process, during which ammonium (NH_4^+) is oxidized to nitrite (NO_2^-) and then nitrate (NO_3^-). The increasing NO_2^- in the soil not only causes soil acidification, but also accumulates in the leaves of crops to affect human health (Meyer & Stitt, 2001). Moreover, compared with NH_4^+ which can be bound to clay minerals, NO_3^- can move freely with the water, thus tending to be leached into groundwater and culminate in eutrophication (Akinnawo, 2023). Nieder & Benbi (2008) estimated that 55Tg of nitrate is leached from agricultural soils every year. The management of soil nitrogen is also highly concerned in greenhouse gas emissions. Extra nitrate can accelerate the process of denitrification, generating nitrous oxide (N_2O) which has a great ozone-depleting ability with 265 times higher global warming potential than CO_2 (IPCC et al., 2014). In Denmark, regulations of the environmental impacts of agriculture have been emphasizing on N reduction (Dalgaard et al., 2014). Thus, mitigating the N loss during agricultural activities is critical in the Danish agricultural soil. A schematic diagram depicting the soil nitrogen cycle is shown in Figure 1.

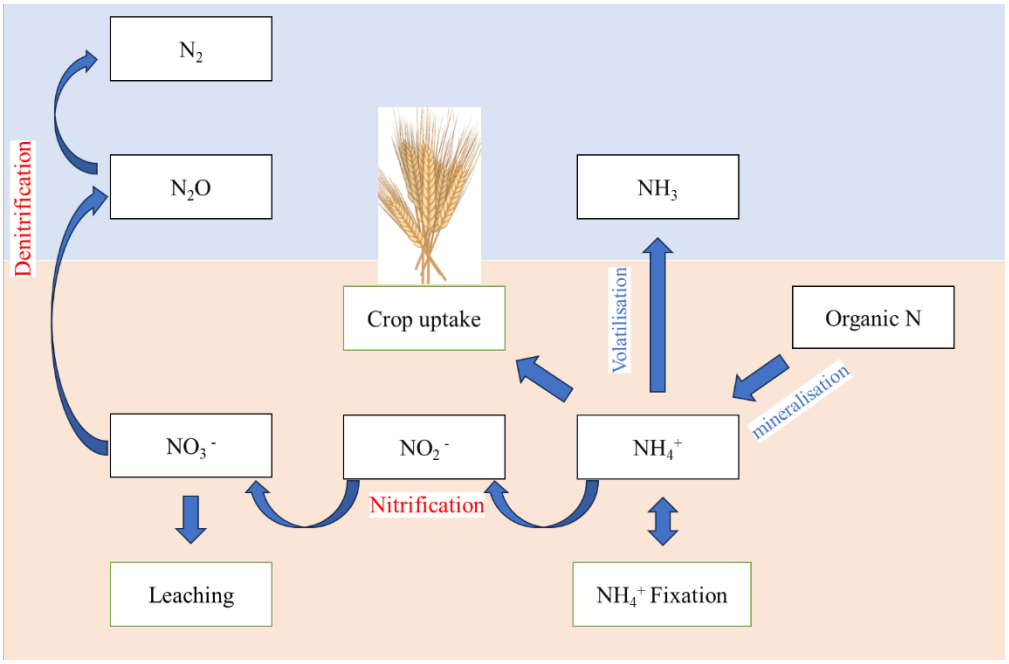


Figure 1 Soil nitrogen cycle.

Both nitrification and denitrification are mediated by microbial activities. Nitrification is an aerobic process, during which ammonia oxidizing bacteria (AOB) and archaea (AOA) oxidize NH_4^+ to NO_2^- through the enzyme ammonia monooxygenase (AMO) encoded by the amoA gene, and the nitrite oxidizing bacteria further transform NO_2^- to NO_3^- (Kuypers et al., 2018). Comammox was recently found to be able to carry out complete nitrification (Daims et al., 2015), and Li et al. (2019) confirmed its abundance and active role of in

nitrification in agricultural soils. On the other hand, in the anaerobic situation, nitrate reducers take the first step to reduce NO_3 to N_2O, and the nitrous oxide reductase encoded by nosZ genes further reduces N_2O to N2 (Philippot et al., 2007).

Despite the inhibition mechanisms of these NIs have been substantially validated, their efficiency in regulating soil N transformations is highly dependent on different environmental factors (Wakelin et al., 2014). A wide range of soil characteristics such as pH (Cui et al., 2021), aeration (Balaine et al., 2015), organic matter content (Singh et al., 2008), temperature (Di & Cameron, 2004) and water content (Di et al., 2014) are found to determine the performance of NIs in soil. In Denmark, while several studies revealed the NIs efficiency pattern via the N_2O measurement (e.g., Peixoto & Petersen, 2023), none of them was looking at the AOM directly. Thus, further research is needed to explore how NIs influence N-cycle-related organisms in the Danish agricultural soil.

A pattern of selective suppression of commercial NIs targeting AOB has been well demonstrated by field experiments and a meta-analysis (Yin et al., 2021; Lei et al., 2022). Prosser et al. (2020) Pointed out that the specific niche specialization contributed to the varying sensitivity of soil AOB to different NIs. This variability suggests that the NIs currently used in agriculture may not be operating at optimal efficiency. Therefore, the implementation of different dosages and types of NIs in the experiment field is very helpful in understanding the underlying mechanisms.

Although there have been many studies on the effect of either DMPP or nitrapyrin on the metabolic activity and community composition of targeting AOA, AOB, or comammox (e.g., Li et al., 2020, Papadopoulou et al., 2024), studies on the effects of NIs on nontarget microorganisms or soil fauna are limited. Since most soil microorganisms are not culturable, amplicon sequencing also known as metabarcoding has been used to reveal the soil microbial community (Hirsch et a., 2010). Nevertheless, existing metabarcoding research has displayed contradictory results: Suleiman et al. (2016) and Duff et al. (2022) denied NIs' ability to affect the composition or structure of soil microbial community, whereas NIs were found to be able to either increase (Guo et al., 2023) or decrease (Papadopoulou et al., 2022) the biodiversity of soil microbiota. Thus, there is a critical need for further research to clarify the ecotoxicology of NIs on the broader soil ecosystem, including both microbial communities and soil fauna, to develop more effective and ecologically sustainable nitrification inhibition strategies.

As much as 80% of soil microbes are reported dormant (Blagodatskaya & Kuzyakov, 2013), hence, rather than soil DNA, soil total RNA was used to represent the active taxa in this study. Relative abundance was revealed by amplicon sequencing of the 16S RNA region and the ITS region for prokaryotes and fungi, respectively. On the other hand, real-time quantitative PCR (qPCR) were conducted for absolute quantification of functional genes. Besides the qPCR, nitrogen related functions were annotated by existing database. This inference-based prediction has been endorsed by a few recent microbial ecology studies (e.g., Sansupa et al., 2021).

As an extremely common and dense group in the upper soil, collembola has long been considered as bio-indicators of the soil health and used in a few EU soil monitoring programs (George et al., 2017). Also, the important role of collembola in soil nitrogen cycle was reviewed by Filser (2002). To my knowledge, none of the research has

investigated how DMPP or Nitrapyrin impacts the collembola community. In this study, Collembola was collected and identified by microscopic approach. Later, the taxonomical diversity and functional composition of soil collembola communities were examined against different NI treatments.

Possessing a high density of pig husbandry, Danish agricultural sector has been extensively using pig slurry as the organic fertilizer (Jensen et al., 2016). Yet, most studies examined the efficiency and outcome of NIs alone, few have looked at the combining effect with different fertilizers nor compared different type of NIs. This study aims to determine the efficacy and off-target effects of NIs in combination with organic fertilizer (pig slurry) and chemical fertilizer on the soil microbiota and collembola. Three types of NIs used were Vizura (DMPP active), Instinct (nitrapyrin active), and ENTEC (DMPP active). We hypothesized that:

(i) the application of NIs can reduce the relative and absolute abundance of AOM.

(ii) the high NI dosage can decrease the biodiversity of the general microbial and collembola community and alter the structure of the general microbial community, while the recommended dosage of NI does not have any significant influence.

2. Background

2.1 Abbreviations

AOA: Ammonia oxidizing archaea

AOB: Ammonia oxidizing bacteria

BSA: Bovine Serum Albumin, used as a stabilizer

cDNA: Complementary deoxyribonucleic acid

Comammox: Complete ammonia oxidization

CWM: Community weight means

DMPP: 3,4-Dimethylpyrazole phosphate

eDNA: Environmental deoxyribonucleic acid

eRNA: Environmental ribonucleic acid

Hifi buffer: High-fidelity buffer, a specialized buffer solution used in PCR

ITS: Internal transcribed spacer

NCM: Neutral community model

PCR: Polymerase chains reaction

qPCR: Quantitative PCR

SYBR Green: A fluorescent dye used to detect DNA during qPCR

2.2 Microbiome in arable soil

Soil microbiomes are critical for maintaining soil health and productivity, making them a current focus of agricultural research (Hartmann & Six, 2023). Represented by bacteria and fungi, soil microorganisms contribute to a wide variety of soil processes necessary for crop growth and ecosystem functioning. Due to the sensitivity to environmental and anthropogenic stressors, soil microorganisms are extensively used as bioindicators of soil health (e.g., Ribas et al., 2023). Healthy soils typically exhibit high microbial diversity and activity, indicating robust nutrient cycling, disease suppression, and maintenance of soil structure; In contrast, a reduction in microbial community composition indicates degradations in soil health, such as those caused by pollution, nutrient imbalances, or inappropriate agricultural practices (Nannipieri et al., 2003).

Agricultural activities can significantly affect soil microbial communities and their functions. Soil microbial communities respond to agricultural practices such as fertilizer application (Bai et al., 2020), tillage (Liu et al., 2020), crop rotation (Neupane, 2021), and pesticide use (Tripathi et al., 2020) were extensively reported. Understanding these processes is critical for soil sustainability and food security. Organic fertilizers and amendments, such as compost and manure, can often enhance microbial diversity and activity (Lazcano et al., 2013). On the other hand, synthetic chemical fertilizers have been found to alter the composition and activity of microbial communities—while they provide essential nutrients to crops, overuse can lead to nutrient imbalances and reduced microbial diversity (Pahalvi et al., 2021). In such cases, integrated management practices that mitigate the non-target impact on organisms have to be implemented.

2.3 Nitrification inhibitor (NI)

Nitrification inhibitors (NIs) have been employed for direct inhibition of soil ammonia-oxidizing microorganisms (AOM). The concept of NI dates to the mid-20th century when the adverse effects of nitrate leaching on water quality and the environment began to be recognized. Early research identified several chemicals capable of inhibiting nitrification, with nitrapyrin being one of the first commercial NIs developed and widely used (Prasad & Power, 1995).

Among a large number of chemical compounds that have been identified as potential NIs, dicyandiamide (DCD), 3,4-dimethyl pyrazole phosphate (DMPP), and 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin) are the most studied and widely used in agriculture, for their much slower degradation rates in the field (Di & Cameron, 2018). All three NIs above are considered Cu chelators interfering with AMO, the key enzyme in the first step of nitrification (Ruser & Schulz, 2015). The specific mechanisms were concluded as: (1) NI inhibits the nitrification process by chelating the Cu component of the relevant enzymes in the ammonia oxidation process (Powell & Prosser, 1986); (2) inhibits the nitrification process by inhibiting the activity of cytochrome oxidase, which is essential in electron transfer and regulates the concentration of reducing agents in the process of ammonia oxidation (Zacher,

1990); and (3) inhibits the conversion of ammonium nitrogen to nitrate nitrogen by influencing the activities of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), which inhibit the whole process of nitrification (Di et al., 2010).

Numerous studies have found that NIs can reduce the abundance of AOM and leaching nitrogen in both laboratory and field experiments (e.g., Zaman & Blennerhassett, 2010), with evidence as early as in the 1980s (Owens, 1981). In addition, NIs were also proven effective in inhibiting denitrification and gaseous N losses, indicating its great potential for global sustainability (Shi et al., 2017).

2.4 Environmental DNA

Environmental DNA (eDNA) is genetic material directly extracted from environmental samples, such as soil and water column, without the need to isolate the target organisms. In soil research, eDNA has become a powerful tool to study biodiversity and monitoring ecosystem health, since most soil microorganisms cannot be cultured (Rappé and Giovannoni, 2003). The effect and efficiency of eDNA approaches were endorsed by field (Westgaard et al., 2024), experimental (Marinche et al., 2023), and meta-analysis (Keck et al., 2022) studies across various taxa and environments.

The eDNA approach was initially applied in some aquatic studies (e.g., Ficetola et al., 2008). Later, its use in soil research began with advances in molecular technology that allowed for the extraction and analysis of DNA from complex soil matrices. A recent review paper by Joshua et al. (2022) recognized the importance of eDNA in agricultural research.

By capturing the DNA of all organisms present in a sample, including bacteria, archaea, fungi, plants, and animals, eDNA allows for a comprehensive survey of soil biodiversity. This holistic approach provides a more comprehensive understanding of soil ecosystems than traditional methods, which tend to neglect rare or unculturable species (Taberlet et al., 2012). A study of temporal scales of eDNA in topsoil showed that, while long fragments of DNA break down quickly, short fragments are still detectable days or even years after the appearance of the organisms (Mathieu et al., 2020). Therefore, the amplified short fragments can have a good resolution of the desired soil sample. eDNA can be used to identify functional genes associated with specific ecological processes such as nutrient cycling, decomposition, and pathogen suppression. This helps to understand the functional potential of soil microbial communities and their role in maintaining soil fertility and health (Bardgett & van der Putten, 2014).

The first step in eDNA analysis is to extract DNA from soil samples. This process must be efficient in recovering DNA from a wide range of soil organisms while minimizing inhibitors (e.g. soil humic acid) that may interfere with downstream analyses. A variety of commercial kits and protocols are available for soil DNA extraction (Bissett et al., 2013). Sequence data generated from eDNA samples were analyzed using bioinformatics tools to identify and quantify organisms present in the soil. This includes sequence comparison, taxonomic assignment and functional annotation conducted by bioinformatic software and databases

such as QIIME2, SILVA and UNITE (Bokulich et al., 2013). Massive free packages in R also provided availability for rapid data analysis and visualization.

2.4.1 Amplicon sequencing

Amplicon sequencing, also known as metabarcoding, is a technique that uses high-throughput sequencing to identify and quantify multiple species in a sample by targeting specific genetic markers. This method evolved from traditional DNA barcoding, which involved sequencing short and standardized regions of the genome to identify species (Hebert et al., 2003). With advances in next-generation sequencing technologies, metabarcoding allows multiple samples and species to be analyzed simultaneously, providing a comprehensive view of biodiversity.

Metabarcoding has been widely used to characterize soil microbial communities and understand their response to natural and anthropogenic stressors. By localizing conserved regions of 16S rRNA genes in bacteria and archaea, or ITS regions in fungi, researchers can obtain detailed information on microbial diversity and community composition (Caporaso et al., 2012). Metabarcoding is particularly useful in assessing the impact of agricultural practices (e.g., application of nitrification inhibitors) on soil microbial communities. It allows for the detection of dominant and rare taxa, providing insights into how different microbiota contribute to soil function and how they are affected by external factors. However, it can only detect the variation of relative abundance. To determine the absolute abundance of taxa of interest, qPCR is necessary to carry out as complementary approaches.

2.4.2 qPCR

Quantitative PCR (qPCR), also known as real-time PCR, is a technique that allows for the quantification of DNA or RNA in a sample. qPCR revolutionized molecular biology with the development of PCR by Kary Mullis in 1983, which made possible the amplification of specific DNA sequences (Mullis et al., 1986). qPCR builds on this foundation by incorporating fluorescent dyes or probes, which are proportional to the amount of PCR product produced, allowing real-time monitoring of the amplification process. In soil microbiology, qPCR has become a key tool for quantifying specific microbiota and functional genes (e.g., genes involved in the nitrogen cycle). For example, the archaeal amoA gene encoding ammonia monooxygenase, a key enzyme in nitrification, can be quantified to assess the abundance of ammonia-oxidizing archaea (AOA) in soil samples (Leininger et al., 2006). Similarly, the bacterial amoA gene can be used to quantify ammonia-oxidizing bacteria (AOB) (Rotthauwe et al., 1997). The sensitivity and specificity of qPCR make it ideally suited for studying the kinetics of microbial responses to environmental or anthropogenic stressors. It allows the accurate monitoring of microbial populations over time or under different conditions.

3. Method and Material

3.1 Site setting and soil sample collection

A total of 30 experimental fields consisting of 10 treatments with 3 replicates were sampled at Højbakkegård (sandy clay), which included control (C), pig slurry (PS), pig slurry + recommended dosage of Vizura (PV_1), pig slurry + 3×recommended dosage of Vizura (PV_3), pig slurry+ 10×recommended dosage of Vizura (PV_10), pig slurry + recommended dosage of Instinct (PI_1), pig slurry + 3×recommended dosage of Instinct (PI_3), pig slurry+ 10×recommended dosage of Instinct (PI_10), chemical fertilizer NS (NS) and Chemical fertilizer NS +recommended dosage of ENTEC (NE). A diagram of the experiment design can be seen in Figure 2.

The three NIs consisted of different active compounds: i) DMPP (3,4-dimethyl-1H-pyrazole phosphate) either in the formulation Entec® (EuroChem, Antwerp, Belgium)) where DMPP is coated on granulated NS 26-3 fertilizer (NS) fertilizer with 17% NH_4^+-N, 9% $NO3^-$-N and 13% total S or in the case of pig slurry DMPP in the liquid formulation called Vizura® (EuroChem, Antwerp, Belgium); ii) Nitrapyrin (Corteva Agriscience, Copenhagen, Denmark) with the active ingredient 2-Chloro-6-(trichloromethyl) pyridine as Instinct™. Vizura was added in combination with UAN 32, a liquid mineral N source with 8% NH_4^+-N, 8% NO^3--N, and 16% urea-N (DanGødning, Fredericia, Denmark). The pig slurry (PS) obtained from a farm contained 5.7 kg total N, 3.8 kg ammoniacal N per ton, and 4.0% dry matter. The recommended dosage used was 2 kg ha^{-1}.

Each plot consisted of a 3 m × 3 m area and was cultivated with spring barley. The NIs and fertilizers were deployed on 20th April 2023, and the field sampling was carried out on 28th April 2023 to monitor a short-term effect. Soil samples were collected from each plot at 5 points at a depth of 0-15 cm, and then mixed and frozen in liquid nitrogen. Later, soil samples were transferred to a -80 °C freezer prior to RNA extraction.

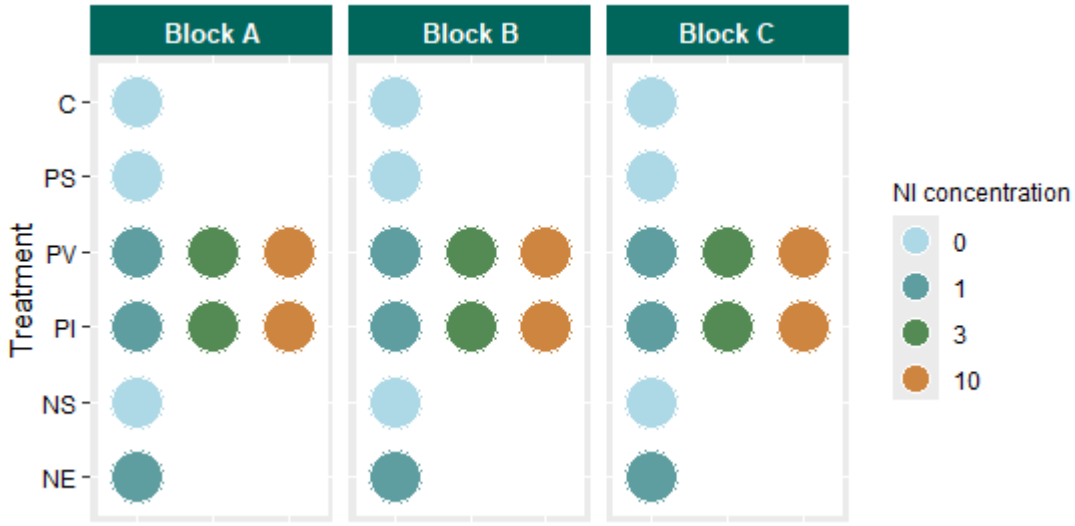


Figure 2. Field design of the soil sampling of 10 treatments and 3 replicates. The color indicates the dosage of nitrification inhibitors. Abbreviations: C (control), PS (pig slurry), PV (pig slurry+Vizura), PI (pig slurry+Instinct), NS (chemical fertilizer NS), NE (chemical fertilizer NS+ENTEC).

3.2 Soil RNA extraction, PCR and high-throughput sequencing

3.2.1 Amplicon Sequencing

The soil RNA was extracted from each sample by Nucleobond soil RNA Kit (Machrey-nagel) after freeze-drying for 48 hours, and then Qubit 3.0 (Thermo Fisher Scientific) was used to determine the concentration of RNA. Subsequently, the extracted RNA was transcribed into complementary DNA by the First Strand cDNA Synthesis Kit (Roche). The primers 341F (5′ – CCTAYGGGRBGCASCAG - 3 ′)/806R (5′ –GGACTACNNGGGTATCTAAT - 3′) and ITS1F (5′ – CTTGGTCATTTAGAGGAAGTAA - 3′)/ITS2R (5′ – GCTGCGTTCTTCATCGATGC - 3′) were used to amplify the V3-V4 region of the bacterial 16S rDNA and the ITS1 region of the internal transcribed spacer (ITS) region of the fungus, respectively.

PCR was performed in 25 μL reactions with 15.25 μL of PCR water, 5 μL of 5 × HIFI Buffer, 0.5 μL forward and reverse primers, 0.5 μL of BSA, 0.25 μL of HIFI polymerase, and 3 μL of template DNA. After denaturing at 94 °C for 5 min, the amplification was carried out with 30 cycles of 30 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C and a final extension step at 72 °C for 10 min. Then, the amplicon libraries were sequenced on an Illumina MiSeq PE250 platform (Sequencing Centre, Aarhus University, Denmark) using standard protocols (http://www.illumina.com/).

3.2.2 Quantitative PCR

The transcripted cDNA were prepared for quantitative PCR through the CFX96 Real-Time PCR instrument (Bio-Rad), and the amplification primers were Arch-amoAF (5' - CTGAYTGGGCYTGGACATC-3 ") and Arch-amoAR (5'-TTCTTCTTTGTTGCCCAGTA - 3') to detect the archaeal amoA gene copies in charge of ammonia oxidation by archaea (Francis et al., 2005). The 20 μL PCR reaction mixture consisted of 7 uL PCR water, 10 uL SYBR Master mix, 0.5 μL forward and reverse primers, and 2.0 μL cDNA . The PCR amplification procedure was as follows: predenaturation at 95°C for 2min, 40 cycles (denaturation at 95°C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s). Negative control and soil DNA samples were repeated 2 times each. The specificity of amplification was confirmed by fusion curve and gel electrophoresis analysis. Positive control containing target genes was ordered from gBlocks gene fragments (Integrated DNA Technologies) and diluted to 5 ng/μL.

3.3 Collembola extraction and identification

Soil collembola was sampled using one cylindrical soil core of 10 cm depth, and each plot was sampled twice. Macfadyen-type high thermal gradient devices were then used to extract collembola from soil core to 90% alcohol (Macfadyen, 1961). Subsequently, collembola was conserved in glycerine and identified to the lowest taxonomical level by a microscope, according to the key book by Fjellberg (2009).

3.4 Data analysis

After sequencing, the obtained raw fastq data were analyzed via the QIIME2 pipeline (http://qiime.org/scripts/assign_taxonomy. html). The primer and index barcode sequences

were first removed and merged into a paired-end sequence. Then, through quality control, denoising and chimerism removal, the optimized bacterial and fungal ASVs were clustered against the Silva database (Release 138) (Quast et al., 2013) and UNITE v7.2 (Full UNITE + INSD datasets) (Koljalg et al., 2005) according to a similarity threshold of 97%. Feature tables with amplicon sequence variants (ASVs) for bacteria and fungi were rarefied to 1890 and 3256 read depths for diversity analyses, respectively. One sample from PI_1 did not have sufficient quality, thus not included for downstream analysis.

3.5 Statistical analysis

All statistical analyses were performed in the R environment (version 4.3.2; R Core Team, 2020). The alpha diversity and beta diversity were estimated by R "microeco" package (Liu et al., 2021): Kruskal-Wallis tests were employed to compare the difference in Shannon index among different treatments; Based on the Bray–Curtis distance, beta diversity was presented through principal coordinates analysis (PCoA) to determine the similarities or differences in microbial communities among different treatments. PCoA can convert the distance matrix into a set of coordinates in a low-dimensional space to capture the most variation in the data. Meanwhile, an adonis (Analysis of variance using distance matrices) test was conducted to examine the significance of the impact on the distance matrix. This test can partition the distance matrix based on the levels of the categorical variable and assess the variation explained by such variables.

Functional metabolic were predicted based on the relative abundance of functional taxa according to FARPROTAX and Funguild database (Louca et al., 2016; Nguyen et al., 2016). ASV matrix was scaled before functional assignment. Furthermore, the Mantel test was used to test the correlation between community distance matrices and the environmental variable distance matrix with the R corrplot package (Wei & Simko, 2021). A significant mantel test with positive mantel's r can indicate that the variation in such environmental variable can drive the variation in the referred community.

The NCM allows to evaluate of the extent to which community assembly in microbial ecosystems is governed by stochastic (random) processes versus deterministic (niche-based) processes (Zhou and Ning, 2017). By applying the NCM to each treatment, it can be determined whether the changes in microbial community composition are due to random dispersal and ecological drift or more influenced by the specific conditions created by the different treatments. A higher R square indicated a better fitness to the neutral community model, thus a more stochastic driven process. Bacterial and fungal community across all treatments were also fitted to the NCM.

For the collembola community, Kruskal-Wallis tests were used to compare the Shannon diversity index across treatments. The taxonomical composition was further converted to functional composition based on the SoilBioStore trait database (http://www.soilbiostore.au.dk/). Two morphological traits (body length and furca) were used from this database: body length was considered as a general performance linked to energy transfer as microbial feeders (Hedde et al., 2012); furca was the most sensible trait for collembola to reflect its preference for soil habitat (Bonfanti et al., 2022). To compare such functional composition, community-weighted mean (CWM) metrics (Garnier et al., 2004) were calculated for both traits across 30 plots as follows:

$$CWM = \sum_{i=1}^{S} P_i \times trait_i$$

Where S is the total number of species in the community, p_i is the relative abundance of species i, and t_i is the trait value of species i. The trait value of furca was assigned to numerical value (1, 2, 3) based on (furca absent, fully developed short furca, and fully developed long furca).

4. Result

4.1 Physicochemical variables of soil in different treatment

Soil physicochemical variables carbon content, nitrogen content, water content and organic matter were compared by Kruskal-Wallis tests (Figure 3), and no significant percentage variation was detected among the 10 treatments, indicating the treatments used in this research did not alter the physicochemical in the soil.

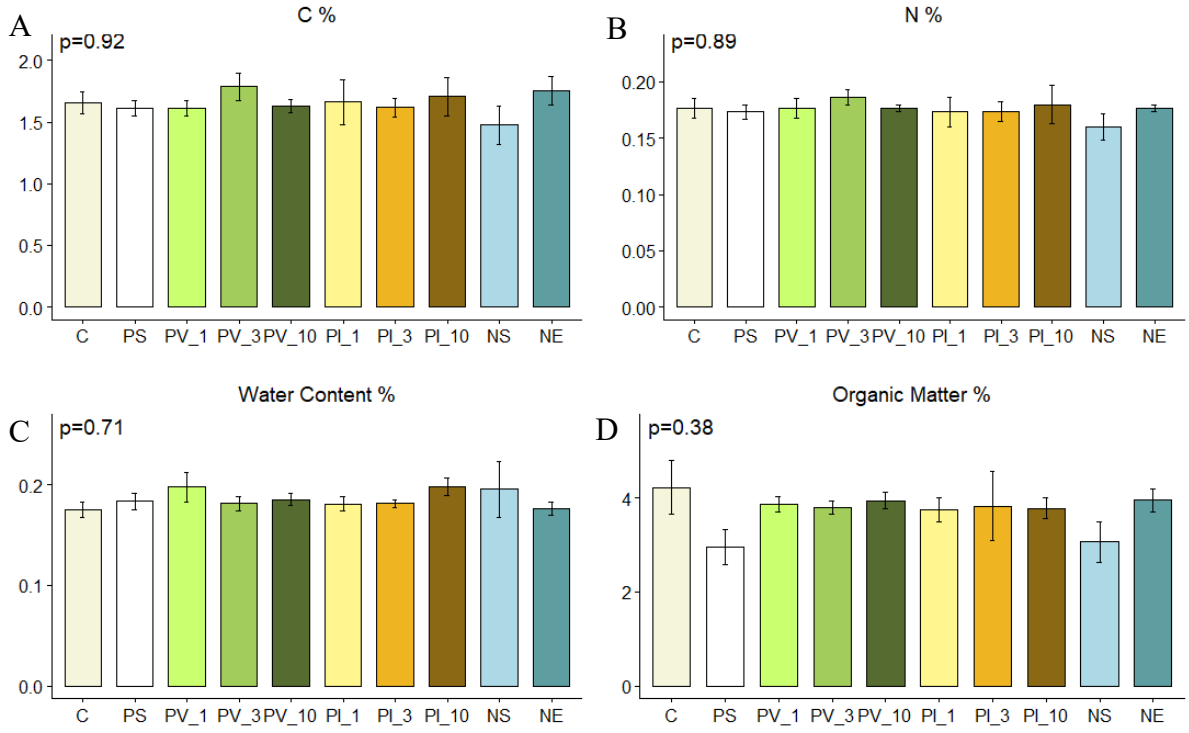


Figure 3 Barplots of the soil physicochemical variables A) carbon content; B) nitrogen content; C) water content; D) organic matter content; among different treatments in the experimental fields. Error bars represent the standard deviation of the mean (n =3).

4.2 Microbial community diversity between different treatment

4.2.1 Community Composition

At the phylum level, the soil bacterial community was dominated by *Actinobacteria* (49.1%) in every treatment, followed by *Firmicutes* with an average proportion of 23.6% (Figure 4).

Notably, the implement of organic fertilizer (i.e. pig slurry) significantly increased the relative abundance of *Firmicutes* (14.1%) compared with non-organic treatments (1.0%). However, there is no clear pattern of compositional variation triggered by nitrification inhibitors.

For the fungal communities, likewise, the utilization of organic fertilizer but not nitrification inhibitor contributed to different patterns of composition. *Mortierellales* and *Pleosporales* were the dominating fungi. In the fields with pig slurry, *Mortierellales* (32.5%) was the most abundant order, especially in PI_1 treatment where it made up for more than half (52.6 %) of the proportion. In the control and chemical fertilizer treatments, *Mortierellale* (16.6%) and *Pleosporales* (19.7%) had a similar relative abundance.

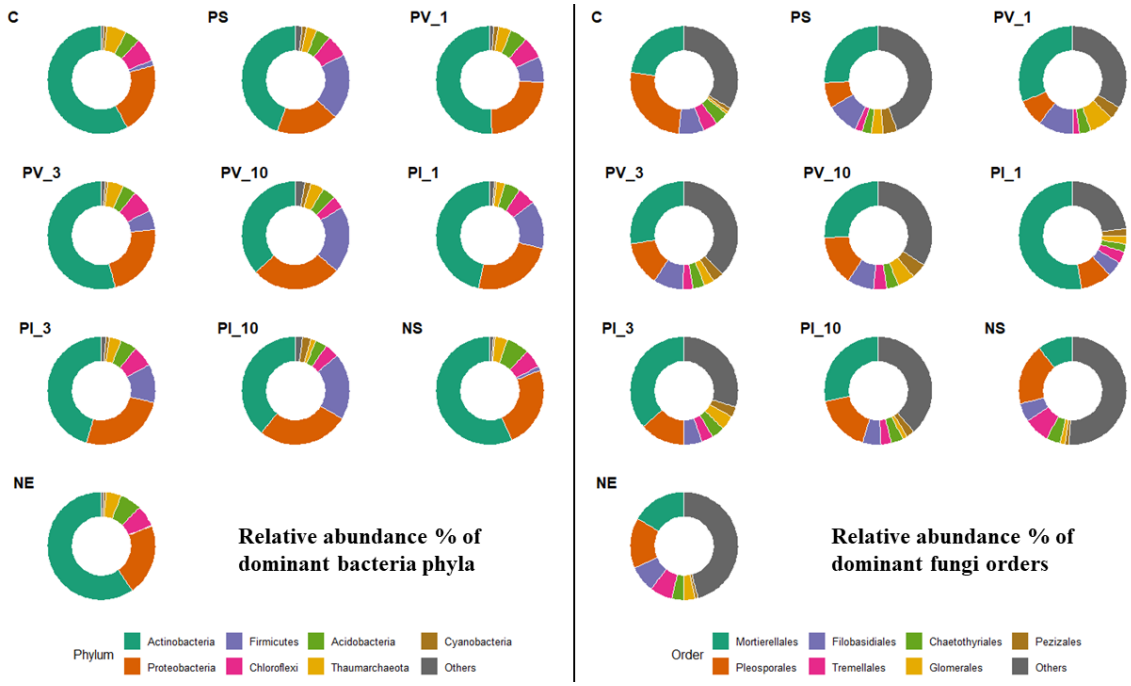


Figure 4. Pie plots displaying dominant bacteria phyla (left panel) and fungi orders (right panel) among 10 treatments.

The petal diagrams in Figure 5 allowed for an intuitive comparison of unique (across all treatments) or shared species among treatments. There were 56 shared bacterial species and 72 fungal species in total. Within all the 10 treatments, PV_10 (82.8%) and PI_10 (82.3%) had the highest percentage of unique bacterial species, while PV_3 (61.7%) had the highest percentage of unique fungal species. Both bacteria (75.6%) and fungi (32.7%) had rather low unique species in the control treatment.

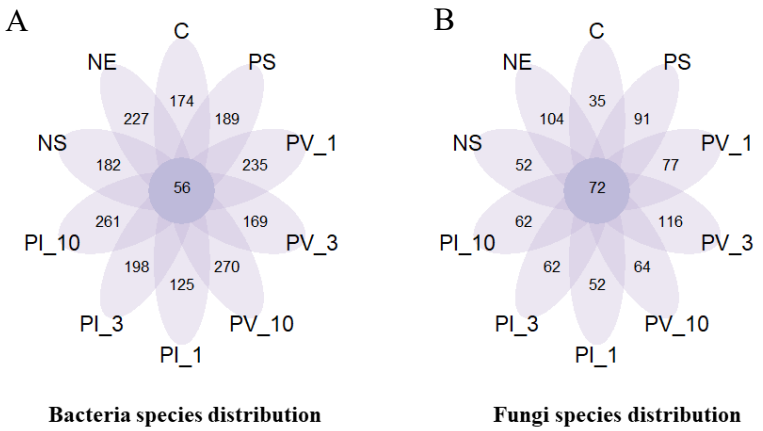


Figure 5 Petal diagrams showing number of shared species and distinct species of each treatment in bacteria (panel A) and fungi (panel B).

4.2.2 Alpha Diversity

To compare the impact of nitrification inhibitor on alpha diversity, the Shannon diversity of fields treated by certain dosage were compared against the NI-free treatment, respectively (Figure 6). The results suggested that the implementation of nitrification inhibitor did not have significant effects on the alpha diversity of bacteria and fungi, no matter in recommended dosage or even by 10 times more.

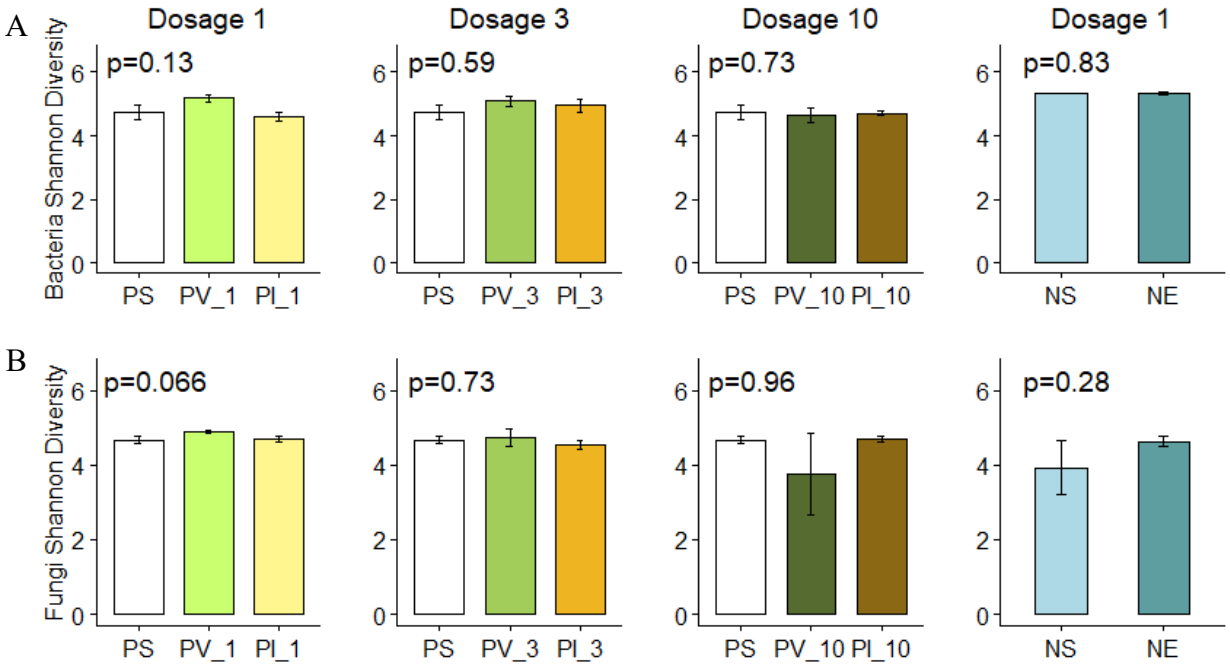


Figure 6 Alpha diversity (Shannon index) of bacterial (panel A) and fungal (panel B) communities in different NI scenarios (n=3).

4.2.3 Beta Diversity

Principal coordinate analysis (PCoA) was used to evaluate the effects of each treatment on the soil bacterial and fungal composition based on the Bray–Curtis distance (Figure 7). Above all, the different treatments significantly impacted the soil bacterial ($p<0.001$) and fungal communities ($p<0.01$), which could explain 45.6% of the soil bacterial community variation and 38.2% for fungi. For both bacterial and fungi communities, the clustering is differentiated between pig slurry and chemical fertilizers. However, it could be seen that the microbial community in the soil was not associated with the type or dosage of nitrification inhibitor.

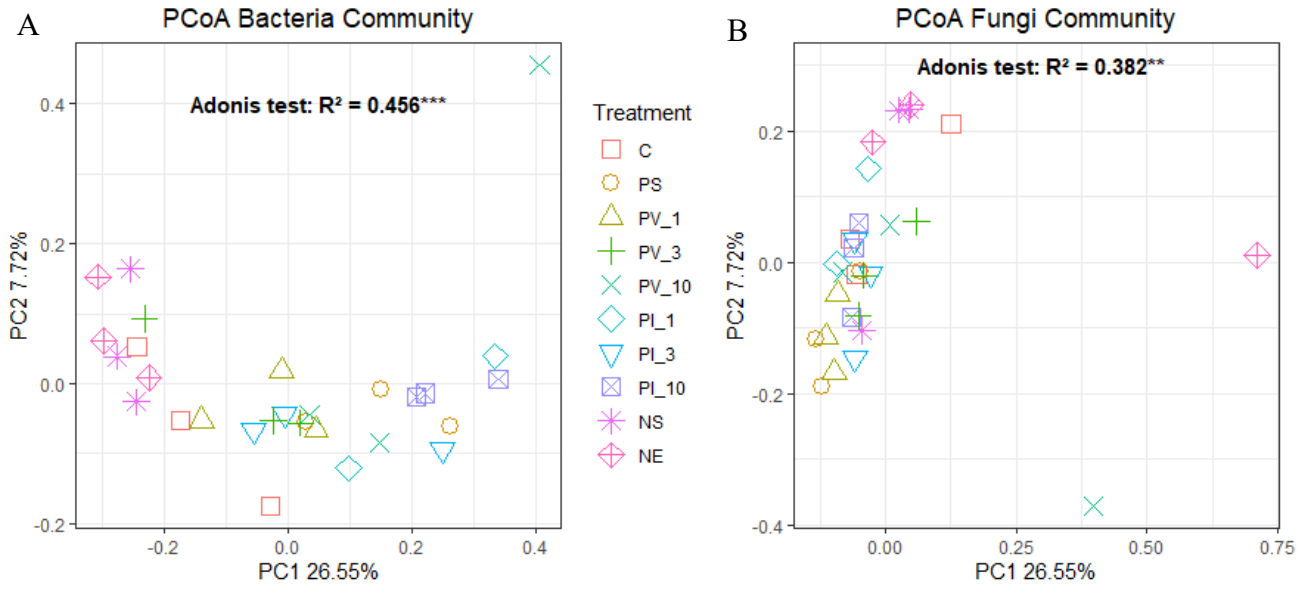


Figure 7 Beta diversity (PCoA) of each treatment (n=3) of bacteria (panel A) and fungi (panel B) community with annotated p values of adonis tests.

4.2.4 Indicator species

Linear discriminant analysis effect size (LefSe) was used to identify the indicator species among different treatments. Nevertheless, after the p-value adjustment, no taxon was found significant in either bacteria or fungi community. This result suggested that there was no specific bacterial or fungal taxon enriched by certain treatments, thus no indicator species was present.

4.3 Microbial Function

4.3.1 Quantitative PCR

The real-time quantitative PCR revealed a range from 1.4×10^3 to 2×10^4copies of the nitrification gene archaeal amoA, though none of the implementation of NIs could induce a significant change (Figure 8). However, chemical fertilizers were found to have less AOA amoA genes than organic fertilizers (Wilcoxon test, $p<0.01$).

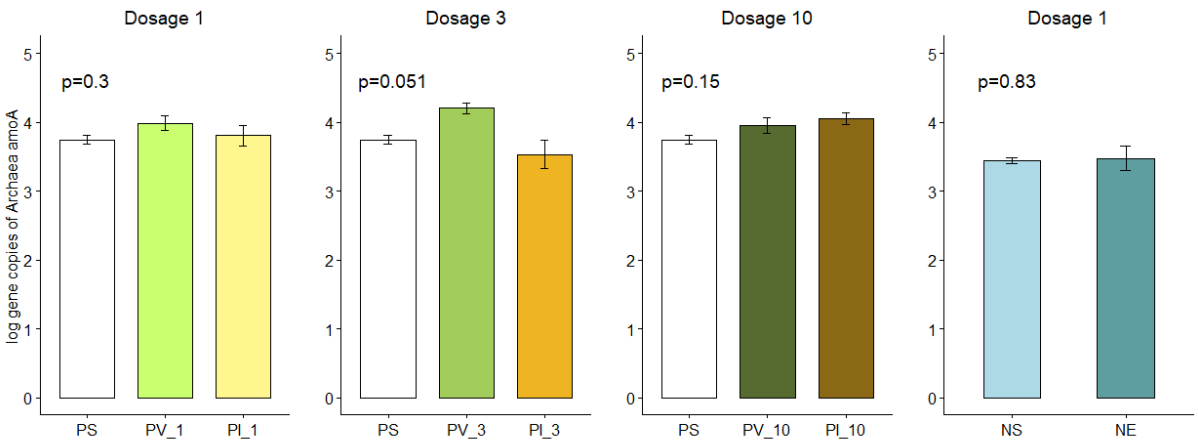


Figure 8 The effect of NIs on the abundance of the archaea *amoA* genes copies (n=3). All values are expressed in log scale.

4.3.2 Function Prediction

Based on the assigned taxonomy of bacteria and fungi, a heatmap of functional prediction was generated through FAPROTAX and Funguild database, respectively (Figure 9). The control treatment possessed the most functional bacteria groups related to N-cycle except for nitrogen fixation. The treatment PI_10 inhibited the nitrifiers most effectively, while NE had the most inhibition on denitrifiers.

On the other hand, the fungal functional group was hardly influenced by different treatments, with Saprotrophic and Sapro-Sumbiotrophic fungi as the most predominant functional group.

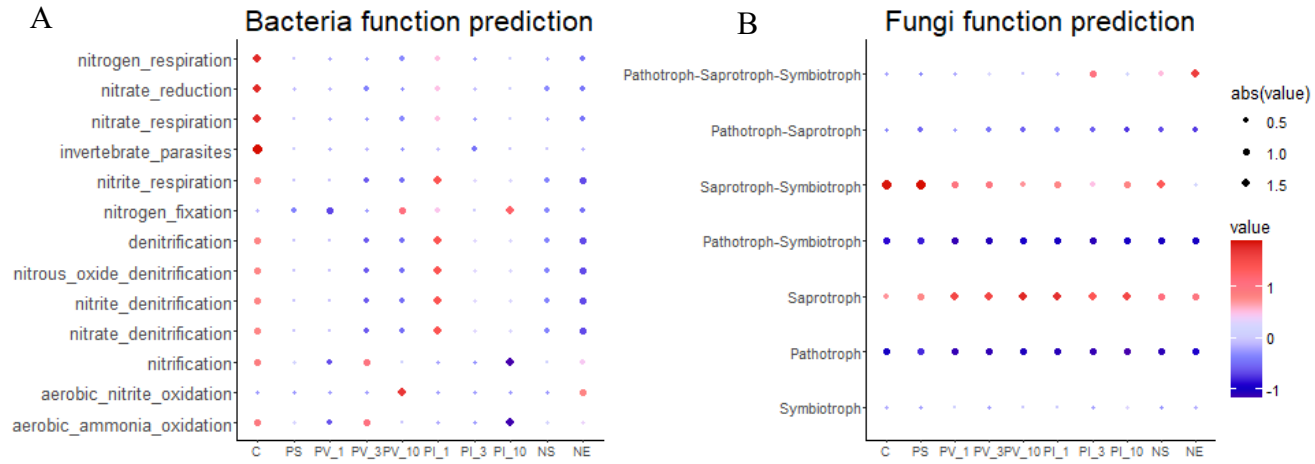


Figure 9 Function prediction of bacteria community (panel A) based on FAPROTAX database and fungi community (panel B) based on Funguild database. The size of each dot indicates the scaled absolute value, and the color indicates positive or negative value.

4.4 Relationships between physicochemical variables and the soil Communities

Although there was no significant variation in physicochemical variables (Figure 3), Mantel test was carried out to examine the linkage between physicochemical variable with the soil bacterial, fungal, and collembola communities of different NI scenarios, since it doesn't rely

on the group structure but rather on the overall relationship between the two sets of distances (Figure 10). Clearly, there was no physicochemical factor that could explain the variation in microbial and collembola communities when the nitrification inhibitor was not used (PS, NS) or used in recommended dosage (PS_1, NE). When the NI dosage came to 3× (PS_3), water content emerged as a significant influencer to the bacterial community (mantel-r ≥ 0.4, $p < 0.05$. Furthermore, the NI dosage of 10× (PS_10) revealed a significantly correlation between water content and the collembola community (Mantel-r ≥ 0.4, $p < 0.05$), and organic matter was significantly correlated with bacterial and fungal community (Mantel-r ≥ 0.4, $p < 0.05$). These findings indicated that under standard or absent usage of nitrification inhibitors, soil physicochemical variables are unlikely to be decisive factors in determining the dynamics of microbial and collembola compositions. However, at higher dosages of nitrification inhibitors, the dynamics between specific physicochemical variables such as water content and organic matter, and community compositions could be altered.

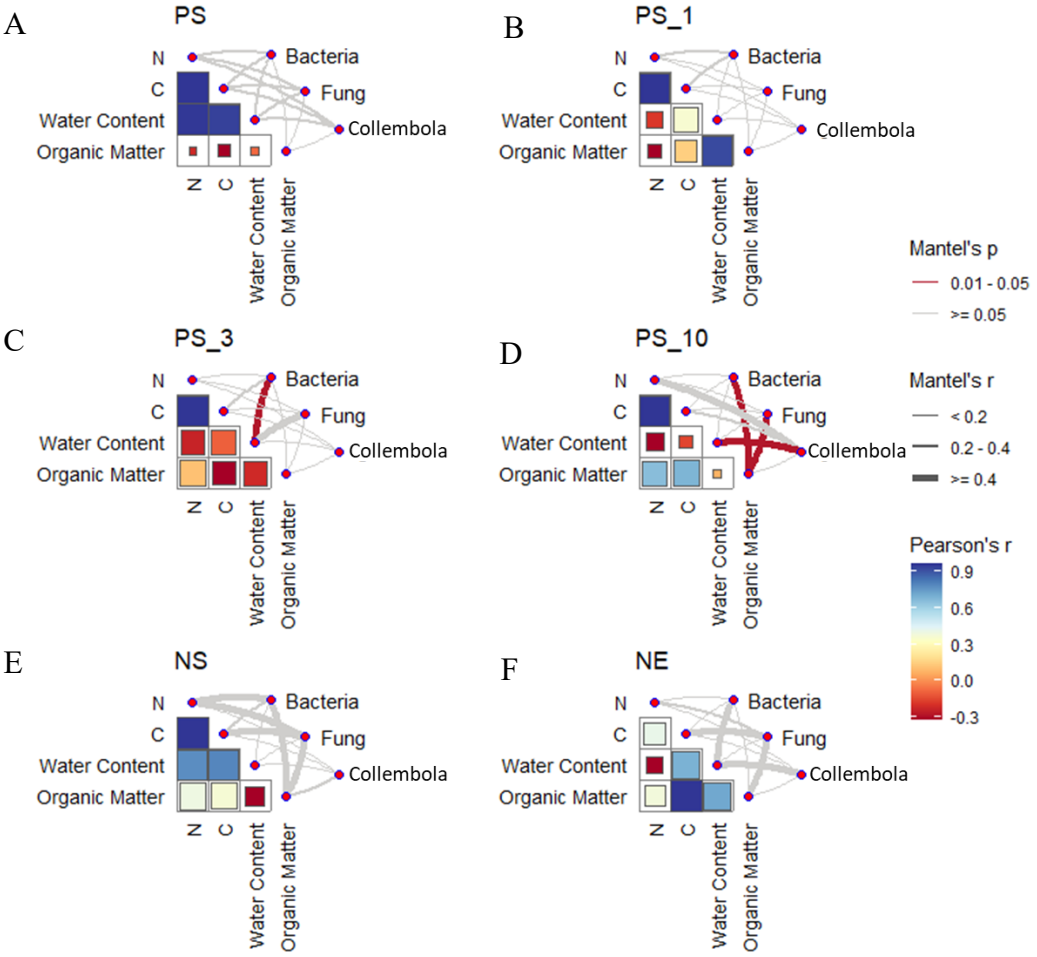


Figure 10 Correlations of the soil physicochemical variables with bacterial, fungal and collembola communities in 6 different NI scenarios A) Pig slurry; B) Pig slurry + 1 dose NI; C) Pig slurry + 3 dose NI; D) Pig slurry + 10 dose NI; E) chemical fertilizer NS; F) chemical fertilizer NS + 1 dose NI. Colour of the line indicated the p value of the mantel test, and the width indicated the mantel's r. Colour of the correlation matrix indicated Pearson's r, with the area indicating the absolute value.

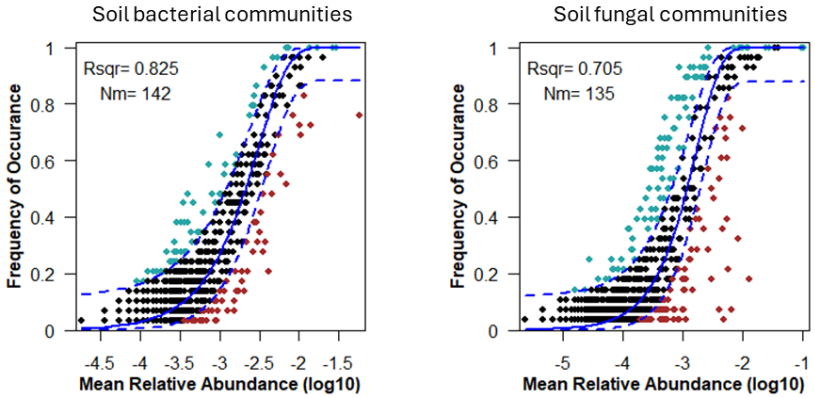
4.5 Neutral community model

The neutral community model (NCM) illustrated the relationship between the occurrence frequency and relative abundance of bacterial and fungal ASVs (Figure 11A). The model

explains a substantial portion of the variance observed in bacterial (R^2=0.825) and fungal community (R^2=0.705), suggesting that stochastic processes (random events) might play a major role in shaping the community structure in general.

Nevertheless, the assembly mechanisms of the microbial communities among different experimental field types were not similar (Figure 11B). For the bacterial community, stochastic processes did not affect the bacterial community assembly except for chemical fertilizer treatments (NS, NE). For the fungal community, fields with pig slurry+Vizura (PV_1, PV_3, PV_3) were significantly shaped by stochastic processes, while they were less common in treatments with Instinct (PI_1, PI_3, PI_10) and not found in chemical fertilizer treatments. These results indicated that whereas deterministic processes governed the controlled treatment, different types of nitrification inhibitor can have different impacts on assembly mechanisms of bacteria and fungi.

A



B

	Treatment	C	PS	PV_1	PV_3	PV_10	PI_1	PI_3	PI_10	NS	NE
R square	Bacteria	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.212	0.157
	Fungi	<0.1	<0.1	0.460	0.537	0.492	<0.1	0.201	0.272	<0.1	<0.1

Figure 11. Fitting neutral community model to bacterial and fungal communities in general (Panel A) and in different treatments (Panel B). The blue dash lines represent the upper and lower confidence intervals (95%) for the predicted occurrence frequencies.

4.6 Collembola communities

In total, 22 collembola species were identified, and Tullbergiinae is the most abundant group. We recorded on average 10611 ± 5933 individuals m^{-2}. Still, no evidence suggested that the implementation of NIs had significant effects on neither alpha diversity nor functional composition (Kruskal-Wallis test).

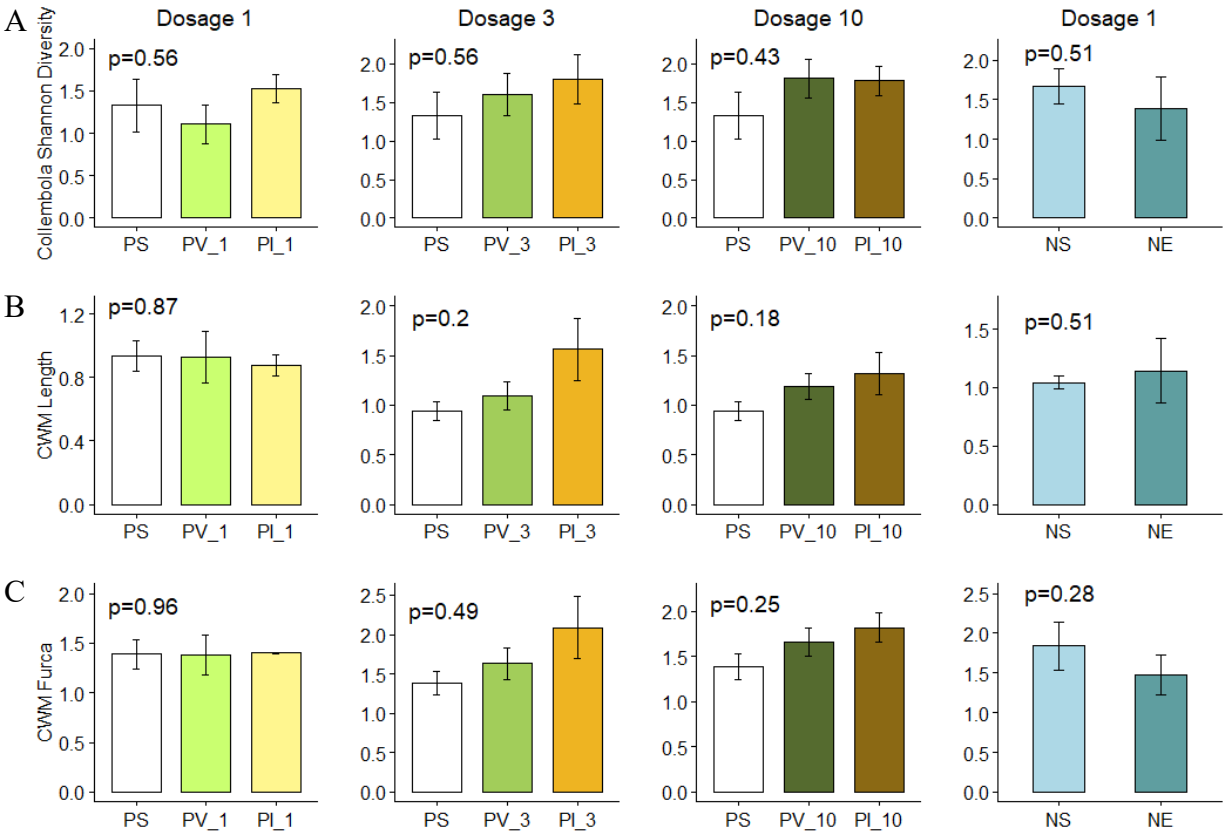


Figure 12 Shannon index (panel A), community-weighted mean of body length (panel B) and furca development (panel C) of collembola communities in different NI scenarios (n=3).

5. Discussion

5.1 Impacts of NIs on target group

The quantitative PCR analysis of the archaeal amoA gene did not identify any significant effects of nitrification inhibitors (NIs) on ammonia-oxidizing archaea (AOA) (Figure 8). This finding is consistent with Shen et al. (2013), who reported that chemical nitrification inhibitors such as DMPP and nitrapyrin have minimal inhibitory impact on AOA. Similarly, functional annotation based on 16S rRNA gene metabarcoding did not detect any significant effects of NIs on nitrifiers or denitrifiers (Figure 9). This pattern suggested nitrification inhibitors were not effective on the soil of research in a short-term monitoring scheme.

Recent research by Duff et al. (2022) supports our findings, showing no significant effects of NIs on either AOA or AOB. However, they reported significant effects of NIs on complete ammonia oxidizers (comammox) for the first time. To date, several studies implied that comammox can be the dominating nitrifier in soil rather than AOA or AOB (Liu et al., 2019; Bai et al., 2024). In our study, functional annotation using FAPROTAX did not reveal any clear patterns, possibly because comammox bacteria, which belong to the *Nitrospira* genus, were discovered relatively recently and not included by current FAPROTAX databases yet. Further quantitative PCR analysis of the comammox amoA gene is necessary to determine the activity of comamox in this context.

Furthermore, McGeough et al. (2016) found that the efficacy of NIs is negatively impacted by soils with high clay and organic matter content, for their strong sorption capacity. In our experiment, the presence of substantial winter barley residue resulted in high organic matter, and the soil type was sandy clay. These soil properties likely reduced the effectiveness of the nitrification inhibitors, making them unsuitable for this specific agricultural context.

5.2 Impact of NIs on non-target group

At the recommended dosage, no effect of NIs was observed on microbial or collembola community diversity, structure or function. This pattern was consistent with our hypothesis and a few recent studies (Suleiman et al., 2016; Duff et al., 2022). Nevertheless, we also hypothesized that the application of high-dosage NIs can alter the composition and functioning of microbial communities in soil. In the case of DMPP and nitrapyrin, unexpectedly, no effect of NIs was observed on microbial or collembola structure and diversity, even at 10 folds greater than the recommended dosage.

There were noticeable variations in microbial composition and structure (Figure 4, 7) among different treatments. However, these variations are clearly attributable to the choice of organic or chemical fertilizer rather than to the use of nitrification inhibitors. For example, the dramatic expansion of *Firmicute* in treatments with organic fertilizers can be attributed to its large composition (76.2%) in pig slurry (Kumar et al., 2020). Still, observed changes at the phylum level do not translate into large effect sizes at lower taxonomic levels which can be detected by Lefse.

All the results above suggested that the soil microbial communities in this experiment are highly resilient and exhibit a high degree of functional redundancy. This resilience implies that the application of different concentrations of NIs does not selectively enrich or suppress specific microbial taxa to a statistically significant extent. Such functional redundancy is common in soil ecosystems, where multiple microbial taxa can perform similar ecological roles, thereby buffering the community against disturbances (Louca et al., 2018).

Moreover, such resilience pattern was well explained by the good fitness to the neutral community model, which assumes that community composition is shaped by random demographic events rather than strong deterministic selection pressures (Figure 11A). The high R^2 value indicates that neutral processes explain a larger proportion of the variation in community composition for both bacterial and fungal communities.

Our study reveals that the neutral community model fits the bacterial communities better in chemical fertilizer treatments, whereas it fits the fungal communities better in organic fertilizer treatments (Figure 11B). These differential fitness highlights distinct ecological dynamics and responses of bacterial and fungal communities to different types of fertilizers. Organic fertilizers can introduce a variety of complex organic compounds into the soil, which creates diverse niches and selective pressures for microbial communities (Bardgett & van der Putten, 2014). Compared with bacteria, fungi are well-equipped to decompose complex organic matter, thus, the application of organic fertilizer has a less deterministic effect on the fungal community. On the other hand, in the case of chemical fertilizer which typically provides specific nutrients, bacteria can quickly exploit these readily available nutrients through their faster growth rates and more flexible metabolic capabilities (Geisseler & Scow,

2014). This resulted in a rather homogeneous bacterial community with more stochastic processes.

This general stochastic pattern was further evidenced by our mantel test (Figure 10), where most soil physiochemical variables indicated no significance with the dissimilarity of microbial and collembola communities. This lack of significant correlations suggests that at low NI concentrations, these inhibitors do not substantially alter the microbial ecosystem or its response to the measured environmental factors, implying that the microbial communities maintain their structure and function without major disruptions at these lower inhibitor levels. Intriguingly, when the NI dosage was enhanced to 10 folders greater than the recommended, soil organic matter was found to be significant with both bacterial and fungal communities. It can imply that high concentrations of nitrification inhibitors may impose stronger selection pressures on microbial communities, favouring taxa that can utilize organic matter more efficiently or are more tolerant to the chemical environment created by the inhibitors. Previous studies have suggested that soil organic matter can mitigate the effects of nitrification inhibitors (McGeough et al., 2016). This buffering effect is particularly important under high NI conditions.

6. Conclusion

In this study, we aimed to enhance the limited knowledge on the targeted and non-target impacts of nitrification inhibitors on the composition and functions of microbial and collembola communities in arable soils cultivated with spring barley. By examining soils where NIs had been applied alongside organic or chemical fertilizers, we assessed the effects of NIs implementation through a combination of microscopic identification, qPCR and amplicon sequencing.

This study has shown that nitrification inhibitors did not have significant effect on target ammonia oxidizing groups. Meanwhile, non-target microbial and collembola communities were not significantly affected by nitrification inhibitors with short-term usage (even at tenfold dosage), while the fertilizing choice (organic/chemical) has significant effects. This non-significant pattern can be explained by the highly stochastic process in the experiment soil. Further investigation focusing on complete ammonia oxidizers (comammox) is suggested for a more comprehensive understanding.

7. Reference

Akinnawo, S. O. (2023). Eutrophication: Causes, consequences, physical, chemical and biological techniques for mitigation strategies. *Environmental Challenges*, 100733.

Balaine, N., Clough, T. J., Kelliher, F. M., & van Koten, C. (2015). Soil aeration affects the degradation rate of the nitrification inhibitor dicyandiamide. *Soil Research*, *53*(2), 137-143.

Bardgett, R. D., & van der Putten, W. H. (2014). Belowground biodiversity and ecosystem functioning. Nature, 515(7528), 505-511.

Blagodatskaya, E., & Kuzyakov, Y. (2013). Active microorganisms in soil: critical review of estimation criteria and approaches. *Soil Biology and Biochemistry*, *67*, 192-211.

Bonfanti, J., Hedde, M., Cortet, J., Krogh, P. H., Larsen, K. S., & Holmstrup, M. (2022). Communities of Collembola show functional resilience in a long-term field experiment simulating climate change. *Pedobiologia*, *90*, 150789.

Cui, L., Li, D., Wu, Z., Xue, Y., Xiao, F., Zhang, L., ... & Cui, Y. (2021). Effects of nitrification inhibitors on soil nitrification and ammonia volatilization in three soils with different pH. *Agronomy*, *11*(8), 1674.

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., ... & Wagner, M. (2015). Complete nitrification by Nitrospira bacteria. *Nature*, *528*(7583), 504-509.

Dalgaard, T., Hansen, B., Hasler, B., Hertel, O., Hutchings, N. J., Jacobsen, B. H., ... & Vejre, H. (2014). Policies for agricultural nitrogen management—trends, challenges and prospects for improved efficiency in Denmark. *Environmental Research Letters*, *9*(11), 115002.

Di, H. J., & Cameron, K. C. (2004). Effects of temperature and application rate of a nitrification inhibitor, dicyandiamide (DCD), on nitrification rate and microbial biomass in a grazed pasture soil. *Soil Research*, *42*(8), 927-932.

Di, H. J., & Cameron, K. C. (2018). Ammonia oxidisers and their inhibition to reduce nitrogen losses in grazed grassland: a review. *Journal of the Royal Society of New Zealand*, *48*(2-3), 127-142.

Di, H. J., Cameron, K. C., Podolyan, A., & Robinson, A. (2014). Effect of soil moisture status and a nitrification inhibitor, dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. *Soil Biology and Biochemistry*, *73*, 59-68.

Duff, A. M., Forrestal, P., Ikoyi, I., & Brennan, F. (2022). Assessing the long-term impact of urease and nitrification inhibitor use on microbial community composition, diversity and function in grassland soil. *Soil Biology and Biochemistry*, *170*, 108709.

Filser, J. (2002). The role of Collembola in carbon and nitrogen cycling in soil: Proceedings of the Xth international Colloquium on Apterygota, České Budějovice 2000: Apterygota at the Beginning of the Third Millennium. *Pedobiologia*, *46*(3-4), 234-245.

Fjellberg, A. (2007). The collembola of Fennoscandia and Denmark, part II: Entomobryomorpha and Symphypleona. In *The Collembola of Fennoscandia and Denmark, Part II: Entomobryomorpha and Symphypleona*. Brill.

Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E., and Oakley, B. B. (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc. Natl. Acad. Sci. U. S. A. 102, 14683–14688. doi: 10.1073/pnas.0506625102

Garnier, E., Cortez, J., Billès, G., Navas, M. L., Roumet, C., Debussche, M., ... & Toussaint, J. P. (2004). Plant functional markers capture ecosystem properties during secondary succession. *Ecology*, *85*(9), 2630-2637.

Geisseler, D., & Scow, K. M. (2014). Long-term effects of mineral fertilizers on soil microorganisms – A review. Soil Biology and Biochemistry, 75, 54-63.

Ghaly, A. E., & Ramakrishnan, V. V. (2015). Nitrogen sources and cycling in the ecosystem and its role in air, water and soil pollution: A critical review. *Journal of Pollution Effects & Control*, *3*(2), 1-26.

Guo, T., O'Connor, P. J., Zhao, X., Zhou, T., Wang, Y., & Zhang, M. (2023). The win-win effects of nitrification inhibitors on soil-crop systems: Decreasing carbendazim residues but promoting soil bacterial community diversities and stabilities and crop yields. *Journal of Hazardous Materials*, *451*, 131175.

Hedde, M., Van Oort, F., & Lamy, I. (2012). Functional traits of soil invertebrates as indicators for exposure to soil disturbance. *Environmental Pollution*, *164*, 59-65.

Hirsch, P. R., Mauchline, T. H., & Clark, I. M. (2010). Culture-independent molecular techniques for soil microbial ecology. *Soil Biology and Biochemistry*, *42*(6), 878-887.

Jensen, J., Larsen, M. M., & Bak, J. (2016). National monitoring study in Denmark finds increased and critical levels of copper and zinc in arable soils fertilized with pig slurry. *Environmental pollution*, *214*, 334-340.

Kõljalg U, Larsson KH, Abarenkov K, Nilsson RH, Alexander IJ, Eberhardt U, Erland S, Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AF, Tedersoo L, Vrålstad T, Ursing BM. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol. 2005 Jun;166(3):1063-8. doi: 10.1111/j.1469-8137.2005.01376.x. PMID: 15869663.

Kumar, H., Jang, Y. N., Kim, K., Park, J., Jung, M. W., & Park, J. E. (2020). Compositional and functional characteristics of swine slurry microbes through 16S rRNA metagenomic sequencing approach. *Animals*, *10*(8), 1372.

Kuypers, M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews Microbiology*, *16*(5), 263-276.

Lei, J., Fan, Q., Yu, J., Ma, Y., Yin, J., & Liu, R. (2022). A meta-analysis to examine whether nitrification inhibitors work through selectively inhibiting ammonia-oxidizing bacteria. *Frontiers in Microbiology*, *13*, 962146.

Li, C., Hu, H. W., Chen, Q. L., Chen, D., & He, J. Z. (2019). Comammox Nitrospira play an active role in nitrification of agricultural soils amended with nitrogen fertilizers. *Soil Biology and Biochemistry*, *138*, 107609.

Li, S., Chen, D., Wang, C., Chen, D., & Wang, Q. (2020). Reduced nitrification by biochar and/or nitrification inhibitor is closely linked with the abundance of comammox Nitrospira in a highly acidic sugarcane soil. *Biology and Fertility of Soils*, *56*, 1219-1228.

Liu, C., Cui, Y., Li, X., & Yao, M. (2021). microeco: an R package for data mining in microbial community ecology. *FEMS microbiology ecology*, *97*(2), fiaa255.

Liu, T., Wang, Z., Wang, S., Zhao, Y., Wright, A. L., & Jiang, X. (2019). Responses of ammonia-oxidizers and comammox to different long-term fertilization regimes in a subtropical paddy soil. *European journal of soil biology*, *93*, 103087.

Louca, S., Parfrey, L.W., Doebeli, M. (2016) - Decoupling function and taxonomy in the global ocean microbiome. Science 353:1272-1277

Louca, S., Polz, M. F., Mazel, F., Albright, M. B., Huber, J. A., O'Connor, M. I., ... & Parfrey, L. W. (2018). Function and functional redundancy in microbial systems. *Nature ecology & evolution*, *2*(6), 936-943.

Macfadyen, A. (1961). Improved funnel-type extractors for soil arthropods. *The Journal of Animal Ecology*, 171-184.

McGeough, K. L., Watson, C. J., Müller, C., Laughlin, R. J., & Chadwick, D. R. (2016). Evidence that the efficacy of the nitrification inhibitor dicyandiamide (DCD) is affected by soil properties in UK soils. *Soil Biology and Biochemistry*, *94*, 222-232.

Meyer, C., & Stitt, M. (2001). Nitrate reduction and signalling. In *Plant nitrogen* (pp. 37-59). Berlin, Heidelberg: Springer Berlin Heidelberg.

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., ... & Kennedy, P. G. (2016). FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology*, *20*, 241-248.

Owens, L. B. (1981). *Effects of nitrapyrin on nitrate movement in soil columns* (Vol. 10, No. 3, pp. 308-310). American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America.

Pachauri, R. K., Allen, M. R., Barros, V. R., Broome, J., Cramer, W., Christ, R., ... & van Ypserle, J. P. (2014). *Climate change 2014: synthesis report. Contribution of Working Groups I, II and III to the fifth assessment report of the Intergovernmental Panel on Climate Change* (p. 151). Ipcc.

Papadopoulou, E. S., Bachtsevani, E., Katsoula, A., Charami, C., Lampronikou, E., Vasileiadis, S., & Karpouzas, D. G. (2024). Nitrification inhibitors impose distinct effects on comammox bacteria and canonical ammonia oxidizers under high N fertilization regimes. *Applied Soil Ecology*, *199*, 105417.

Papadopoulou, E. S., Bachtsevani, E., Papazlatani, C. V., Rousidou, C., Brouziotis, A., Lampronikou, E., ... & Karpouzas, D. G. (2022). The effects of quinone imine, a new potent nitrification inhibitor, dicyandiamide, and nitrapyrin on target and off-target soil microbiota. *Microbiology spectrum*, *10*(4), e02403-21.

Peixoto, L., & Petersen, S. O. (2023). Efficacy of three nitrification inhibitors to reduce nitrous oxide emissions from pig slurry and mineral fertilizers applied to spring barley and winter wheat in Denmark. *Geoderma Regional*, *32*, e00597.

Philippot, L., Hallin, S., & Schloter, M. (2007). Ecology of denitrifying prokaryotes in agricultural soil. *Advances in agronomy*, *96*, 249-305.

Prosser, J. I., Hink, L., Gubry-Rangin, C., & Nicol, G. W. (2020). Nitrous oxide production by ammonia oxidizers: physiological diversity, niche differentiation and potential mitigation strategies. *Global Change Biology*, *26*(1), 103-118.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Opens external link in new windowNucl. Acids Res. 41 (D1): D590-D596.

Ruser, R., & Schulz, R. (2015). The effect of nitrification inhibitors on the nitrous oxide (N2O) release from agricultural soils—a review. *Journal of Plant Nutrition and Soil Science*, *178*(2), 171-188.

Sansupa, C., Wahdan, S. F. M., Hossen, S., Disayathanoowat, T., Wubet, T., & Purahong, W. (2021). Can we use functional annotation of prokaryotic taxa (FAPROTAX) to assign the ecological functions of soil bacteria. *Applied Sciences*, *11*(2), 688.

Shen, T., Stieglmeier, M., Dai, J., Urich, T., & Schleper, C. (2013). Responses of the terrestrial ammonia-oxidizing archaeon Ca. Nitrososphaera viennensis and the ammonia-oxidizing bacterium Nitrosospira multiformis to nitrification inhibitors. *FEMS microbiology letters*, *344*(2), 121-129.

Shi, X., Hu, H. W., Zhu-Barker, X., Hayden, H., Wang, J., Suter, H., ... & He, J. Z. (2017). Nitrifier-induced denitrification is an important source of soil nitrous oxide and can be inhibited by a nitrification inhibitor 3, 4-dimethylpyrazole phosphate. *Environmental microbiology*, *19*(12), 4851-4865.

Singh, J., Saggar, S., Giltrap, D. L., & Bolan, N. S. (2008). Decomposition of dicyandiamide (DCD) in three contrasting soils and its effect on nitrous oxide emission, soil respiratory activity, and microbial biomass—an incubation study. *Soil Research*, *46*(7), 517-525.

Suleiman, A. K., Gonzatto, R., Aita, C., Lupatini, M., Jacques, R. J., Kuramae, E. E., ... & Roesch, L. F. (2016). Temporal variability of soil microbial communities after application of dicyandiamide-treated swine slurry and mineral fertilizers. *Soil Biology and Biochemistry*, *97*, 71-82.

Taiyun Wei and Viliam Simko (2021). R package 'corrplot': Visualization of a Correlation Matrix (Version 0.92). Available from https://github.com/taiyun/corrplot

Wakelin, S., Williams, E., O'Sullivan, C. A., Cameron, K. C., Di, H. J., Cave, V., & O'Callaghan, M. (2014). Predicting the efficacy of the nitrification inhibitor dicyandiamide in pastoral soils. *Plant and soil*, *381*, 35-43.

Zaman, M., & Blennerhassett, J. D. (2010). Effects of the different rates of urease and nitrification inhibitors on gaseous emissions of ammonia and nitrous oxide, nitrate leaching and pasture production from urine patches in an intensive grazed pasture system. *Agriculture, Ecosystems & Environment*, *136*(3-4), 236-246.

Zhou J., Ning D. (2017). Stochastic Community Assembly: Does It Matter in Microbial Ecology?. Microbiol Mol Biol Rev81:10.1128/mmbr.0000217. https://doi.org/10.1128/mmbr.00002-17