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Effects of urease inhibitor 2-NPT and nitrification inhibitors DMPP and MPA on soil microbial communities: an incubation study

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Abstract

Background Urease inhibitors and nitrification inhibitors delay nitrogen fertilizer transformations in soil to reduce nitrogen losses and increase nitrogen use efficiency. While new inhibitor compounds are constantly being developed, little is known about non-target effects on the soil microbiome. This is the first study to investigate non-target effects of the urease inhibitor 2-NPT and the nitrification inhibitor MPA on the soil microbiome. In addition, the more established nitrification inhibitor DMPP was investigated. Target effects and potential non-target effects on the function and composition of microbial communities in three soils from Germany were assessed.

Results Soil microcosms were treated with practically relevant doses of inhibited and non-inhibited fertilizer products. Effects on soil nutrients and enzyme activities from nutrient cycling were analyzed. Gene abundances of bacterial and archaeal *amoA* as well as 16S rRNA and ITS marker genes were quantified using the QIAcuity nanoplate digital PCR. The bacterial and fungal community compositions were analyzed via amplicon sequencing of 16S rRNA and ITS marker genes. Significant reductions of target enzyme activities were found for 2-NPT and DMPP but not MPA. Effect size of inhibition was soil-dependent. Ammonium and nitrate concentrations were significantly affected by the inhibitors in one of the three soils. The non-target enzyme activities of phosphatase, beta-glucosidase, and arylsulfatase were not affected by the inhibitors. Both nitrification inhibitors primarily targeted bacterial ammonia oxidizers, as bacterial but not archaeal *amoA* genes were reduced. Overall bacterial and fungal communities were not clearly affected. Observed abundance shifts of soil microorganisms were linked to indirect effects driven by nutrient availability rather than direct effects of the inhibitors.

Conclusion Our study suggests that the newer inhibitors 2-NPT and MPA as well as DMPP do not directly affect the function and composition of the soil microbiome in the short-term. Significant target effects of the inhibitors change the availability of mineral N which causes indirect effects with minimal non-target microbial community shifts. Further tests involving other soil organisms and long-term field studies are required to further improve the environmental risk assessment of inhibitors.

Keywords Nitrogen fertilization, AOA, AOB, dPCR, Chip-based digital PCR, Sustainable agriculture

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Introduction

The extensive use of nitrogen (N) fertilizers in agriculture is well-known to increase ammonia volatilization, nitrous oxide emissions, and nitrate leaching [1–3]. Such N losses do not only hamper crop N uptake and thus reduce N use efficiency substantially but are also drivers of climate change [4, 5] and biodiversity loss [6–8]. Thus, preventing N losses is important from an economical as well as an ecological perspective. Urease inhibitors (UIs) and nitrification inhibitors (NIs) can be added to fertilizers to delay the transformation processes of nitrogenous compounds in soil. By inhibiting the urease enzyme, UIs delay the hydrolysis of urea fertilizer into ammonia, while NIs delay ammonia oxidation by inhibiting the ammonia monooxygenase enzyme (AMO). This shall give more time for crop plants to take up N, reduce N losses, and increase N use efficiency.

An increasing use of UIs and NIs can be expected in the future because they are easy to apply on a large scale and benefit farmers as well as the environment. Countries like Germany that have strong ammonia emission reduction targets (derived from the European Union National Emission Ceilings Directive 2016/2284/EU) support the use of UIs by farmers via legislative regulations. The German Fertilizer Application Ordinance (DüV) amended in 2017 demands urea fertilizers to be applied with urease inhibitors if not incorporated into the soil within four hours [9]. Germany was the first country in the world to regulate the use of UIs and it successfully reduced ammonia emissions [10]. Other countries from and outside of EU could also cut their ammonia emissions with the use of UIs, a study found [11]. Similar regulations with regards to NIs are conceivable in the future in order to reduce N₂O emissions and nitrate leaching.

While there are also biological UIs [12] and biological NIs [13], synthetic inhibitors are relevant to the market and have been used for decades, with new compounds still being developed [14–16]. Their intended effects have been confirmed in several laboratory and field studies [17–21], and their efficiency was found to depend on many local parameters, such as soil properties and climate conditions.

Despite their increasing use, there are knowledge gaps regarding the environmental risks of UIs and NIs. The scientific literature comprises only few studies that investigated potential non-target effects. Although the compounds are intentionally applied to soils to negatively affect organisms and ecological functions, inhibitors are not regulated in the way that pesticides are. Therefore, little information about the environmental consequences of the use of inhibitor compounds is available. The reported non-target effects and unwanted environmental fate of NIs and UIs highly depend on the individual

compound. For example, an inhibition of the methane monooxygenase enzyme, which might indirectly lead to an increase in methane emissions, was reported for the NI Nitrapyrin [22], but not for dicyandiamide (DCD) and 3,4-Dimethyl-1H-pyrazole phosphate (DMPP) [21, 23]. DMPP was found to shift bacterial community composition [24, 25], impair soil microbial respiration [26], and negatively affect soil nitrogenase activity [27]. Catabolism of different substrate types and microbial growth were affected only at higher doses [28]. Other studies have reported no effects of DMPP on non-target microbial enzymatic activities [29] and community composition [30–32] at normal doses. However, “normal doses” of inhibitors by means of active compound per mass of soil are not consistent across studies because they depend on several factors such as fertilizer application rate and soil density. In addition, since inhibitor compounds are designed to target specific functional groups of microorganisms, they might have more subtle effects and disrupt microbial interaction networks without major shifts in overall diversity.

Some compounds are more mobile than others and can be taken up by plants, like NBPT [33], DMPP and DMPBS [34], and DCD [35]. Consequently, residues of DCD in milk from pastures treated with DCD containing fertilizers caused a ban of DCD in New Zealand [36]. These studies showed that inhibitor compounds can potentially lead to unwanted effects that are relevant for environmental and human health. Especially for newer compounds, such as the NI N-((3(5)-methyl-1H-pyrazol-1-yl)methyl)acetamide (MPA) and the UI N-(2-Nitrophenyl)phosphoric triamide (2-NPT), such information is scarce, although these compounds are already on the market. MPA and 2-NPT are mostly used in Germany [37]. Environmental monitoring of surface waters in Lower Saxony, Germany, in 2019 by the Niedersächsischer Landesbetrieb für Wasserwirtschaft, Küsten- und Naturschutz found no contaminations of MPA or 2-NPT but 1,2,4-Triazole and DCD. Based on the information in their REACH registration dossiers (details see Supplementary Material S1), a high mobility and quick degradation of MPA and 2-NPT can be assumed in soil. Elucidating the environmental behavior of MPA and 2-NPT is subject of currently ongoing research. Until now, no scientific publications are available that investigate potential non-target effects of MPA and 2-NPT. Our study is a first step towards narrowing this knowledge gap.

In this study, we aimed to assess the impact of UIs and NIs on the soil microbiome to check for potential non-target effects on its function and composition. Here, we focused on the less well studied compounds 2-NPT and MPA as well as the more popular nitrification inhibitor

DMPP. In a laboratory incubation study, we applied six fertilization treatments and compared three different soil types. We investigated whether the inhibited fertilizers show their intended target effects on enzymatic activities and gene abundances of target organisms, whether there are non-target effects on enzymatic activities that are not related to the N cycle or on the community composition of bacteria and fungi, and how these effects differ between soils of differing edaphic properties. This is the first comprehensive study to assess the non-target effects of the newer inhibitors 2-NPT and MPA on the soil microbiome.

Material and methods

Soil microcosms

Three soils of different properties have been sampled from the top 20 cm with a spade (details presented in Supplementary Material S2) from fields in Germany (Table 1). The soils were air-dried as needed, sieved to 2 mm, and stored at +4 °C in loosely sealed buckets for no longer than a month (with one exception, see Supplementary Material S2). From each site, two sampling events took place and the batches were used in two experimental series (Exp I, Exp II) with soil microcosms set up identically as presented below.

Soil microcosms were set up with 180 g soil dry weight (DW) in a 600 mL glass beaker and set to 40% of its maximum water holding capacity (WHC) with distilled water. Then, 0.299 g of shredded wheat straw (ground using a swing mill, sieved to 0.1–1 mm) was added. The straw amount corresponds to 0.166 w% or 3990 kg straw ha⁻¹

[38] and was added to stimulate microbial growth and to generate an N demand before application of N-fertilizers. Soil microcosms were homogenized with a spatula and sealed with laboratory film (Parafilm “M”) to reduce water loss. Sufficient gas exchange was verified in preliminary tests using fiber optic oxygen sensors (DP-PSt7-10, PreSens). Microcosms were pre-incubated for 7 days in the dark at 20 °C.

Fertilizer applications

Six fertilization treatments were applied after 7 days of preincubation (Table 2). Treatments consisted of a non-fertilized control (CTR) and five inhibited or non-inhibited mineral fertilizers at an application rate of 100 kg N ha⁻¹ (15 cm depth and 1.2 g cm⁻³ density). Since the amount of NI and UI contained is not specified on the products, the stated amounts of NI and UI are based on the minimum inhibitor contents according to German fertilizer ordinance [39] and have not been measured. The fertilizers were used within 1 year after purchase to avoid inhibitor degradation.

To apply the fertilizers, the granules were dissolved in water and the freshly prepared solution was applied with additional distilled water as needed to restore 40% WHC. CTR received distilled water only. All microcosms were mixed thoroughly with spatulas and sealed with fresh parafilm. Duplicate microcosms were set up for each soil-treatment combination.

We conducted two experimental series, whereby one considered soil microbial function and the other community composition. In experimental series I, microcosms

Table 1 Soils origin and properties

Name	Sand soil	Silt soil	Clay soil
Site	Lat 49.312629, Lon 8.327040 D-67374 Hanhofen, Germany	Lat 52.238250, Lon 10.870889 D-38154 Königslutter, Germany	Lat 49.205383, Lon 8.051040 D-76833 Siebeldingen, Germany
Provided by	LUFA, Speyer	Thünen Institut, Braunschweig	LUFA, Speyer
Sampling dates	27 Oct 2023 (Exp I) 11 Mar 2024 (Exp II)	14 Mar 2024, from 0N plot (Exp II) 21 May 2024, from ASN plot (Exp I)	15 Jan 2024 (Exp I) 18 Mar 2024 (Exp II)
Management	Meadow No fertilization	Wheat 0N plot: 0 kg N ha ⁻¹ ASN plot: 206 kg N ha ⁻¹ , split 12 Mar and 10 Apr 2024	Fallow No fertilization
Texture	Loamy sand 73% sand, 17% silt, 10% clay	Clayey silt 6% sand, 80% silt, 14% clay	Silty clay 22% sand, 37% silt, 41% clay
pH (0.01 M CaCl ₂)	5.6	6.8	7.3
Carbon	1.82% C _{org}	1.19% C _{total}	1.58% C _{org}
Nitrogen	0.19% N	0.11% N (sampled 28 Feb 2024)	0.18% N
Water holding capacity (g kg ⁻¹)	442 (soil from 27 Oct 2023) 489 (soil from 11 Mar 2024)	338 (soil from 14 Mar 2024)	431 (soil from 15 Jan 2024) 471 (soil from 18 Mar 2024)

Details on soil sampling and characterization are provided in Supplementary Material S2

Soils were used in two experimental series (Exp I, Exp II)

0N, non-fertilized control; ASN, Ammonium sulfate nitrate (the plot was fertilized with ASS 26 (+ 13S), EuroChem Agro)

Table 2 Treatment groups details

Treatment group	Treatment name	Fertilizer product	Manufacturer	Application rate (per kg soil DW)
CTR	Non-fertilized control	NA	NA	NA
ASN	Ammonium sulfate nitrate	Ammonsulfatsalpeter 26 (+ 13S)	EuroChem Agro	40.6 mg NH ₄ ⁺ -N 15.0 mg NO ₃ ⁻ -N
ASN + NI	Ammonium sulfate nitrate with nitrification inhibitor DMPP	ENTEC 26 (+ 13S)	EuroChem Agro	39.5 mg NH ₄ ⁺ -N 16.0 mg NO ₃ ⁻ -N 0.316 mg DMPP
U	Urea	PIAGRAN	SKW Piesteritz	55.6 mg urea-N
U + UI	Urea with urease inhibitor 2-NPT	PIAGRAN pro	SKW Piesteritz	55.6 mg urea-N 0.022 mg 2-NPT
U + UI + NI	Urea with urease inhibitor 2-NPT and nitrification inhibitor MPA	ALZON neo-N	SKW Piesteritz	55.6 mg urea-N 0.022 mg 2-NPT 0.028 mg MPA

were sampled for measuring soil nutrients and enzyme activities 4 days after application of fertilizers. For experimental series II, new microcosms were set up identically and sampled for DNA extraction 14 days after application of fertilizers (for details see Supplementary Material S2).

Mineral N analysis

For extraction of mineral N at four days after the application of fertilizers, 1 g of soil fresh weight (FW) was mixed with 5 mL of 0.01 M CaCl₂ solution in a 15 mL centrifuge tube and inverted for 30 min (two technical replicates per microcosm). After centrifugation (2900 g, 5 min), aliquots of the supernatant were frozen at - 20 °C until mineral N analysis.

Ammonium was analyzed via a Berthelot reaction (based on [40]). In three technical replicates, 150 µL of sample, 75 µL of fresh color reagent (0.6 g L⁻¹ sodium nitroprusside dihydrate and 85 g L⁻¹ sodium salicylate in 0.15 M NaOH) and 30 µL of a fresh 1 g L⁻¹ sodium dichloroisocyanurate dihydrate solution were mixed. After 30 min at room temperature, the absorbance was read at 650 nm against a 0.01 M CaCl₂ blank (Synergy MX, BioTek). The concentration of ammonium was calculated via a linear regression of 0.05–6.4 mg NH₄⁺-N L⁻¹.

Nitrate was analyzed via reduction to nitrite and a modified Griess reaction (based on [41, 42]). In three technical replicates, 120 µL of sample were mixed with 100 µL of VCl-Griess reagent (5 parts of 8 g V(III)Cl L⁻¹ in 3.1% HCl, 1 part of 2 g N-(1-naphthyl)ethylenediamine dihydrochloride L⁻¹, and 1 part of 20 g sulfanilamide L⁻¹ in 10% HCl). After incubation at 45 °C for 60 min and cooling down, the absorbance was read at 540 nm against a 0.01 M CaCl₂ blank. For nitrite, the procedure was identical but using Griess reagent without V(III)Cl. The concentrations of nitrate and nitrite were calculated

via a linear regression of 0.1–3.2 mg NO₃⁻-N L⁻¹ and 0.02–0.64 mg NO₂⁻-N L⁻¹ respectively. To determine the concentration of nitrate, the sample's own nitrite concentration was subtracted.

pH

To determine the pH of the soil (similar to DIN 10390 [43]), 1 g of soil FW were mixed with 5 mL of 0.01 M CaCl₂ in a 15 mL centrifuge tube and inverted for 60 min at room temperature (two technical replicates per microcosm). After sedimentation for 60 min, pH was measured with an electrode (FiveEasy FE20, Mettler Toledo).

Enzyme activities

To determine potential enzyme activities, the product yield of samples incubated with the appropriate substrate were compared to a blank sample incubated without substrate. From each soil microcosm, two samples and two blank samples were prepared for each of the enzyme activity assays four days after the application of fertilizers.

Nitrification rate was determined with a modified protocol (based on [44]). 5 g soil FW was mixed with 20 mL of 1 mM ammonium sulfate (or distilled water for blank samples) in 100 mL conical flasks. The flasks were covered with parafilm and incubated at 28 °C in the dark on a horizontal shaker for 6 days. For extraction of mineral N, 5 mL of 0.01 M CaCl₂ was added and shaking continued for 5 min. An aliquot from the suspension was centrifuged (2900 g, 5 min) and the clear supernatant was stored at - 20 °C until nitrate analysis.

Urease activity was determined with a modified protocol (based on [44]). 5 g soil FW was mixed with 1.5 mL of 80 mM urea solution (or distilled water for blank samples) in 100 mL conical flasks. The flasks were covered with parafilm and incubated at 28 °C in the dark for 2 h. After incubation, 1.5 mL distilled water (or urea solution

for blank samples) and 25 mL of 0.01 M CaCl_2 were added and the flasks were shaken horizontally for 30 min at room temperature. After centrifugation (2900 g, 5 min), the clear supernatant was stored at -20°C until ammonium analysis.

Acid phosphatase activity was determined with a modified protocol (based on [40]). 1 g soil FW was mixed with 4 mL modified universal buffer (“buffer”) at pH 6.5 and with (or without for blank samples) 1 mL of p-nitrophenyl phosphate (26.5 mM in buffer) in a 15 mL centrifuge tube. The tubes were shaken at 32°C in the dark lying on a horizontal shaker for 1 h. The samples from silt soil were incubated at 29.5°C instead due to a technical defect. Then, 4 mL 0.5 M NaOH and 1 mL 0.1 M CaCl_2 were added (as well as 1 mL of p-nitrophenyl phosphate solution to the blank samples) and the tubes inverted manually. After centrifugation (2000 g, 5 min), the absorbance of the clear supernatant was read at 420 nm in three technical replicates using buffer as blank. The concentration of p-nitrophenol was calculated via a linear regression of 2.5–80 mg p-nitrophenol L^{-1} against a buffer blank including NaOH and CaCl_2 in respective amounts.

The protocol for determining beta-glucosidase activity was identical to the acid phosphatase protocol but using the buffer at pH 6.0 and a 25 mM p-nitrophenyl-beta-D-glucopyranoside substrate solution instead.

The protocol for determining arylsulfatase activity was identical to the acid phosphatase protocol but using a 0.5 M acetate buffer at pH 5.8 and a 20 mM p-nitrophenyl sulfate substrate solution instead.

DNA extraction

One soil sample per microcosm was taken 14 days after the application of fertilizers and frozen at -20°C until DNA extraction. After thawing, two separate extractions were performed from each soil sample using the NucleoSpin Soil Kit (Macherey–Nagel, Germany) according to the manufacturer’s instructions. In short, we used 0.5 g soil FW, 700 μL lysis buffer SL1 and 150 μL Enhancer SX. The lysis step was assisted by a swing mill (30 Hz, 5 min, Retsch, Germany). DNA purity was measured with NanoDrop 2000 (ThermoFisher, USA) and quantified with Quant-iT PicoGreen dsDNA Kit (ThermoFisher, USA) according to the manufacturer’s instructions in technical duplicates. Until dPCR and DNA sequencing was performed, eluted DNA samples were stored at -20°C for only up to a few months to prevent degradation and potential downstream effects.

Gene quantification with digital PCR

Gene quantification was performed via nanoplate digital PCR (dPCR) using a QIAcuity One (Qiagen, Germany).

The abundances of ammonia oxidizer genes were quantified targeting the bacterial *amoA* gene [45] for ammonia oxidizing bacteria (AOB) and the archaeal *amoA* gene [46] for ammonia oxidizing archaea (AOA). Abundances of total bacteria and archaea were assessed by targeting the V4 region of the 16S rRNA gene [47–49], while fungi were assessed by targeting the ITS region [50, 51]. Technical duplicates of dPCR reactions were prepared with the extracted DNA (diluted if necessary), respective primers, QIAcuity EvaGreen Master-Mix, and DNA/RNAase-free water. Final volumes of 12 μL were transferred into the QIAcuity 8.5 k 96-well nanoplates. Two non-template controls (NTCs) were included with each nanoplate, containing the DNA extraction kit elution buffer instead of DNA sample. Details including reaction mix, primer sequences and dPCR cycling conditions are provided in Supplementary Material S3. There, we also provide detailed information about method development because this is one of the first studies analyzing the abovementioned target genes from soil DNA with QIAcuity. The data was analyzed using the QIAcuity Software Suite 3.0.0 (Qiagen). Positive and negative partitions were separated by the software and checked for each individual reaction. NTCs contained no more than 2 positive partitions (out of 8500 partitions per reaction). Copy numbers determined in NTCs were subtracted from the samples to correct for contamination. With the DNA amount used per reaction and the DNA yield from the DNA extraction, the number of copies per g soil DW were calculated.

DNA sequencing and data analysis

The V4 and ITS region were sequenced to analyze the prokaryotic and fungal community composition using the exact same primers as for the dPCR analysis. The extracted DNA was sent to StarSEQ (Mainz, Germany) and the library preparation was performed with a one-step PCR reaction [52]. DNA sequencing was performed on an Illumina MiSeq platform (300 nt, paired-end, V3 chemistry, including PhiX). The data was provided in FASTQ files.

The bioinformatic analysis of this data was performed in R (versions 4.4.2–4.5.0, [53]). Primers were removed using cutadapt (version 4.9 with Python 3.13.0, [54]). DADA2 [55] was used to process the sequences, filter them according to quality criteria, remove chimeras, and determine amplicon sequencing variants (ASVs, [56]). For details, see Supplementary Material S4. The taxonomic assignment of these sequences was carried out using the DADA2-formatted training set from SILVA database (Nr99 v138.2 to Species, 15 Nov 2024, [57, 58]) for the bacteria, and using the UNITE database (UNITE general FASTA release for Fungi, Version 19.02.2025,

[59]) for the fungi with the naive Bayesian classifier method [60] in DADA2. ASVs were not filtered or agglomerated according to their taxonomic assignment and data processing continued on ASV level. Singletons (ASVs with a total count of 1) were removed. The rarefaction curves of all samples reached the plateau, indicating sufficient sequencing depths for V4 and ITS region. All samples were rarefied without replacement to an even depth (90% of the minimum count: 15,874 counts for V4, 15,098 counts for ITS, using 123 as seed) using phyloseq version 1.48.0 [61], resulting in 6992 bacterial, 77 archaeal, and 2576 fungal ASVs. Taxonomic assignment at species level was “NA” for 96% of bacterial and 71% of fungal ASVs. The relative abundances of ASVs from DNA sequencing (percentages) were weighted with the absolute gene abundance of V4 or ITS marker genes from dPCR in each sample to overcome compositionality [62]. This was possible because the exact same primers were used for DNA sequencing and dPCR. Details of abundance calculations are provided in Supplementary Material S3. Analyses of diversity and community composition were performed separately for each soil type using phyloseq [61] and vegan (version 2.6–8, [63]) and visualized using ggplot2 (version 3.5.1, [64]) and cowplot (version 1.1.3, [65]). Permutational multivariate analysis of variance (PERMANOVA) was performed using vegan to statistically test if the fertilization treatments significantly altered the overall structure of the microbial communities. Pairwise comparisons of treatments were performed using pairwiseAdonis (version 0.4, [66]) with Benjamini–Hochberg correction for multiple comparisons.

Analysis of differentially abundant bacterial and fungal ASVs across fertilizer treatments was performed in R using DESeq2 (package version 1.48.1, [67]). The raw sequencing counts were used as input as required by this analysis. In order to handle the zero-inflation, ASVs were filtered by a minimum abundance cutoff of at least 10 counts in at least 2 samples. This kept 52–56% of bacterial ASVs and 34–38% of fungal ASVs while retaining 97–99% of total counts within each of the six datasets (2 DNA regions \times 3 soil types). Significantly differentially abundant ASVs were identified by an adjusted p-value of < 0.1 (FDR: Benjamini-Hochberg).

Statistical significance testing

Data from mineral N analysis, pH, enzymatic activities, gene abundances, and alpha diversity measures were tested for statistical significance between treatments (separately for each soil type) via one-way analysis of variance (ANOVA) and post hoc Tukey’s Honest Significant Difference test using a significance level of 0.05. The analyses were performed in R using the stats package (version 4.5.0). For comparing the target effect sizes of the two

nitrification inhibitors, eta squared (η^2) values were calculated for a one-way ANOVA model including only the inhibited treatment (ASN+NI or U+UI+NI) and the reference treatment (ASN or U+UI, respectively) using the lsr package (version 0.5.2, [68]) in R.

Results

Soil nutrients

The concentrations of ammonium and nitrate in the non-fertilized CTR were low in all three soil types (Fig. 1). While there was almost no ammonium present, there were 6 and 14 mg NO_3^- -N g^{-1} soil DW in sand and clay soil, respectively, whereas nitrate was below the limit of quantification in silt soil. Comparing ASN and ASN+NI, levels of ammonium and nitrate were quite similar in sand and clay soil, while in silt soil there was significantly more ammonium and less nitrate with the NI. The UI did not affect the ammonium or nitrate contents in any of the three soils. The combination of both inhibitors in U+UI+NI significantly increased ammonium and reduced nitrate levels in comparison to U and U+UI in silt soil only. Thus, four days after application, the mineral N levels in all three soils were increased due to the fertilization, but the UI did not affect ammonium or nitrate levels, while significant effects of both NIs (DMPP and MPA) were found in silt soil only.

Enzyme activities

Despite the ammonium and nitrate levels being less affected by the NIs and UI, the inhibitors clearly affected the potential activity of their respective target enzyme. The UI significantly reduced the urease activity in all three soils by more than 50% (Fig. 1). Inhibition of nitrification by NIs was found in all three soils, but to different extents. The already low nitrification rate in sand soil was reduced to about 0 with both DMPP (in ASN+NI) and MPA (in U+UI+NI). In silt soil, nitrification was completely inhibited with DMPP, but only slightly reduced with MPA. However, this slight reduction was sufficient to significantly affect ammonium and nitrate levels. In clay soil, nitrification inhibition was not significant but the effect of DMPP was stronger than the effect of MPA.

The enzymes for phosphorus, carbon and sulfur cycling showed different activity levels in the three soils (Fig. 2). Most notably, the phosphatase and arylsulfatase activities were much higher in sand soil than in silt and clay soil. In general, however, the fertilization treatments hardly affected the enzyme activities of phosphatase, beta-glucosidase, and arylsulfatase as they were mostly comparable to CTR. Some statistically significant differences between treatments were found due to little standard error but absolute activity

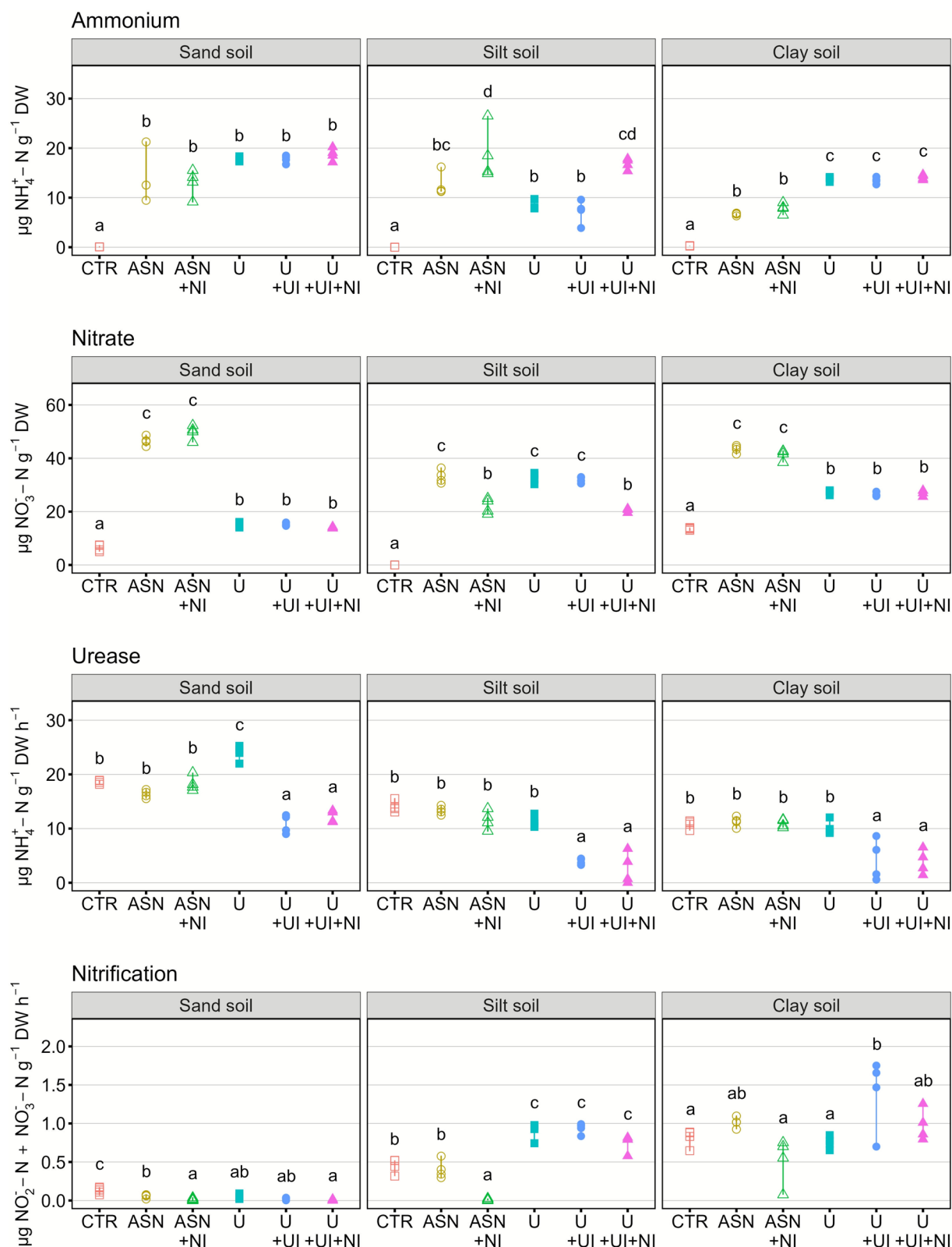


Fig. 1 Nitrogen cycle-related endpoints in three different soils four days after application of six fertilization treatments. Treatments sharing a letter are not significantly different ($p < 0.05$, Tukey's HSD, comparing treatments against each other within one soil but not across soils). CTR, non-fertilized control; ASN, ammonium sulfate nitrate; U, urea; NI, nitrification inhibitor; UI, urease inhibitor. $n = 4$

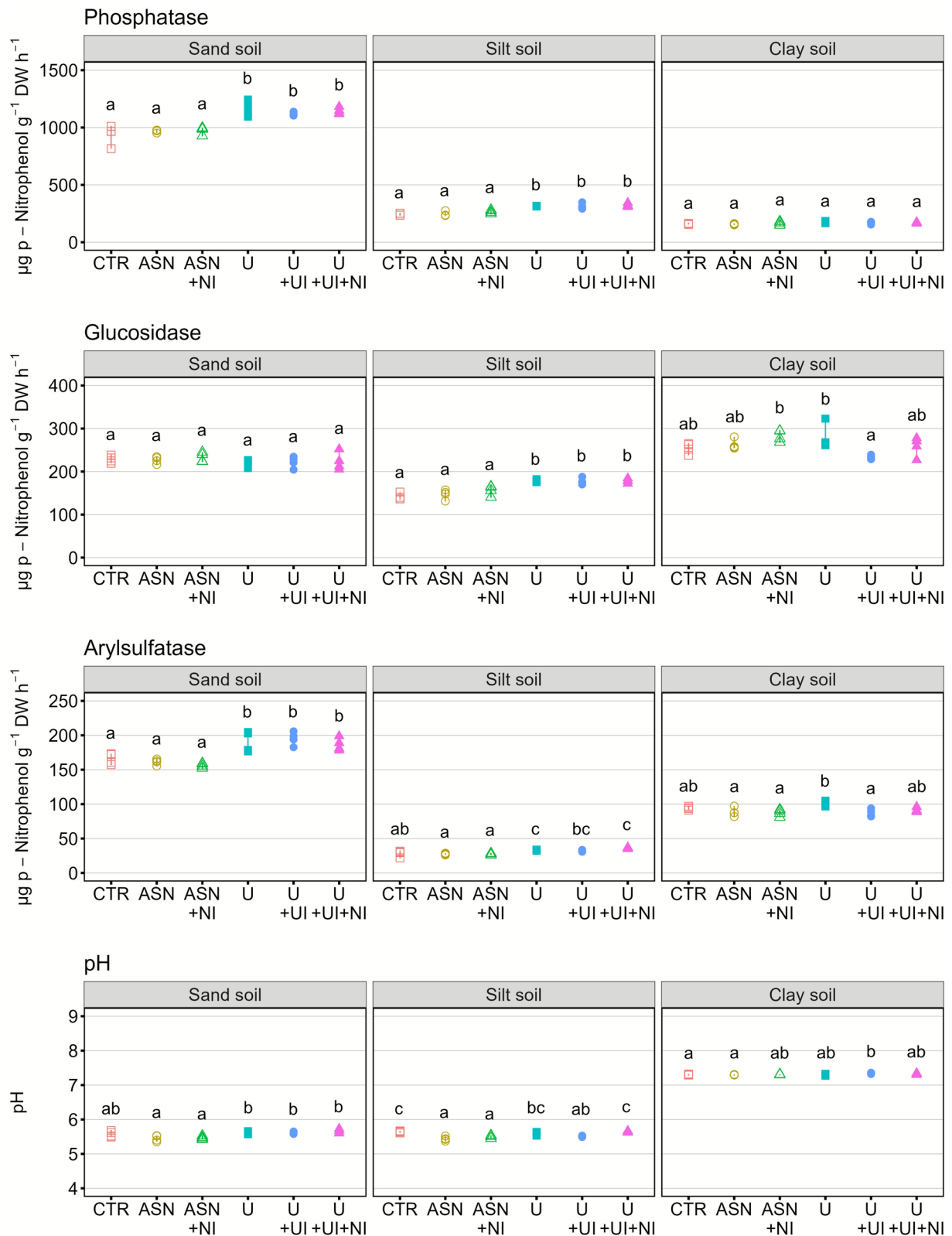


Fig. 2 Enzymatic activities for phosphorus, carbon and sulfur cycling as well as soil pH in three different soils four days after application of six fertilization treatments. Treatments sharing a letter are not significantly different ($p < 0.05$, Tukey's HSD, comparing treatments against each other within one soil but not across soils). CTR, non-fertilized control; ASN, ammonium sulfate nitrate; U, urea; NI, nitrification inhibitor; UI, urease inhibitor. $n=4$

rates remained on a very similar level. Thus, no clear effects of the NIs and UI were found on non-target enzyme activities.

The pH levels were around 5.6 for the sand and silt soil and around 7.2 for the clay soil. The silt soil was found to have a lower pH than initially reported (pH 6.8), probably due to acidification in wheat rhizosphere in the field. The fertilization treatments did not have a pronounced effect on the pH in any soil at four days after application.

Even statistically significant treatment effects on pH were within 0.2 pH units.

Ammonia oxidizer gene abundance

The gene abundances of AOA and AOB were very different in the three soils, with the lowest copy numbers found in the sand soil (Fig. 3). AOA were similarly abundant in the silt and clay soil, but AOB were more abundant in the silt soil. The treatment effects of the fertilizers

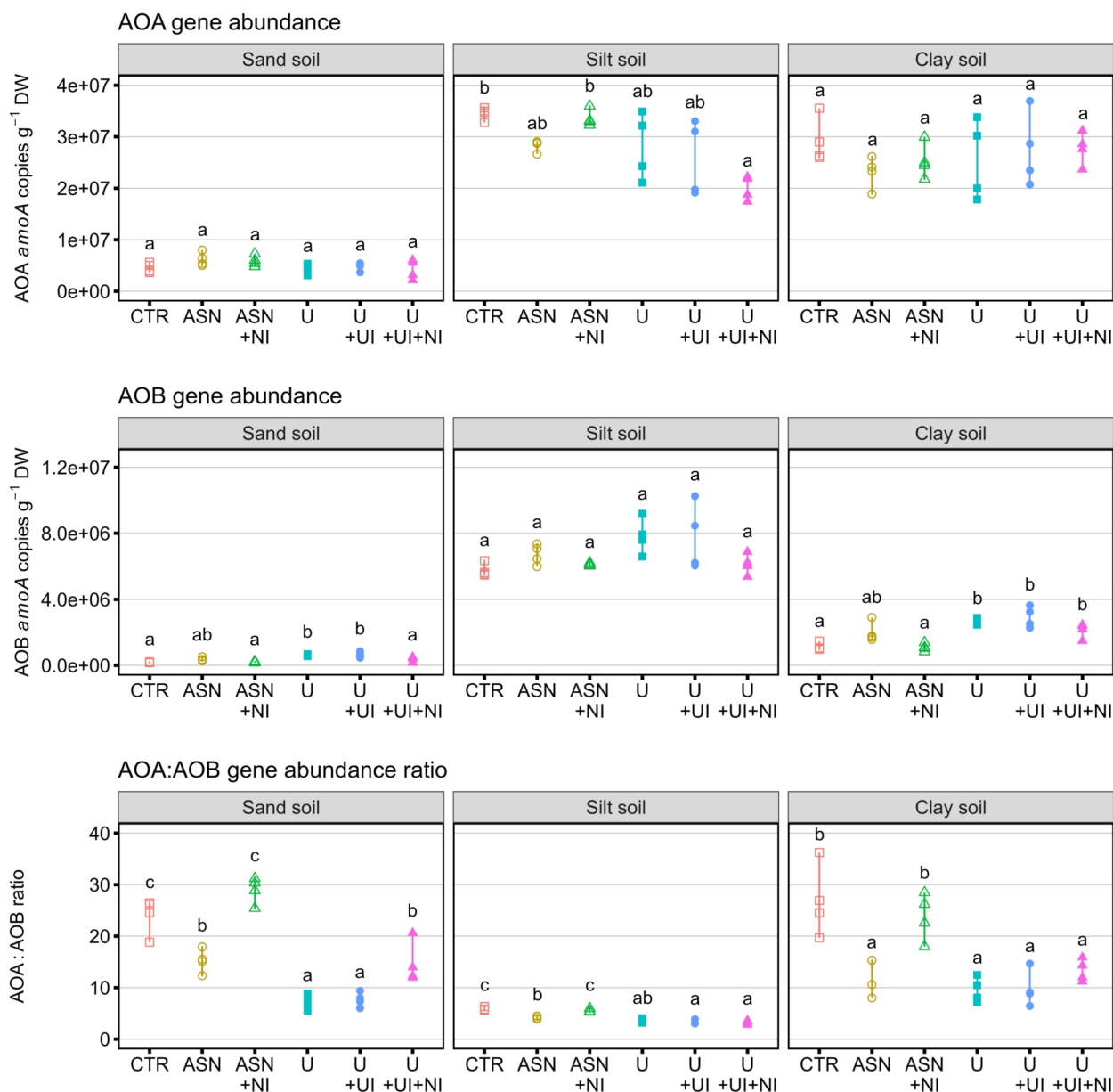


Fig. 3 Gene copy numbers of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) as well as their ratio in three different soils 14 days after application of six fertilization treatments. Treatments sharing a letter are not significantly different ($p < 0.05$, Tukey's HSD, comparing treatments against each other within one soil but not across soils). CTR, non-fertilized control; ASN, ammonium sulfate nitrate; U, urea; NI, nitrification inhibitor; UI, urease inhibitor. DW, soil dry weight. $n = 4$

as well as the inhibitors on AOA gene abundances were non-significant in all cases when comparing to the respective reference treatment (e.g., ASN vs. ASN+NI). AOB were more sensitive to the fertilizers and increased (not always significantly) in ASN, U, and U+UI treatments. However, when the fertilizer contained a NI, the AOB copy numbers increased much less and often were similar to the non-fertilized CTR. The ratio of AOA to AOB copy numbers showed significant treatment effects and clearly showed that AOA were stronger in CTR and NI-containing treatments, while AOB profited from non-inhibited ammonium in ASN, U, and U+UI treatments. Effect sizes of DMPP on AOB abundance and AOA:AOB ratio were greater than those of MPA in all three soils (Supplementary Material S5). A negative correlation between the AOA:AOB ratio and the nitrification rate was found for silt and clay soil (Supplementary Material S6). This indicated that a dominance of AOB coincided with a higher potential nitrification rate.

Microbial community composition

The three different soils were inhabited by bacterial and fungal communities of very different compositions (Supplementary Material S7, S8). Still, their richness (the number of observed ASVs) and diversity (Simpson's index) were in a similar range (Fig. 4). In general, the bacterial communities were more diverse than the fungal communities. The most abundant bacterial ASV in each sequencing sample accounted for not more than 3% of total bacterial counts. Actually, the two most abundant ASVs from 16S rRNA V4 sequencing in silt and clay soil were archaea of unknown genus from the family of *Nitrososphaeraceae*. In contrast, fungal communities were strongly dominated by single ASVs. In clay soil, one ASV accounted for 23–46% of total fungal counts in each clay soil sample (genus *Hormiactis*, species unknown). The exceptionally high abundance of this ASV resulted in lower Simpson's index values for some samples in this soil (Fig. 4). The most abundant fungal ASV in sand soil was assigned to *Humicola quadrangulata* (from the class of *Sordariomycetes*) with 12–28% of counts, and in silt soil, it was *Podila humilis* (from the class of *Mortierellomycetes*) with 13–25% of counts. Thus, three very different microbial communities present in the three soils were exposed to the fertilizer treatments in this study.

The effects of the fertilization treatments on the bacterial and fungal alpha diversity were analyzed and visualized for each soil separately (Fig. 4). Richness and Simpson's index of the fertilized soils were mostly within range of the unfertilized CTR soil. A non-significant tendency towards lower fungal richness in ASN-based fertilizers compared to CTR was visible for all three soils, but

it resulted in a significantly lower Simpson's index in sand soil only.

Beta diversity of bacteria and fungi was not clearly affected by the fertilization treatments according to NMDS ordinations (Fig. 5). The variation within groups was often larger than between groups. The ordination plots of the bacteria indicated a separation of the CTR from the fertilized soils for sand and silt soil but not in clay soil. In clay soil, a separate clustering of the urea-based treatments in comparison to ASN-based treatments was found. However, these separations and clusters were not supported by hierarchical clustering analyses based on Bray–Curtis dissimilarity (Supplementary Material S9). The fungal communities did not show separate clusters in the NMDS ordinations. To statistically test for significant treatment effects on the community level, PERMANOVA was performed (see Supplementary Material S10). It was found that fertilization treatment was a statistically significant explanatory variable for the fungal communities of all three soils and for the bacterial community of silt soil. To identify which of the treatments were significantly different from each other, pairwise comparisons were performed. These however, found that none of the pairwise comparisons were statistically significantly different. Thus, shifts on the overall community level of bacteria and fungi were found but seemed to be minor.

Since the V4 primers can also amplify ASVs belonging to archaea, we also investigated the 77 ASVs classified as archaea. Despite the relatively low number of archaeal ASVs ($n=77$; 1.1% of total V4 ASVs), some of these were very abundant (ranks 1, 2, 12, 24, 27, and 70 within the top 100 most abundant ASVs). From the 77 archaeal ASVs, 60 belonged to the *Nitrososphaeraceae* family. No clear effects of the fertilization treatments on archaeal alpha and beta diversity were found across the three soils (Supplementary Material S11).

DESeq2 analysis was used to identify differentially abundant ASVs across treatments (Fig. 6). Lists of the differentially abundant ASVs and their taxonomic assignments are provided in the Supplementary Material S12. The majority of differentially abundant ASVs were not affected by a single treatment only but significantly affected (positively or negatively) by two or more treatments. It was found that many ASVs showed inverse responses to non-inhibited and inhibited fertilization. Multiple ASVs increased in abundance when fertilizers were non-inhibited, but increased less or not at all when the fertilizer was inhibited. Vice versa, several other ASVs were less abundant in non-inhibited fertilizer treatments, but were less negatively affected when fertilizers were inhibited. These patterns were observed in all three soils and for both bacteria and fungi.

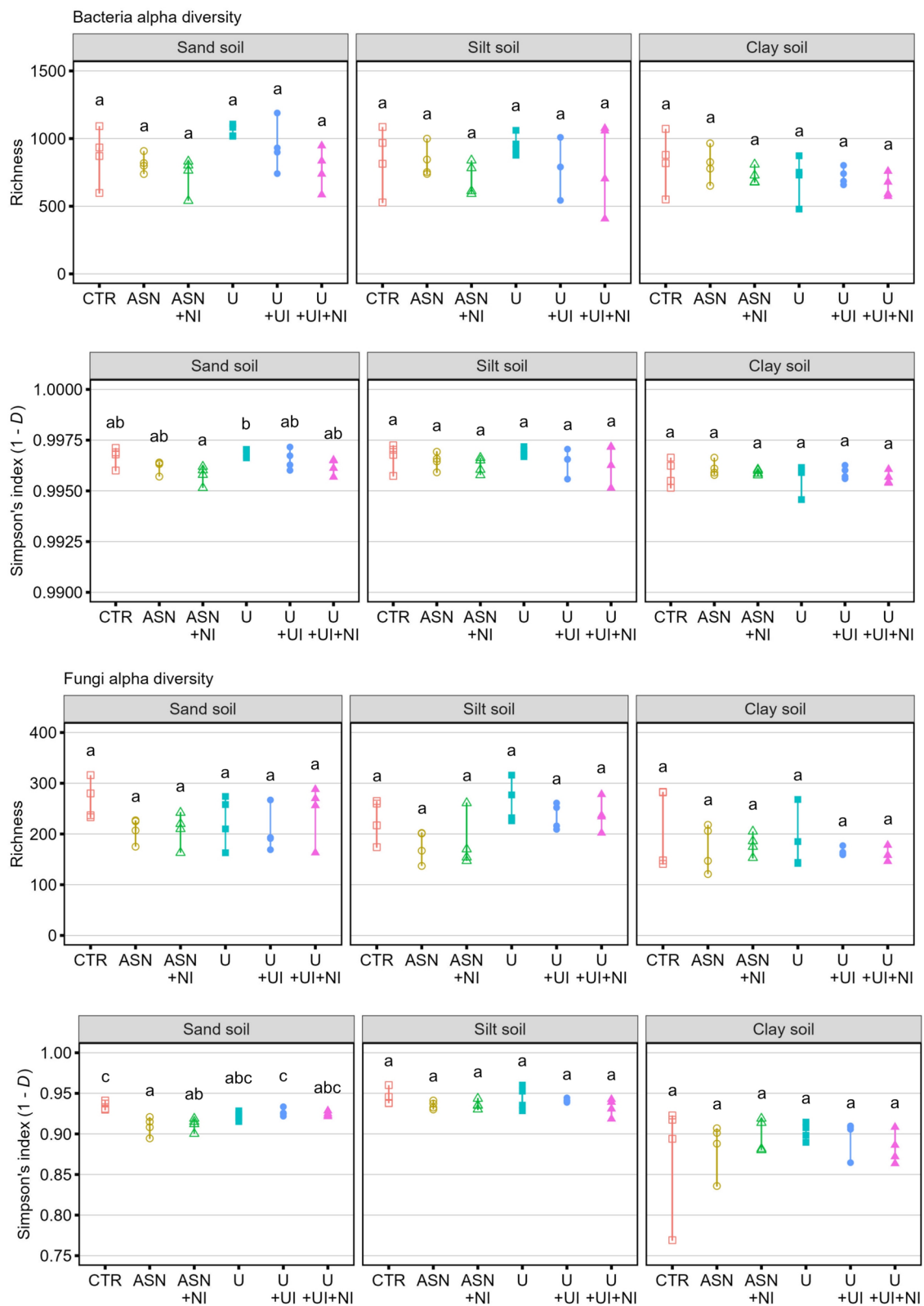


Fig. 4 Bacterial (top) and fungal (bottom) alpha diversity in three different soils 14 days after application of six fertilization treatments. Treatments sharing a letter are not significantly different ($p < 0.05$, Tukey's HSD, comparing treatments against each other within one soil but not across soils). CTR, non-fertilized control; ASN, ammonium sulfate nitrate; U, urea; NI, nitrification inhibitor; UI, urease inhibitor. $n = 4$

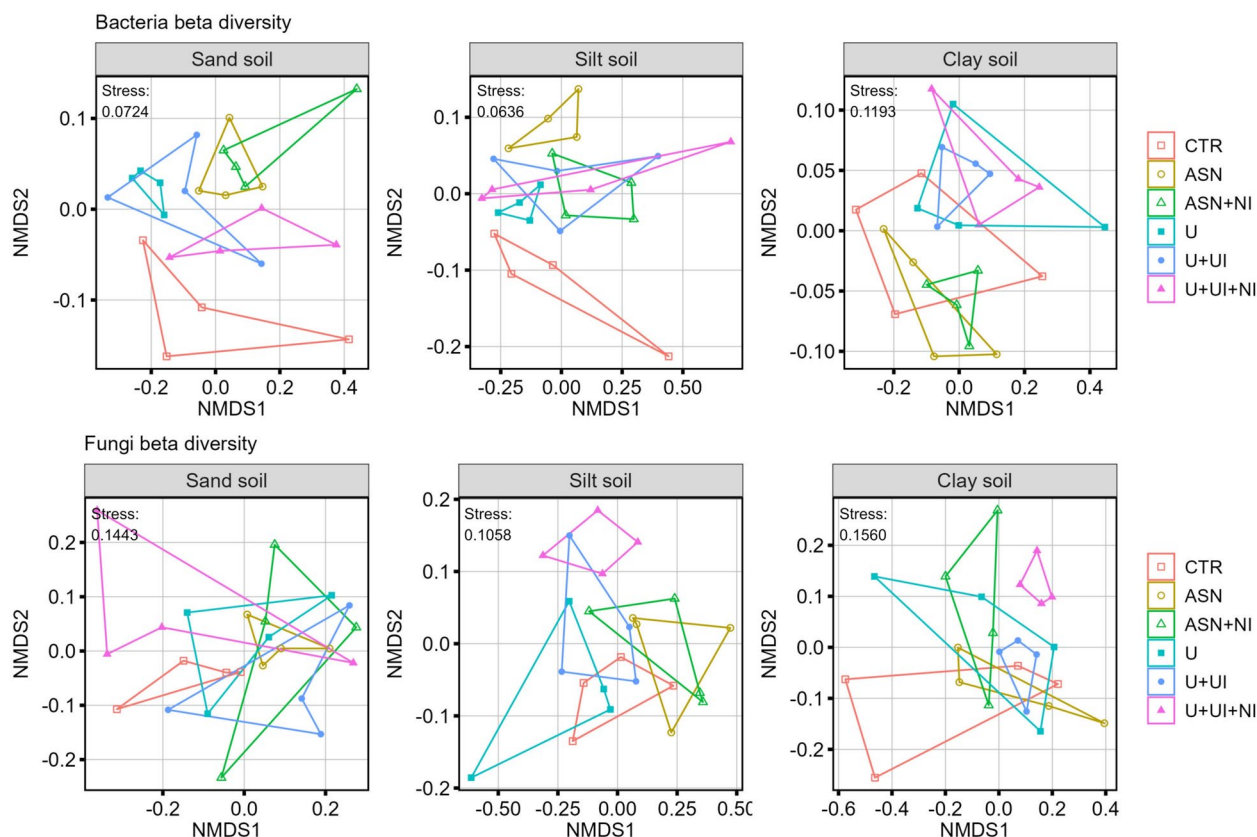


Fig. 5 Bacterial (top) and fungal (bottom) beta diversity in three different soils 14 days after application of six fertilization treatments. Non-metric multidimensional scaling (NMDS) of Bray–Curtis dissimilarities is shown. CTR, non-fertilized control; ASN, ammonium sulfate nitrate; U, urea; NI, nitrification inhibitor; UI, urease inhibitor. $n=4$

The abundance of the nitrifying bacteria *Nitrosospira* (species unknown) was increased with ASN and U compared to CTR in sand and clay soil, but decreased when comparing ASN+NI vs. ASN. Thus, the application of the nitrification inhibitor DMPP affected the abundance of some nitrifying bacteria negatively. The other nitrification inhibitor MPA did not cause significant changes to abundances of nitrifiers from *Nitrosomonadaceae* family. Some ASVs that were negatively affected by the presence of MPA (U+UI+NI vs. U+UI) were taxonomically assigned to genera like *Pseudoduganella*, *Gemmatimonadaceae*, and *Ferruginibacter*, which are known to contain members that are involved in other steps of the N-cycle. However, the missing taxonomic assignment on species level hinders further clarification. Regarding the urease inhibitor 2-NPT, there were only very few ASVs that were negatively affected (U+UI vs. U), clearly less than for the nitrification inhibitors.

The fungi's response to the ASN and U fertilization was more balanced than for the bacteria, including less positive and more negative responding ASVs in all three soils. The largest taxonomic group of significant responders

(across all different treatment comparisons) were ascomycetes from the class of *Sordariomycetes*. More than 60% of the significantly responding ASVs in sand and silt soil belonged to this class. But the response to the treatments within this family was not consistent, as different species were positively or negatively affected by the provision of N or the inhibitors. In clay soil, the groups of significantly responding ASVs were taxonomically more diverse and comprised several ASVs in each of the classes of *Dothideomycetes*, *Leotiomycetes*, *Pezizomycetes*, *Sordariomycetes*, and *Mortierellomycetes*. Overall, as for the bacteria, it was often observed that ASVs that responded positively to the ASN or U treatments responded negatively to the inhibited treatments and vice versa.

Discussion

Fertilization effects in three different soils

The fertilizer applications (ASN and U) increased the mineral N content as expected. In sand soil, urease activity was high but nitrification rate low, which caused high ammonium but low nitrate levels at 4 days after urea-based fertilization. Microbial N

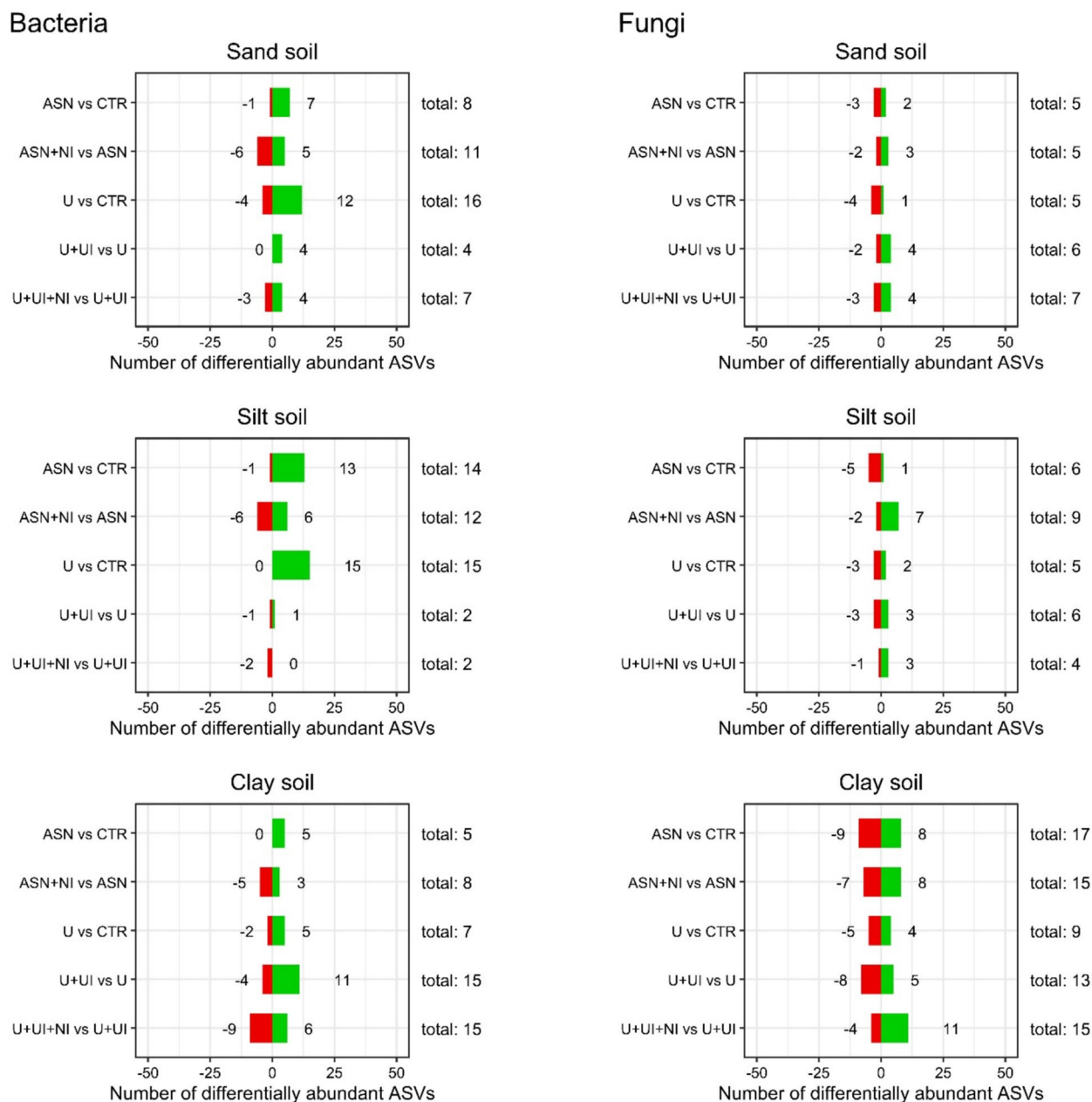


Fig. 6 Differentially abundant ASVs of bacteria (left) and fungi (right) according to DESeq2 analysis in three different soils 14 days after application of six fertilization treatments. Each treatment is compared to its respective reference treatment. ASVs that are significantly more (green, positive x-axis) and less abundant (red, negative x-axis) are listed with their taxonomy in the Supplementary Material S12. CTR, non-fertilized control; ASN, ammonium sulfate nitrate; U, urea; NI, nitrification inhibitor; UI, urease inhibitor

transformations from urea to nitrate was fastest in silt soil, showing the largest amount of gained nitrate (additional nitrate in U in comparison to CTR). In clay soil, nitrate gained from U was lowest despite a solid urease activity level and the highest nitrification rate of the three soils. This was attributed to the high clay content which binds ammonium and hampers nitrification.

Thus, total ammonium levels in clay soil likely were higher than what was extracted, while the extracted amounts rather reflected the concentration that was readily bioavailable for microbial nitrification. Overall, the fertilizer type (urea or ASN) as well as the soil texture and microbial functions markedly influenced the resulting ammonium and nitrate levels.

Our test systems required a realistic fertilizer application rate (100 kg N ha^{-1}) in order to be able to study the effects of a realistic inhibitor dose. However, since fertilizers are intended for plant nutrition and our system did not include plants, we artificially induced an N demand by straw addition. This successfully prevented an N overdose. At the same time, we aimed at not fully depleting mineral N with the straw in the non-fertilized CTR soils. The straw addition successfully caused low concentrations of ammonium and nitrate in non-fertilized CTR treatments in sand and clay soil but lead to a complete depletion of mineral N in silt soil. This might have been due to this soil originating from a wheat field with a more active microbial community, causing a faster degradation of the straw and a stronger N decline compared to the meadow and fallow soils. But the fact that the ammonium and nitrate levels at four days after application of the fertilizer treatments were in the same range in all soils showed that there was an overall accurate balance of nutrient and straw addition achieved for all three soils.

Archaeal *amoA* copies outnumbered bacterial *amoA* copies in all three soils. It is worth noting that AOB *amoA* copies per genome is up to three [69], while AOA have mostly one, sometimes two [70]. Ammonia-oxidizer communities in soils are shaped by availability of ammonium and soil pH [71, 72]. AOAs are more relevant in oligotrophic soils while AOB are more relevant in fertilized soils [73–75]. Because AOA have a 2600-fold higher affinity for ammonium than AOB [71], they outcompete AOB in low ammonium concentrations. However, because AOB have higher growth rates, they dominate in eutrophic conditions [76]. Consequently, AOA:AOB ratios were much higher in the sand soil and clay soil, which were managed as meadow and fallow, respectively, and received no N fertilization in the past five years. In the CTR treatments of the sand and clay soil, archaeal outnumbered bacterial *amoA* copies about 25 times. In the silt soil, which was sampled from a wheat field that received N fertilization every year, AOA still outnumbered AOB but only about 7 times in the CTR treatment, indicating a higher relevance of AOB here. The provision of available ammonium via ASN or even more via U fertilization strongly reduced AOA:AOB ratios because AOB quickly utilized the provided resources and outcompeted AOA, which showed non-significantly reduced copy numbers in silt and clay soil. In sand soil, archaeal *amoA* copies were not reduced in ASN and U treatment, maybe due to the overall lower presence of AOB in this soil type. Thus, interactions of AOA and AOB became visible in the fertilizer treatments and will be further discussed below regarding the effects of the inhibitors.

Effects of ASN or U fertilization on alpha diversity measures of bacteria were non-significant in all three

soils, but ASN caused a significant reduction of fungal Simpson's diversity in sand soil. Fungal richness was also (non-significantly) reduced in sand and silt soil after ASN treatment. Species that quickly utilize the provided nutrients for growth and replication outnumber and replace other species which reduces the richness and diversity. The ecological significance of this depends on the resilience of the community to recover to the original state when nutrient levels decline again. Ecosystem functioning might be affected when species with important functions are lost and the loss is not covered by functional redundancy [77, 78].

Beta diversity of bacteria and fungi was less affected by the fertilizations according to the partly overlapping clusters in NMDS as well as the non-significant pairwise PERMANOVAs. This indicated that the positive and negative responses of some ASVs were minor in comparison to the inherent variability of the communities within the treatments. More statistical power (a higher number of replicate samples) would have allowed to better resolve these slight community shifts. The PERMANOVAs, however, indicated that the treatment explained a greater variability of the communities in silt and sand soil than in clay soil. This might be due to the high clay content of 41%. Clay minerals of type 2:1 can adsorb ammonium [79] and thus lower the nutrient concentrations microorganisms are exposed to. While the exact clay mineral composition of the clay soil is not known, the markedly lower increase of the sum of extractable mineral N in clay soil in comparison to sand and silt soil suggested that ammonium-fixing clay minerals were present. Furthermore, activity levels of community members might have been changed after fertilization but were not resolved by our DNA-based approach. Overall, effects of the fertilizers ASN and U were detected for functional groups of ammonia-oxidizers as targeted by dPCR and were also visible when looking at specific ASVs with the differential abundance analysis. However, at the full community level, effects were weaker.

Target effects of inhibitors on N cycle

The intended inhibitory effects of the UI and NIs on the urease activity and nitrification rate, respectively, were clearly found. This confirmed that the test systems and methods used were suitable for displaying effects of the investigated test substances. However, while the UI and NIs showed clear target effects on enzyme activities, the actual contents of ammonium and nitrate were less affected (significant effects in silt soil only). The enzymatic activities determined in our study were potential activity rates, measured after incubation at elevated temperatures with substrate addition. This allowed to better resolve the inhibitory effect of the inhibitors but does

not necessarily reflect the actual enzymatic activities in the soil microcosms from which the ammonium and nitrate levels were determined. Thus, the actual inhibition effect in the microcosms might have been lower than the inhibition effect detected for the potential enzyme activity, leading to less affected ammonium and nitrate concentrations in soil. An additional explanation could be that the sampling at 4 days after fertilization was not suitable to show the maximum inhibition effect on mineral N contents and that additional sampling times might have been required for a better assessment. Nevertheless, the fact that mineral N levels were significantly affected in silt soil but not in sand and clay soil showed that the efficiency of inhibitors is different in different soils. Thus, our results emphasize that the effects and benefits of the use of inhibitors are soil-dependent.

The two nitrification inhibitors DMPP (in ASN+NI) and MPA (in U+UI+NI) showed different effect sizes on target endpoints. Overall, both reduced the nitrification rate in all soils, but the effect size of DMPP on nitrification, AOB abundance, and AOA:AOB ratio was stronger in all three soils (data shown in Supplementary Material S5). However, the amount of active ingredient applied to the soils with ASN+NI was 0.316 mg DMPP kg⁻¹ soil, while it was only 0.028 mg MPA kg⁻¹ soil in U+UI+NI according to the regulatory minimum inhibitor contents of the fertilizer products [39]. In addition, the amount of urea-N applied in U+UI+NI is higher than the amount of ammonium-N in ASN+NI. Therefore, while differences in the effects of the two NI-containing fertilizers were found, additional data on dose–response relationships would be required for a proper comparison of their efficiency.

Target effects of inhibitors on gene abundances of ammonia oxidizers

Ammonia-oxidizers are not direct targets of urease inhibitors, but the UI could have had an indirect negative effect on AOA and AOB abundances by delaying the provision of ammonium in comparison to the U treatment. However, abundances of AOA and AOB were similar in the U and U+UI treatments in all three soils since their ammonium concentrations were also similar.

The presence of NIs strongly influenced the ammonia oxidizers. The inhibition of the ammonia monooxygenase cuts their energy supply chain, which leads to reduced cell activity and, eventually, reduced cell numbers. While DMPP reduced AOB abundances in this study only non-significantly, significant effects on AOB abundance 14 days after DMPP application have been reported for other soils [80]. However, in the cited study, the DMPP dose of 1.54 mg kg⁻¹ soil was higher than in our study (0.316 mg kg⁻¹). Nevertheless, our data indicated that

DMPP effectively prevented the increase of AOB despite the application of relevant amounts of ammonium and kept the AOA:AOB ratio similar to the CTR. MPA also reduced AOB abundances in all three soils, even significantly in sand soil, but did not maintain the CTR level of AOA:AOB ratio in U+UI+NI treatments, as these were significantly lower in all three soils. A higher dominance of AOB (as indicated by lower AOA:AOB ratio) correlated with a higher potential nitrification rate in silt and clay soil (Supplementary Material S6). This indicated that AOB might be the major contributor to the ammonium-oxidation potential of the soil after fertilizer addition. A similar observation was made in a field study in Utah that found AOB to be more responsive to ammonium fertilization and suggests to focus on inhibiting AOB shortly after fertilization to control nitrification in soil [81]. However, the reductions of AOB abundances by the NIs cannot fully explain the inhibition of nitrification observed in our study. For example, AOB abundance was overall higher in silt soil than in clay soil but the nitrification rate was higher in clay soil. Additional factors such as activity, nitrite-oxidizers, and soil conditions influence the final nitrification rate.

The observation that gene abundances of some ammonia-oxidizers were significantly reduced by NIs was supported by the differential abundance analysis of the DNA sequencing data (Supplementary Material S12). *Nitrospira* was identified as one of the significantly reduced ammonia oxidizers in sand and clay soil upon treatment with DMPP. This genus was also identified by other studies to be affected by DMPP in a silt loam [25] and in a tropical soil [82]. However, different studies report different species that are involved in the nitrification process to be affected by DMPP, including also comammox *Nitrospira* [83] and nitrite-oxidizing *Nitrobacter* [80]. Additionally, it seemed to be soil-dependent which species are inhibited [83]. Apparently, inhibition of nitrifiers is a complex process that is determined by various factors and there are still knowledge gaps regarding the mechanistic understanding.

It remains to be clarified whether AOA were directly inhibited by the NIs. Our data suggested that AOB but not AOA were inhibited by NIs. Because replication times of AOA can be 14 days or longer, an effect on AOA might have become visible only at a later stage. A study with an agricultural soil from Spain found AOB and AOA to have reduced *amoA* copy numbers in a urea+DMPP fertilization treatment compared to urea only after 60 days [84]. However, this pattern was already significant at day 1 after fertilization although less pronounced. Another study that investigated the effect of DMPP on AOA and AOB gene abundances after 14, 21, and 56 days in a loamy rice field soil from Greece found

that DMPP with ammonium sulfate decreased AOB and increased AOA gene abundance compared to the application of ammonium sulfate alone at all three time points [85]. While gene abundance does not necessarily indicate gene expression and cell activity because primers can also bind to relic DNA [86], many studies report that DMPP only inhibits AOB but not AOA [87–91]. This might be due to structural differences in the AMO enzyme [92], substrate affinities [71] and mixotrophic growth of AOA [89, 93]. However, others report AOA inhibition in soils [94–96] or inhibition of AOA in pure cultures at higher DMPP concentrations only [97]. The general consensus is that current NIs do not inhibit all nitrifiers equally well and there is room for improving the efficiency of inhibitor compounds [98].

Non-target effects of inhibitors

Enzyme activities of other nutrient cycles in soil were measured to check for non-target effects of the inhibitors on microbial soil functions. In general, the three different soils had different activity levels of acid phosphatase, beta-glucosidase and arylsulfatase, confirming the different microbial functional properties of the soils. The effects of the N-fertilizers on these enzyme activities were minor and there were no effects of the inhibitors found that were consistent across the three soils (Fig. 2). This result suggests that the inhibitors do not affect other microbially driven nutrient cycles in soil. However, it has been shown that some NIs can also affect the methane monooxygenase [99–101] that is structurally similar to AMO [102, 103]. This might lead to increased methane emissions and could counteract the climate-positive effect of the use of NIs. Within our study, 18 ASVs were assigned to families that are known to include methane oxidizers, such as *Methylobacteriaceae* and *Methylophilaceae*. In silt soil, ASV_364 from family *Methylobacteriaceae* (unknown genus and species) indeed was present in all four samples from CTR and ASN treatment but absent in half of the samples from ASN+NI treatment. While this might hint to a negative effect of DMPP on methane oxidizers, it was not significant according to the DESeq2 analysis. Since the other 17 ASVs of potential methane oxidizers did not show abundance changes or were present in too little samples to tell, our data did not suggest an inhibition effect of DMPP on methanotrophs in the soils investigated here. This is in line with measurements from field experiments [21, 23]. Thus, the investigated inhibitors do not seem to affect other nutrient cycles in agricultural soils. However, the abundance and activity of methanotrophs in fertilized agricultural soils is often lower than in other soils [104, 105]. A targeted analysis of methane monooxygenase genes or their transcription would have been required

to better resolve potential treatment effects on this functional group of microorganisms. In addition, further investigations of other microbial parameters are required to exclude that the use of inhibitors leads to unacceptable side effects on soil functions in the long-term.

Changes of ASV abundances due to the presence of inhibitors often seemed to be caused by the differences in available N rather than a direct (toxic or growth-promoting) effect of the inhibitor itself. Several ASVs seemed to benefit from available nutrients as they were more abundant in fertilized than CTR treatment and less abundant in inhibited fertilizer treatment. Other ASVs, seemingly nutrient-sensitive ones, had reduced abundances in fertilized soil but benefited from inhibitors. This supports observations from another study that concluded that the application of NIs “partially alleviated the negative effect of fertilizer on several oligotrophic taxa” from the kingdom of bacteria [24]. With our study, we showed that this is also true for fungal taxa. Thus, such abundance shifts seemed to be directly linked to the preferences of species for ammonium or nitrate. Ammonium is generally considered the preferred inorganic N source for soil microorganisms because it can be assimilated directly whereas nitrate has to be reduced to ammonium first [106]. However, nitrate is often more abundant than ammonium in soils and there are microbial strains that prefer ammonium while others prefer nitrate [107]. Since the inhibitors affect the dynamics of inorganic N after fertilization in soil, the responses of some community members will depend on their preferences and their ability to utilize the available N sources. This might eventually lead to abundance shifts that become detectable with DNA sequencing. Such abundance shifts of some community members can have further indirect effects and consequences for other community members of the microbial interaction network. Another recent study also assigned microbial community shifts upon NI applications to indirect effects due to changes in pH, nutrient availability and rhizodeposition [25]. In general, our results indicate that effects of NIs as well as UIs on non-target microbial taxa are predominantly indirect effects.

Assessing the alpha and beta diversity of the microbial soil communities, no clear effects of the inhibitors could be found. The overall community composition was not shifted, despite the clear effects of NIs on the gene abundance of the target group of ammonia oxidizers. Apparently, although the inhibitors target certain groups of microorganisms, their shifts are not visible when looking at the full community level. However, the ecological consequences can go far beyond compositional changes. For example, the enzyme activity measurements showed that the inhibitors strongly reduced important soil functions, as intended. Therefore, the absence of shifts in the

composition of the community does not mean that the function of the community is not altered. Such changes can cause indirect effects on the community level, which might take more time to become visible. For example, changes in bacterial co-occurrence networks have been reported from a study that investigated the effects of DMPP in soil over several months and with multiple fertilizer applications [24]. Another study reported strong effects of DMPP on community structure of bacteria and fungi but used a 5-times higher DMPP dose [80]. Investigations on soils treated with DMPP over multiple years are required to exclude chronic effects.

Comments on study limitations

In this study, the assays for potential phosphatase, aryl-sulfatase and beta-glucosidase activities in silt soil were conducted at 29.5 °C instead of 32 °C due to a technical issue. Thus, the reported values probably slightly underestimate the potential activities in silt soil, which is important to note for study comparisons. However, this did not impede the conclusions of this study since our assessment of treatment effects is based on the comparisons of treatments within each soil type.

We focused on investigating short-term effects in many soil-fertilizer-combinations for this study, so the study design lacked additional later sampling time points. These would have been valuable to investigate effect duration, to check for effects on slower-replicating microorganisms, and to check if the community composition shifted more at a later time point. However, the microbial dynamics in a laboratory incubation deviate from the field situation with increasing deviation the longer the incubation lasts due to the unrealistically constant and optimal incubation conditions. In this study, we preferred to check for short-term effects of multiple fertilizers in multiple soils over investigating multiple time points, so the results may be useful as a first screening to identify the need for more detailed research.

This study relied on DNA-based metrics and assessed the functional activity of the soil microbiome only via selected enzyme activity measurements but not at the mRNA level or with an untargeted protein analysis. Thus, our DNA-based approach may not have captured short-term physiological responses. Future studies should include an RNA-based assessment of treatment effects, e.g., via reverse transcription qPCR of *amoA* genes or metatranscriptomics. Assessing active microbial populations could resolve more subtle effects and help to clarify the mechanisms of potential inhibitor effects.

In ecotoxicological studies, treatment dosing is key for the study conclusions. Because we required the full homogeneity of our test systems for reproducible sampling, we dissolved the fertilizer granules for application.

Thus, the nutrients and inhibitors were immediately diluted within the full soil volume. In the field situation, granules are distributed and slowly dissolve only within the moist soil that is in direct contact. This leads to exceptionally high nutrient and inhibitor concentrations on a small local scale at first, which are distributed and diluted over time via diffusion and water infiltration due to rain events. Therefore, it is possible that these initially high concentrations cause stronger effects and also non-target effects on the soil microbiome that is present around the granule than what we observed in our study. Future studies should investigate whether such effects are transient or if they have unacceptable effects on the composition and function of the soil microbiome as a whole.

Conclusions

We conclude that fertilizers which contain the urease inhibitor 2-NPT and the nitrification inhibitors DMPP and MPA show significant target effects but do not cause strong and direct non-target effects on soil microbial composition and functions in the short-term. Instead, indirect effects driven by the inhibitor-affected availability of mineral N were found. This is the first study of potential non-target effects of 2-NPT and MPA. With regards to DMPP, we confirm the conclusion of an older study that DMPP causes no negative effects on soil microbes and soil metabolism at the recommended concentration [29], using modern molecular techniques. Observed treatment effects clearly differed across the three soil types of different texture, pH, management, and initial microbial community. Our study contributes insights into the target and non-target effects of different inhibitor compounds on soil microbial communities. Still, there are knowledge gaps regarding long-term effects under field conditions and ecotoxicological effects towards other soil functions and other organisms. Further studies are required to provide such insights for a comprehensive environmental risk assessment of urease and nitrification inhibitors to protect soil health and improve sustainability of N fertilization.

Abbreviations

2-NPT	N-(2-Nitrophenyl)phosphoric triamide
ANOVA	Analysis of variance
ASN	Ammonium sulfate nitrate
ASV	Amplicon sequencing variant
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
CTR	Control (non-fertilized treatment)
DMPP	3,4-Dimethyl-1H-pyrazole phosphate
dPCR	Digital polymerase chain reaction
DW	Dry weight
FDR	False discovery rate
FW	Fresh weight
ITS	Internal transcribed spacer (within the fungal ribosomal RNA gene cluster)
MPA	N-((3(5)-methyl-1H-pyrazol-1-yl)methyl)acetamide

NMDS	Non-metric multidimensional scaling
PERMANOVA	Permutational multivariate analysis of variance
U	Urea
UI	Urease inhibitor
V4	Variable region 4 (of the prokaryotic 16S ribosomal RNA gene)
WHC	Water holding capacity

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12302-026-01334-7>.

Supplementary Material 1.

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Author contributions

Martin Siedt, Joost T. van Dongen, Damien R. Finn and Christoph C. Tebbe contributed to the study conception and design. Material preparation, data collection and analysis were performed by Martin Siedt, Amaru B. Ponton Paul, Matthias Franck, Inga Brill, Larissa Drews, Alexander Nothbaum, Siraz Kader, Wouter H.G. Hubens and Damien R. Finn. The first draft of the manuscript was written by Martin Siedt and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

Sequence data have been deposited in the NCBI repository with the BIOproject accession number PRJNA1304361. Further data is provided within the supplementary information file.

Declarations

Ethical approval and consent to participate

This is not applicable.

Consent to publication

This is not applicable.

Competing interests

The authors declare no competing interests.

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