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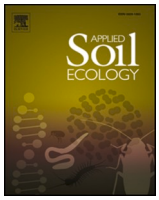

Research paper

Impact of plant protection product applications on soil microbial nitrogen cycle function not fully captured by gene quantification

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ABSTRACT

The widespread use of plant protection products (PPPs) in agriculture raises concerns about their long-term impact on soil health and nitrogen (N) cycling. Current regulatory assessments focus mostly on single active ingredients and microbial mineralisation, ignoring the complexities of formulated PPPs and their influence on microbial functions. We investigated the effects of realistic PPP application scenarios on soil N cycling using a controlled incubation experiment with increasing PPP intensities, measuring potential nitrification (PN), denitrifying enzyme activity (DEA), and N_2O reduction capacity (NRC), alongside molecular analyses of key microbial genes involved in N-cycling. Functional assays were more sensitive to PPP exposure than gene abundances, indicating severe disruptions to N cycling. Among measured processes, PN was the most PPP-sensitive, showing substantial reductions across treatments. DEA and NRC were also strongly inhibited, exhibiting complex temporal patterns. While gene abundances were less responsive, there were significant positive correlations between the gene abundance of archaeal and bacterial ammonia monooxygenase (amoA) and PN, as well as between nitrite reductase (nirK) and DEA. Our findings underscore the importance of updated risk assessments that integrate both molecular and functional indicators. We propose a tiered approach, using gene quantification as an initial screening tool, followed by functional assays to capture biologically relevant changes. Post-registration monitoring of PPP mixtures under field conditions is likewise essential to address cumulative and long-term impacts. Overall, this study highlights the vulnerability of soil N cycling to PPP exposure and provides a framework to enhance environmental risk assessments aimed at safeguarding soil ecosystem functions.

1. Introduction

The rising food demands of the increasing global human population with its current food spectrum are placing continuous pressure on the productivity of agricultural ecosystems. Safeguarding food production by protecting crops from weeds, pests, and pathogens is crucial, as it is estimated that pests and disease cause yield losses ranging from 17 to 30 % (Savary et al., 2019). To improve crop yields and address other agricultural challenges, the use of plant protection products (PPP) has increased substantially over the years. Compared to the 1990s, the applied PPPs have risen by nearly 50 % from 1.2 to 1.8 kg/ha or a total of 2.7 million tonnes of active ingredients during this period (FAO, 2022).

Unfortunately, a significant proportion of the applied PPPs fail to reach their intended targets (Pimentel, 1995) and accumulate in the environment, predominantly in the soil. Once there, PPPs persist longer than anticipated (Riedo et al., 2021, 2023a) and commonly occur as mixtures of multiple active ingredients and their degradation products (Franco et al., 2024; Silva et al., 2019). These residues raise concerns about their potential long-term impact on soil health and the ecosystem services (ES) soil organisms support. As the soil health concept can vary (Lehmann et al., 2020), for the scope of this work, we define soil health as the capacity of soil organisms to perform their ES under natural, undisturbed conditions. These ES include processes such as nutrient cycling and biodiversity which have also been named as specific protection goal options by the European Food Safety Authority (EFSA)

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Panel on PPPs and their Residues (2017).

Given the critical role microorganisms play in supporting these ES, understanding their responses to PPPs is essential. Microorganisms have been widely used to assess PPP-induced stress on soil health due to their high sensitivity to environmental changes (Karas et al., 2018; Karpouzas et al., 2022a; Nielsen et al., 2002; Schloter et al., 2003; Thiele-Bruhn et al., 2020). Their activity reflects the functioning of soil components and processes (Nielsen et al., 2002; Schloter et al., 2003), making them invaluable for assessing PPP impacts on soil ecosystems (EFSA PPR Panel et al., 2017; Karpouzas et al., 2022a).

Despite their critical role in sustaining soil health, risk assessment guidelines evaluating PPP impact on soil microorganisms still rely on the OECD guideline No. 216 (2000), which evaluates microbial nitrogen (N) mineralisation. This approach is highly limited as it focuses on a single endpoint, failing to capture the complexity and broader dynamics of microbial activity that underpin soil health (Karpouzas et al., 2022a). However, the N cycle presents a compelling target for identifying indicators of PPP-impact due to its high sensitivity to PPP-induced stress (Johnsen et al., 2001) and its essential role in agriculture by supporting plant growth and soil fertility while also contributing to ecosystem stability (Koch and Sessitsch, 2024). At the same time, the N cycle is a source of environmental concern, as excessive N inputs in intensive agriculture often lead to substantial losses (Liu et al., 2020), including greenhouse gas emissions such as N_2O (Hénault et al., 2019) and eutrophication of aquatic ecosystems through nitrate leaching (Andersen et al., 2014).

Given its dual role – both its sensitivity to PPPs and its agricultural and environmental significance – the N cycle (Fig. 1) has emerged as a valuable indicator of PPP-induced stress. Research has focused on nitrification and denitrification processes to assess these effects. For nitrification, studies often quantify ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) levels over time, alongside molecular endpoints, such as abundances of genes coding for of microbial enzymes like archaeal and bacterial *ammonia monooxygenase (amoA)* (akter et al., 2022; Cycoń et al., 2013; Riedo et al., 2023b; Rose et al., 2018; Vasileiadis et al., 2018). The *amoA* gene has been consistently recognised as a robust stress indicator e.g. (Karas et al., 2018), demonstrating PPP-sensitivity, also in field settings (Walder et al., 2022). Most studies report a negative impact of PPPs on nitrification and *amoA* gene abundance, although specific results vary by compound.

Research on the influence of PPPs on denitrifying microbial communities and their contributions to N gas emissions often relies on genomics and quantification of key genes, such as *nitrate reductase (nap* and *nar), nitrite reductase (nir), nitric oxide reductase (nor),* and *nitrous oxide reductase (nos*). However, studies that additionally incorporate direct measurements of N_2O and N_2 production, offering comprehensive insights into functional impacts, are less frequently employed (Joly et al., 2015; Kinney et al., 2005; Su et al., 2019, 2020; Yang et al., 2023; Zhang et al., 2018b). Results commonly indicate that PPPs inhibit denitrification while decreased N_2O reduction simultaneously increase emissions of N_2O, a potent greenhouse gas, underscoring the importance of robust assessments.

Moreover, complete ammonia oxidizers (comammox) have emerged as an important third player in nitrification. Bi et al. (2024) provided evidence that comammox communities occur widely in vegetable soils across China and can contribute significantly to N_2O emissions, suggesting that future ecotoxicological studies should also track comammox-specific markers alongside *amoA* to fully capture PPP impacts on the entire ammonia-oxidizing guild.

Until now, inconsistencies in methodology persist, particularly in the definition and measurement of endpoints such as “nitrification rate”. To address this, Karpouzas et al. (2022b) and EFSA (2017) have recommended the use of standardised assays, improving comparability across studies and providing a more reliable evaluation of microbial responses to PPP treatments. Moreover, while molecular markers, such as functional gene abundances, are widely used to assess microbial responses, their presence or abundance does not necessarily indicate active functioning (Lennon et al., 2018), and their connection to microbial activity is rarely examined (Rocca et al., 2015).

Another critical limitation of current research is the predominant focus on active ingredients in isolation, often ignoring the complexities of commercial PPP formulations. These products typically contain multiple active ingredients alongside so-called inert formulation ingredients (Ghosson et al., 2023), which can influence their

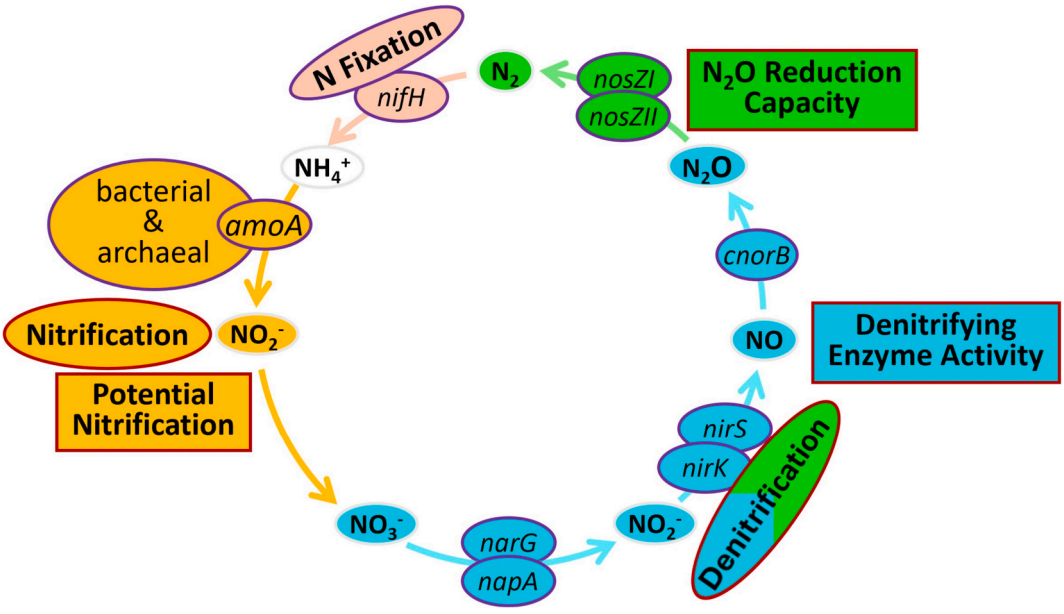


Fig. 1. Overview of the nitrogen (N) cycle, adapted from Cardenas et al. (2018). Nitrification is shown in yellow, denitrification in blue and green, and N_2O reduction green. Functions assessed by gene qualification of involved microbial guilds have a red border, those only assessed by gene qualification a purple one. The employed functional assays are marked with red-bordered rectangles and all qualified genes have a rounded purple borders. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

environmental behaviour, bioavailability, and toxicity (Monkiedje and Spiteller, 2002; Prudnikova et al., 2021). Most studies to date have investigated the effects of individual active ingredients, while far fewer have considered formulated products (Sim et al., 2022), even more rarely in mixtures. One example of such a study was performed by Joly et al. (2012), who found that a mixture of only two herbicides applied at field rate had deleterious effects on soil microbial communities, whereas the individual products produced only minor changes.

Building on these insights, this study aimed to use the PPP-sensitive soil N cycle as an indicator of complex but realistic PPP-induced stress. To achieve this, we conducted a 29-day soil incubation experiment under a range of realistic PPP exposure scenarios, assessing the effects of commercially available products rather than isolated active ingredients to better reflect field conditions. The scenarios we used varied in intensity, from an oats' spray plan comprising only a few PPP compounds to a full-scale potatoes' spray plan involving 18 active ingredients. To evaluate PPP-induced effects, we measured potential nitrification (PN) using ISO 15685 (2012) to assess ammonia oxidation, denitrifying enzyme activity (DEA) using ISO/TS 20131–1 (2018) to capture nitrate reduction, and N_2O reduction capacity (NRC) with ISO/TS 20131–2 (2018) to quantify the conversion of N_2O to N_2.

With this approach, we sought to answer three key research questions: (i) does the soil N cycle respond sensitively to PPP stress, with effects increasing with application intensities? (ii) Do functional assays differ from gene abundance measurements in capturing PPP-induced effects? (iii) Are the observed effects transient or persistent over time? Our findings aim to inform the development of improved risk assessment frameworks to mitigate potential long-term consequences of PPPs on essential soil functions. We hypothesised a dose-dependent inhibition of nitrification and denitrification by PPP mixtures, detectable by both functional assays and the abundances of their key microbial genes, but with the assays proving more sensitive. Moreover, we anticipated some PPP effects to be transient, while some could persist or possibly grow stronger over time.

2. Materials & methods

The experiment involved four treatments: control, low, medium, and high-intensity PPP treatments, two exposure durations (8 and 29 days), and was conducted in two blocks, each block consisting of three technical replicates, resulting in 48 experimental systems (SI Fig. 1A).

2.1. Soil sampling & storage

In June 2022, topsoil (0–20 cm) was collected from a grassland field belonging to an organically managed farm in Zurich, northern Switzerland, adhering to the guidelines of the Federation of Swiss Organic Farmers (Bio Suisse, 2025). This site was selected since it had a Cambisol, a widespread soil type in Switzerland, and a low background contamination with PPPs (Riedo et al., 2021).

The soil was then processed by removing plant material, drying for three days at room temperature, and sieving (2 mm). After these processing disturbances the soil was equilibrated for nine weeks at 4 °C in the dark. This extended cold pre-incubation dissipates the transient respiration and nutrient flush that follows disturbance and restores microbial activity to a steady baseline, as recommended by Meyer et al. (2019) and other long-term cold-storage studies.

2.2. Soil physicochemical properties

The determination of the soil physicochemical properties followed the reference methods of the Swiss Federal Agricultural Research Station (FAL, 1996). Exchangeable Ca^{2+}, Mg^{2+}, K^+ and Na^+ were obtained by saturating the soil with 0.1 M $BaCl_2$ buffered at pH 8.1. Ca^{2+} and Mg^{2+} in the $BaCl_2$ extract were quantified by flame atomic-absorption spectroscopy, and K^+ and Na^+ by flame atomic-emission spectroscopy. Exchangeable H^+ was obtained also using the $BaCl_2$-solution and titrating the displaced protons. Potential cation exchange capacity (CEC_{pot} = T-value), the base-cation sum (S-value) and base saturation (100 × S/T) were calculated from these data. Textural fractions (clay < 2 µm, silt 2–50 µm, sand > 50 µm) were determined with the pipette method. Total C and N were measured by Dumas method with elemental analysis; organic C was assessed by oxidation with dichromate and humus content derived as $C_{org} \times 1.72$. Soil pH was recorded in deionised water (soil: solution = 1: 2.5). The resulting triplicate means are compiled in SI Table 1.

2.3. Experimental set-up and acclimatisation

After equilibration, the soil was partitioned into 48 experimental microcosms in 600 mL glass beakers, 200 g of fresh soil each, and covered with perforated aluminium foil (SI Fig. 1.A). The soil moisture content was established gravimetrically using a Kern DBS moisture analyser, which measures the change in weight while heating a subsample of 5 g of soil to 105 °C. The systems were incubated in a climate chamber at 20 °C with 80 % humidity in the dark for one week to acclimatise and maintained at 50 % water-holding capacity (WHC; range: 46–54 %) by weekly gravimetric measurement and adjustment with a spray bottle throughout the exposure period.

2.4. Treatment application

For each experimental block, conducted two days apart, soil samples were pooled from three replicates and two sampling time points, thoroughly mixed, and evenly spread into stainless steel trays (SI Fig. 1.B). Pooling was necessary to ensure feasibility given the workload constraints while maintaining methodological consistency. This process was repeated independently for each of the four treatment groups:

1. Control: Water only.
2. Low-intensity treatment: PPP application based on a winter oats spray plan (one product containing three active ingredients; Table 1).
3. Medium-intensity treatment: PPP application based on a potatoes' spray plan in which every second fungicide application was omitted, representing a schedule advised for drier-than-average seasons with low late-blight pressure (Table 2).
4. High-intensity treatment: PPP application based on the same potatoes' spray plan with all recommended fungicide applications, reflecting a wet season with high late-blight risk (Table 2).

These treatments were chosen to reflect typical Swiss agricultural practices, where crops like oats generally need minimal crop protection, whereas potatoes are highly disease-prone and growers scale fungicide frequency up or down according to weather-driven infection risk. PPP

Table 1
Oats' PPP application = low-intensity treatment.

product name	action	intensity / amount of product to apply		active ingredient	amount of active ingredient in product	amount of product / system
Constar	herbicide	1.0	L/ha	fluroxypyr-meptyl metsulfuron-methyl thifensulfuron-methyl	0.51 % 3.03 % 13.60 %	0.557 µL

Table 2
Potatoes' PPP applications = medium- and high-intensity treatments.

product name	action	intensity / amount of product to apply				active ingredients	amount of active ingredient in product			amount of product / system	
		medium		high							
Boxer Syngenta	herbicide	2.5	L/ha	2.5	L/ha	prosulfocarb	800	g/L	78.40 %	1.392	µL
Bandur Bayer	herbicide	2.0	L/ha	2.0	L/ha	aclonifen	600	g/L		1.113	µL
Firebird Plus	herbicide	2.0	L/ha	2.0	L/ha	pyraflufen-ethyl	11	g/L		1.113	µL
Spotlight Plus	herbicide	1.0	L/ha	1.0	L/ha	carfentrazone-ethyl	60	g/L	6.53 %	0.557	µL
Sencor	herbicide	0.5	L/ha	0.5	L/ha	metribuzin	600	g/L		0.278	µL
Polyram DF BASF	fungicide	2.5	Kg/ha	8.5	Kg/ha	metiram	700	g/kg	70.00 %	4.732	mg
Cymoxanil WG Schneiter	fungicide	0.3	Kg/ha	0.8	Kg/ha	cymoxanil			45.00 %	0.417	mg
Revus Top	fungicide	1.2	L/ha	1.8	L/ha	mandipropamid	250	g/L	21.90 %	1.002	µL
						difenoconazol	250	g/L	21.90 %		
Infinito	fungicide	1.6	L/ha	3.2	L/ha	propamocarb-hydrochlorid	625	g/L	55.60 %	1.781	µL
						fluopicolide	63	g/L	5.56 %		
Signum BASF	fungicide	0.2	Kg/ha	0.6	Kg/ha	pyraclostrobin (F 500®)	67	g/kg	6.70 %	0.334	mg
						boscalid	267	g/kg	26.70 %		
Ibiza SC	fungicide	0.8	L/ha	0.8	L/ha	fluazinam	500	g/L	38.40 %	0.445	µL
Gazelle SG	insecticide	0.2	Kg/ha	0.2	Kg/ha	acetamiprid			20.00 %	0.111	mg
Audienz	insecticide	0.1	L/ha	0.1	L/ha	spinosad	480	g/L		0.056	µL
Silwet L 77	insecticide	0.2	L/ha	0.2	L/ha	polyalkyleneoxid-modified heptamethyltrisiloxane			85.00 %	0.111	µL
Axcela	molluscicide	11.0	Kg/ha	11.0	Kg/ha	metaldehyd	30	g/kg	3.00 %	6.123	mg

application plans were sourced from the extension service of LANDI Furt- und Limmattal Genossenschaft, Adlikon b. Regensdorf.

PPP products were suspended in water and serially diluted to achieve the desired concentrations the day before application. Concentrations were calculated based on the surface area of the glass beakers, ensuring the field-recommended doses were accurately applied. The solutions were shaken at room temperature overnight and wrapped in aluminium foil to protect them from UV degradation. From the prepared solutions, 9.6 mL was applied per treatment tray, corresponding to 1.4 mL per experimental system (scaled across six systems). The PPP solution was thoroughly worked into the soil by hand for 12 min to ensure even distribution.

Following treatment application, soil moisture content was measured gravimetrically (Kern DBS moisture analyser). Treated soils were redistributed into individual glass beakers for further incubation. Soil moisture content was maintained at 50 % WHC throughout the exposure period by weekly spraying with water.

2.5. Destructive sampling after exposure period

After 8 and 29 days of exposure, destructive sampling was performed. Before sampling, heterogeneity was reduced by thoroughly mixing each experimental system with a spoon. Then, subsamples of each sampled microcosms were taken for the PN assay and DNA extraction. The rest of the soil was stored at 4 °C for 6 days for the two assays assessing denitrification, DEA and NRC.

2.6. Functional assays

2.6.1. Micro-scale potential nitrification rate (PN)

The assay, also called potential NH_4^+ oxidation, was performed according to Annex B of the ISO 15685 (2012) guideline. This test reflects the current microbial capacity to convert NH_4^+ into NO_2^-. In brief, 2.5 g of fresh soil was weighed into a Falkon tube and 10 mL of the test medium was added, providing diammonium sulphate as an NH_4^+ source and sodium chlorate to inhibit the bacterial oxidation of NO_2^- to NO_3^-. The soil slurries were then incubated on a shaker for 2 and 6 h. The resulting extracted nitrite (NO_2^-) solution was stored at −20 °C until analysis. The NO_2^- content of the samples was determined by measuring the absorbance at 540 nm using a Synergy™ H1 multi-mode microplate reader and the nitrification rate over time was calculated according to the guidelines.

2.6.2. Ammonium (NH_4^+) & nitrate (NO_3^-)

According to section "7.5 Extraction of soils" of the ISO 14238 (2012) guideline, NH_4^+ and NO_3^- were extracted only after 29 days of exposure. For this, 15 g of fresh soil was weighed into a 250 mL Erlenmeyer flask, and 5 mL of 1 M KCl solution per 1 g dry mass equivalent of soil was added. The soil slurries were then incubated at RT and 150 rpm/min for 1 h. Subsequently, 30 mL of the samples were taken, centrifuged, and filtered to remove soil particles. The extracts were stored at −20 °C until analysis. The samples' extracted NH_4^+ and NO_3^- contents were determined by measuring the absorbance using the Hach Lange LCK304 and LCK339 cuvette tests, respectively, and the Hach Lange Dr. 6000 for the readings.

2.6.3. Denitrifying enzyme activity (DEA)

To determine DEA (ISO/TS 20131–1, 2018), 20 g of soil was added to a 100 mL serum bottle and S1 medium added as NO_3^- source, glucose as a carbon source, and chloramphenicol to block denitrifying enzyme de novo synthesis. The serum bottles were then closed, and an anaerobic atmosphere was generated by flushing with N_2. Next, the samples were treated with acetylene to inhibit N_2O to N_2 reduction and capture all N_2O produced. The samples were incubated on a shaker for 2 h and samples from the gas atmosphere were taken after 0 h, 1 h, and 2 h. These gas samples were stored in evacuated 12 mL glass vials with

double wadded caps until analysis. The quantification of produced N_2O is expressed in μg N_2O-N·g^{-1} soil and was calculated according to the guideline.

2.6.4. Soil N_2O reduction capacity (NRC)

To determine NRC (ISO/TS 20131–2, 2018), from each microcosm, two 100 mL serum bottles received 10 g of fresh soil. Then, to each serum bottle 10 mL S2 medium was added, providing a nitrate source. The serum bottles were then closed, and an anaerobic atmosphere was generated by flushing with N_2. Next, half of the samples were treated with acetylene to inhibit N_2O to N_2 reduction. The soil slurries were then incubated on a shaker at 20 °C for 7 days. A sample of the gas atmosphere was taken after 24 h, 96 h, 168 h. These gas samples were stored in evacuated 12 mL glass vials with double-wadded caps until analysis.

Again, the quantification of produced N_2O is expressed in μg N_2O-N·g^{-1} soil and was calculated according to the guideline and subsequently used the area under the curve of N_2O levels over the 7-day incubation period. NRC r_{max} and NRC index were also calculated. NRC r_{max} is the maximum ratio of the accumulated N_2O (N_2O produced during incubation without acetylene) to the denitrified NO_3^- (N_2O produced during incubation with acetylene). NRC index is r_{max} multiplied by incubation time t over which N_2O accumulates in the bottle during the incubation without acetylene, i.e. the production rate is higher to the consumption one. If the index is higher than 50, the soil poorly reduces N_2O, and emissions of N_2O in this situation are especially suspected to be very large.

2.6.5. N_2O gas analysis

N_2O concentrations in headspace samples were determined using an Agilent 7890 N gas chromatograph (Agilent, Wilmington, Delaware) equipped with a 12 ft. Porapak Q 100/120 column run isothermally at 80 °C using 90 % Ar / 10 % CH_4 as carrier gas. N_2O was detected on an electron capture detector operated at 330 °C, without additional makeup-gas.

2.7. DNA extraction

For DNA extraction, soil samples were shock-frozen in liquid N_2, lyophilised for 24 h, and stored at −80 °C until further processing. DNA was extracted from 100 mg of dry soil using the NucleoSpin 96 Soil kit (MACHEREY-NAGEL, Germany), following the manufacturer's protocol with the inclusion of an additional step to enhance extraction efficacy by repeating the extraction with Enhancer SX buffer. DNA concentrations were quantified using the PicoGreen method with an Agilent Varian Cary Eclipse Fluorescence Spectrometer Microplate Reader and subsequently diluted to 2 μg/μL.

2.8. Gene quantification

To quantify different microbial guilds in the soil samples, the gene copy numbers of specific marker genes were analysed. For prokaryotes, the ribosomal marker *16S* was used, while the internal transcribed spacer (*ITS*) was employed to quantify fungi. Quantification of the genes associated with archaeal and bacterial ammonia monooxygenase (*amoA*), nitrate reductase (*napA* and *narG*), nitrite reductase (*nirK* and *nirS*), nitric oxide reductase (*cnorB*), nitrous oxide reductase (*nosZI* and *nosZII*), and nitrogenase reductase (*nifH*) were employed to quantify the microbial guilds engaged in the different steps of soil N cycling (Fig. 1). All primer sequences are listed in SI Table 2. Absolute quantification of the marker genes was performed using quantitative polymerase chain reaction (qPCR), using the CFX Opus 384 Real-Time PCR System. Each 10 μL reaction mixture contained 2 ng of DNA template, 2 μL 5xHOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia), 500 nM of each primer, and the remaining volume nuclease-free water. For *nosZII*, we used a different qPCR mix, namely Luminaris HiGreen qPCR Master Mix, Low ROX (Fisher Scientific). Each reaction for *nosZII* contained 2 ng of DNA template, 5 μL 1× Luminaris HiGreen qPCR Master Mix, 1 μM of each primer, and the remaining volume of nuclease-free water. The thermal cycling parameters for individual genes can be found in SI Table 3. Gene abundance was quantified using a standard curve created by linear regression of the standards. This curve was used to correct and determine gene abundance in the samples. Both samples and standards were run in triplicates to minimise experimental error and improve precision. The linear regression provided a mathematical relationship between the known quantities and their corresponding measurements, which was applied to the unknown samples. For further analysis, the mean of the sample triplicates was used.

2.9. Statistical analysis

All statistical analyses were performed in R version 4.3.2 (R Core Team, 2023). A linear mixed-effects model (LME) was employed to investigate the influence of treatment and exposure duration on the different endpoints with block as a random effect (Formula 1), using the lme4 package (Bates et al., 2015):

$$y = endpoint \sim treatment \times exposure\ time + (1|block) \quad (1)$$

The anova function was used to perform an analysis of variance (ANOVA) on the fitted model. All ANOVA tables are provided in SI Tables 5 and 6. The normality assumption was assessed using the Shapiro-Wilk test and visualised with a Q-Q plot using the ggpubr package (version 0.6.0) (Kassambara, 2023). The normality assumption was met for all models except the PN model. Although the normality assumption was violated for some of the lower residuals (Shapiro-Wilk $p = 0.002$), it was acceptable to use the linear mixed-effects model because these models are generally robust to such violations, often providing unbiased and accurate estimates even when distributional assumptions are not fully met (Schielzeth et al., 2020). In the DEA data, two outliers were found (Fig. 2.B, in red). One of them was removed from the data set (high-intensity treatment, 29 days of exposure) because it was the result of gas sample leaking out of its glass vial. The other outlier (medium-intensity treatment, 29 days of exposure) was winsorized with a threshold of 0.04 using the datawizard package, as extreme values were not our focus, and this allowed for a normal distribution (Patil et al., 2022).

Subsequently, the emmeans package (version 1.10.0) was used to perform pairwise comparisons of PPP treatment and exposure time means (Lenth et al., 2024). The emmeans package was also used to obtain the estimated marginal means for the treatment and exposure time groups from the linear mixed models. Those were in turn used to calculate the Cohen's *d* for each PPP treatment group compared to the control for each exposure time point (Formula 2).

$$d = \frac{x_1 - x_2}{\sqrt{{s_1}^2 + {s_2}^2/2}} \quad (2)$$

where

x_1 mean of group 1
x_2 mean of group 2
${s_1}^2$ variance group 1
${s_2}^2$ variance group 2

To get insights in how gene abundances are linked to the corresponding functional assay outputs, we performed Spearman rank-based correlation analyses between gene abundances and their corresponding functional assays. Specifically, archaeal and bacterial *amoA* (*AOA* and *AOB*) were analysed for their relationship with PN, while *narG, napA, nirK, nirS*, and *cnorB* were examined for their connection to DEA. For NRC, we evaluated *nosZI* and *nosZII*. We also examined a potential negative relationship between NO_3^- levels after 29 days of exposure and the gene abundances of *narG* and *napA*, as these genes are involved in NO_3^- reduction.

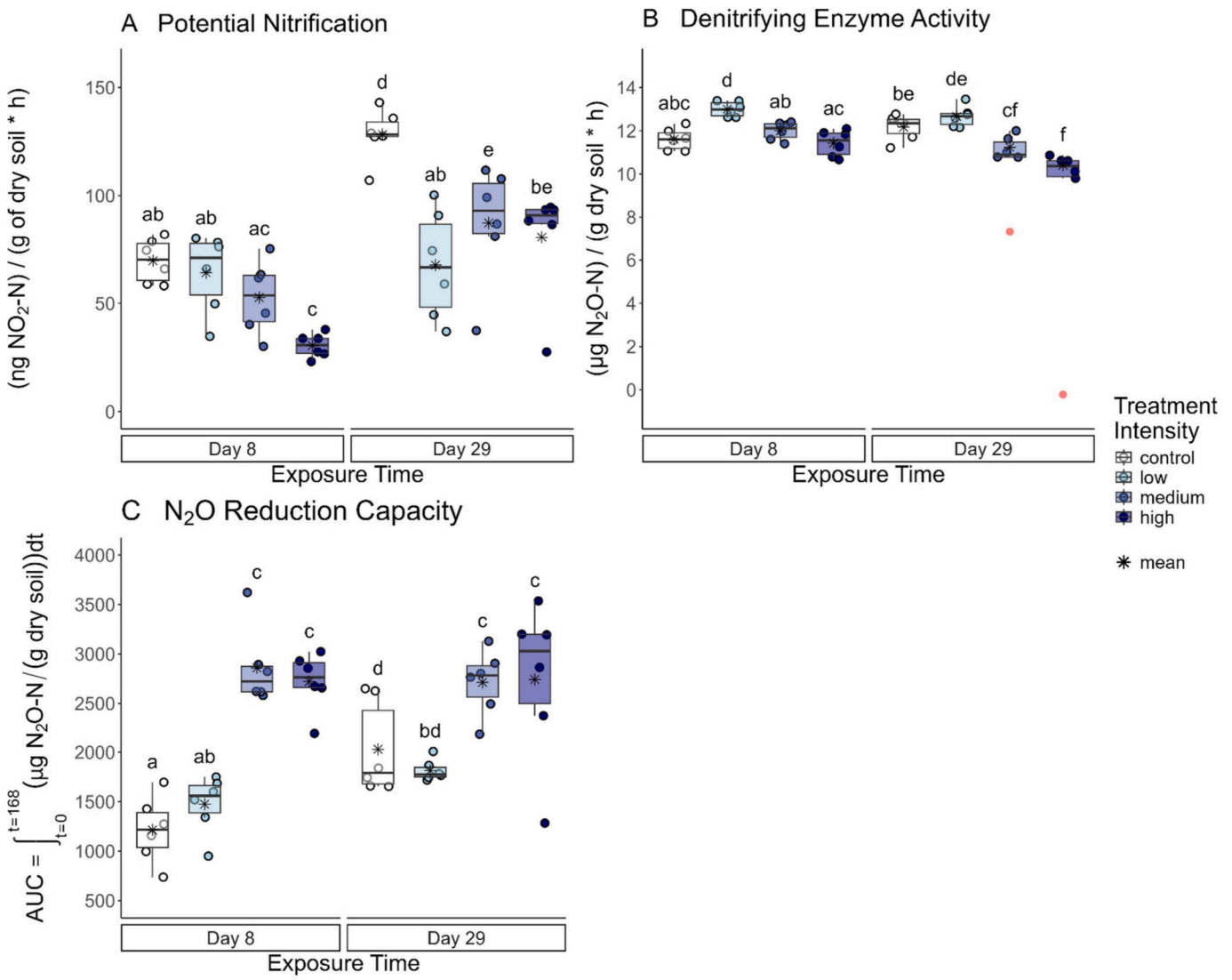


Fig. 2. Functional assays after 8 and 29 days of exposure. A) potential nitrification. B) denitrifying enzyme activity (DEA). Highlighted in red are outliers (see method section). C) N_2O reduction capacity (NRC), displays the level of N_2O in the system over incubation time (hours). As a function of treatment intensity and exposure duration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Functional assays sensitive to PPP treatments

The functional assays revealed significant and mostly intensity-dependent effects of PPP treatments on soil N cycling processes, with impacts becoming more pronounced over the exposure period in most cases.

To assess **nitrification**, we performed the standardised potential nitrification (PN) assay (Fig. 2.A), which revealed a significant reduction in PN following PPP application compared to the control. Both PPP treatment and exposure time, as well as their interaction, had significant effects on PN (SI Table 5). After 8 days of exposure, the high-intensity PPP treatment showed the most pronounced negative effect on PN (SI Table 7). By day 29, PN was strongly reduced in all treatment intensities (Table 3). Notably, while the high-intensity treatment already significantly reduced PN after 8 days, the low-intensity treatment exhibited the largest effect after 29 days, reflecting a delayed but substantial reduction in PN.

To assess **denitrification**, we used denitrifying enzyme activity (DEA) and N_2O reduction capacity (NRC). The DEA assay (Fig. 2.B) demonstrated significant effects of PPP treatment, exposure time, and their interaction (SI Table 6).

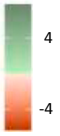
After 8 days of exposure, the low-intensity treatment showed a substantial increase in DEA compared to the control, whereas the other treatments showed no clear effects (SI Table 7). By 29 days, the low-intensity treatment's DEA had converged with that of the control, while both the medium- and high-intensity treatments exhibited markedly lower DEA compared to the control (Table 3).

In the NRC assay (Fig. 2.C) a higher amount of remaining N_2O reflects a decreased NRC. The analysis revealed significant main effects of treatment, exposure time, and their interaction on N_2O reduction capacity (SI Table 6).

After 8 days of exposure, both medium and high PPP treatment intenities vastly reduced NRCs (SI Table 7). Notably, this was the only parameter where the negative impact of the treatment was overall less severe after 29 days compared to 8 days, but both the medium and high treatments were still resulted in substacially lower NRC (Table 3) than the control. In the high treatment intensity group, the curve after 29 days indicated that the peak of denitrification had not fully passed, with ongoing N_2O production exceeding its reduction even after 168 h (= 7 d) of incubation (SI Fig. 2.A). Although the NRC r_{max} and NRC index were calculated as suggested by the guidelines to provide additional insights (SI Fig. 2.B and C), these metrics did not effectively depict the treatment

Table 3
Effect size (Cohen's d) after 29 days of exposure by treatment intensity. Stars and the dot indicate the significance levels of the overall treatment effect across both time points. Significance levels are categorised as follows: ***$p < 0.001$, $p < 0.1$. Values with $p < 0.05$ are shown in bold. PN = potential nitrification, DEA = denitrifying enzyme activity, NRC = N_2O reduction. Both the increase in NO_3^- and NRC indicate higher remaining NO_3^- and N_2O, reflecting reduced denitrification.

Effect size relative to control day 29			low	medium	high
Core Parameters		NH_4^+	-2.24	-2.48	-2.48
		NO_3^- ***	2.22	4.94	7.80
		16S	-0.25	-1.08	-0.06
		ITS	-2.44	-1.02	-1.28
Nitrification	functional	**PN** ***	-6.32	-4.27	-4.96
	potential	*archaeal amoA*	0.54	-1.47	1.12
		bacterial amoA	0.46	-0.13	0.23
Denitrification	functional	**DEA****	1.01	-3.19	-3.36
		NRC**	-1.04	3.33	3.47
	potential	*narG*	-0.77	-1.85	-0.86
		napA	-0.89	-1.52	-0.82
		nirK .	0.21	-2.37	-2.72
		nirS	0.22	-0.64	0.07
		cnorB	0.10	-1.57	-0.66
		nosZI	-1.10	-2.57	-2.56
		nosZII	-0.54	-1.50	-0.96
N Fixation	potential	*nifH*	-0.16	-1.42	-0.38



effects due to the limited sampling points and material constraints, which restricted the accurate determination of true r_{max} and peak timing; therefore, the area under the N_2O levels curve is presented as the primary endpoint.

We also measured NH_4^+ levels after 29 days of exposure (SI Fig. 3.A). Although the effects of PPP treatments on NH_4^+ levels were not statistically significant (SI Table 5), the negative impact of all treatment intensities are noteworthy (Table 3), indicating a decrease trend in NH_4^+ levels compared to the control.

Another way we observed the effects of PPP treatments on N cycling is by examining NO_3^- levels after 29 days of exposure (SI Fig. 3.B). The ANOVA revealed a significant treatment effect on NO_3^- levels (SI Table 5). Specifically, medium and high PPP treatment intensities resulted in substantially higher NO_3^- levels compared to the control (Table 3). These elevated NO_3^- levels suggest that NO_3^- is not being converted to other N forms, indicating a possible disruption in N transformation processes.

Overall, our results for the functional endpoints demonstrate that realistic PPP application treatments can significantly and adversely affect soil N cycling processes in a mostly intensity-dependent manner. This is particularly evident for nitrification and denitrification changes, which may have profound implications for soil health and ecosystem functioning.

3.2. Associations of functional assays with gene abundances

The Spearman rank-based correlation analysis between the functional assay outputs and their corresponding gene abundances revealed significant positive correlations for some pairs, while no significant correlations were observed for others. For **nitrification**, we found a positive Spearman rank-based correlation between PN and archaeal *amoA* (Fig. 3.A) as well as between PN and *AOB* (Fig. 3.B). For **denitrification**, DEA showed a significant positive correlation with *nirK* (Fig. 3.C). However, none of the other gene abundances were significantly associated with DEA (SI Fig. 4.A-D), and neither *nosZI* nor *nosZII* gene abundances were significantly correlated with NRC (SI Fig. 4.G-H). No significant Spearman rank-based correlation was found between either *narG* or *napA* and NO_3^- levels (SI Fig. 4.*E*-F), which could serve as a substrate for the enzyme transcribed form these genes, after 29 days of exposure.

3.3. Gene abundances less sensitive to PPP treatments

While some microbial guilds were positively correlated with their corresponding functional assays, most gene abundances were not significantly affected by PPP treatments compared to the control. The only partial exception were the gene copy numbers of *nirK*, which exhibited a borderline significant treatment effect ($p = 0.053$; SI Fig. 6; SI Table 6). Specifically, *nirK* abundance differed marginally between the low-intensity treatment and the medium- and high-intensity treatments. The analysis also revealed a significant exposure-time effect (SI Fig. 5; SI Table 6) but no interaction with treatment intensity, indicating consistent responses across both time points.

Beyond *nirK*, none of the other investigated genes exhibited statistically significant treatment effects (SI Tables 5 & 6). However, while not significant, effect sizes revealed notable trends reflective of an increased PPP-based inhibition of PN & DEA over exposure time. All gene abundances – *16S, ITS, AOA, AOB, narG, napA, nirK, nirS, nosZI, nosZII,* and *nifH* – showed treatment effect sizes that were more strongly negative after 29 days of exposure (Table 3) compared to the less negative or even positive effects observed after 8 days of exposure (SI Table 7). Further, analysis of exposure time effects revealed that *nirK* was also the only gene to exhibit a significant decrease in abundance over the 29-day period while most other genes (*16S, AOA, AOB, narG, nirS, nosZII,* and *nifH*) showed significant increases over time (SI Fig. 5; SI Tables 5 & 6).

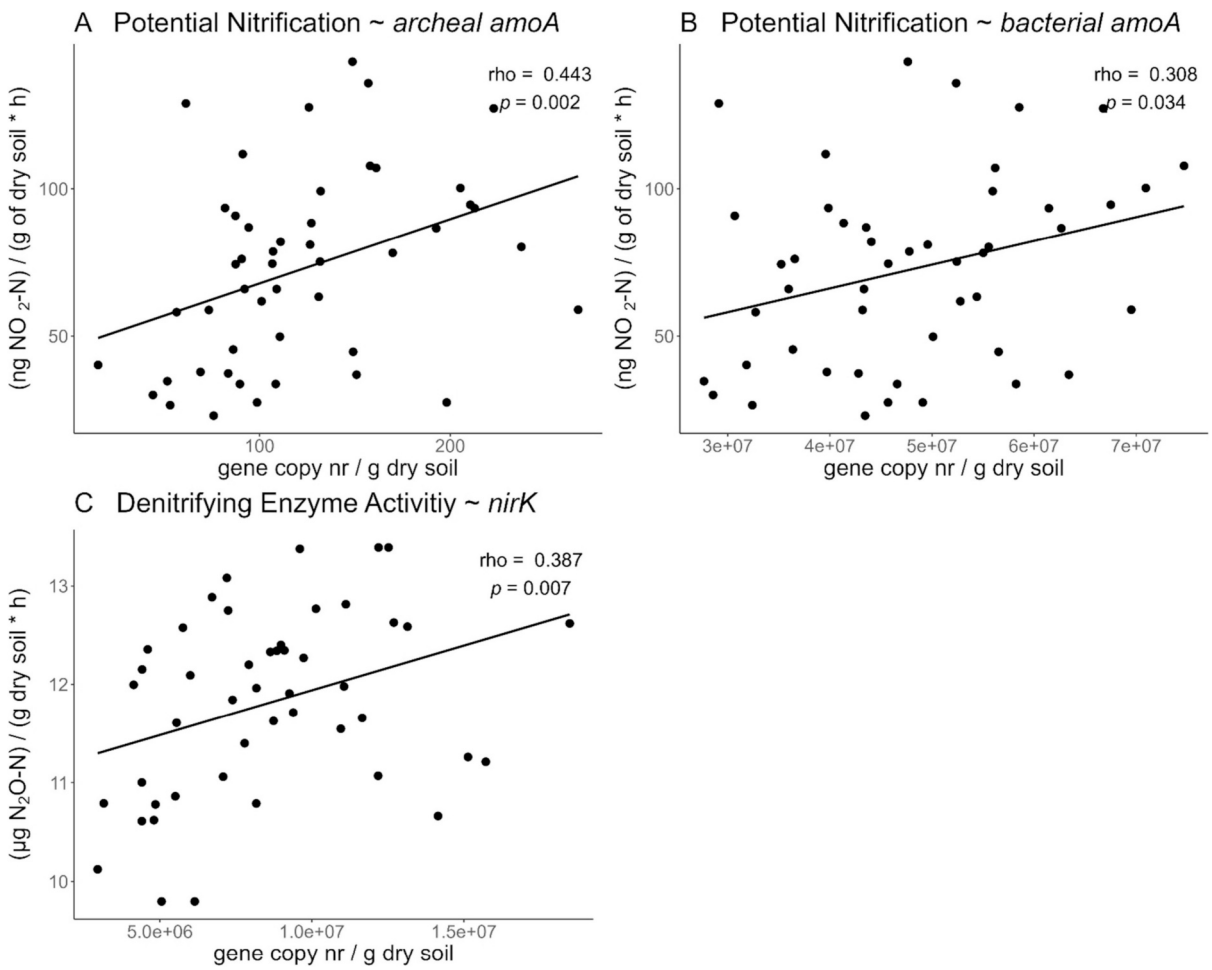


Fig. 3. Spearman correlation between functional assays and abundance of genes involved in these functions with corresponding rho and *p* values in the top right corners. A) Correlation between potential nitrification and archaeal *amoA* gene abundance. B) Correlation between PN and bacterial *amoA* gene abundance. C) Correlation between denitrifying enzymes activity and *nirK* gene abundance.

3.4. What are the most sensitive indicators?

When examining effect sizes across endpoints, functional assays demonstrated stronger and more consistent responses to PPP treatments compared to gene abundances. Among the functional assays, PN was the most sensitive, showing substantial and consistent reductions across all treatment intensities, with pronounced effects by 29 days of exposure (Table 3). DEA also displayed significant treatment effects. Though these varied with time, by 29 days of exposure, both medium- and high-intensity treatments resulted in markedly reduced DEA compared to the control (Table 3). In contrast, NRC exhibited a unique temporal response, with the strongest reductions occurring after 8 days under medium and high intensities (SI Table 7), followed less pronounced but still large effects after 29 days (Table 3). This temporal pattern indicates NRC may reflect short-term impacts, while PN and DEA better capture sustained disruptions to N cycling processes.

For gene abundances, *nirK* was the most responsive marker, exhibiting marginally significant treatment and exposure time effects, with a decline in abundance as treatment intensity and exposure time increased (SI Table 7; Table 3). Other genes showed weaker or inconsistent trends across time points and treatment intensities (SI Table 7; Table 3). These findings reinforce the greater sensitivity of functional assays compared to gene abundances, particularly for capturing intensity-dependent and long-term impacts.

4. Discussion

Reliable indicators are essential to monitor and understand the environmental impact of PPPs. In this study, we targeted the PPP-sensitive N cycle employing functional assays paired with the quantification of gene abundance from relevant microbial guilds to assess the effects of various realistic PPP application scenarios with increasing intensity providing a direct test of our predictions: (i) that PPP mixtures would inhibit nitrification and denitrification in a dose-dependent manner, (ii) more clearly in functional than molecular read-outs, and with (iii) some PPP effects transient and some persistent in nature. Our findings confirm that the soil N cycle is highly sensitive to PPP-induced stress, with both nitrification and denitrification demonstrating significant and intensity-dependent inhibition, particularly at higher real-world treatment levels and prolonged exposure. Functional assays and gene abundances reflected this with varying degrees of impact.

4.1. Impact on nitrification

Potential nitrification (PN) emerged as the most sensitive indicator, demonstrating substantial reductions across all treatment intensities, with effects becoming more pronounced over the 29-day exposure period. This aligns with prior studies that identified PN as highly susceptible to chemical stressors, including PPPs e.g. (Joly et al., 2015; Papadopoulou et al., 2016; Sim et al., 2022; Vasileiadis et al., 2018).

The delayed but substantial inhibition of PN after 29 days exposure

to the low-intensity treatment may result from the toxic effects of transformation products (TPs) rather than the parent PPPs, which degrade with half-lives ranging from 2 to 14 days (Hu et al., 2011; Maznah et al., 2020; Rotam, 2009; Stähler, 2024). This aligns with findings by Vasileiadis et al. (2018), who showed that TPs can exhibit greater toxicity to ammonia-oxidizing microorganisms than their parent compounds, potentially explaining the observed effects. It should be noted, however, that reported PPP half-lives are not always consistent with field observations, where residues have been found to persist far longer than previously assumed (Riedo et al., 2023a; Riedo et al., 2021). PN's sensitivity to even low-intensity PPP treatments underscores its utility as a robust endpoint for detecting subtle disruptions in soil microbial activity.

The strong negative effects as demonstrated by PN were accompanied by positive correlations with the gene abundance of *AOA* and *AOB*. Contrary to our expectations, quantification of these genes failed to fully capture the intensity- and time-dependent impacts indicated by PN, since neither showed significant treatment effects, even though *amoA* has previously been reported as sensitive to PPPs (Karas et al., 2018; Riedo et al., 2023b; Zhang et al., 2018a). For instance, Zhang et al. (2018a) reported significant reductions in *AOA, AOB*, and associated NO_2^-/NO_3^- levels following weekly applications of the fungicide iprodione (1.5 mg/kg) over 28 days. The stronger effects observed in their study were likely influenced by the repeated application design, unlike the single application approach in Riedo et al. (2023b) who found AOA and AOB highly sensitive to a single dose of three fungicides across different soils, with effects detectable at the recommended field application dose levels. However, Riedo, Yokota, et al. (2023b) also reported that sensitivity was strongly dependent on soil type and interactions with exposure duration, which could partly explain discrepancies between their results and ours.

The observed disconnect between functional and molecular indicators has also been reported in other studies, e.g. Papadopoulou et al. (2016) and Vasileiadis et al. (2018). Functional activity can decline in response to PPP treatment without corresponding reductions in gene abundance (Prosser and Nicol, 2008), likely reflecting shifts in enzymatic or transcriptional regulation, including post-transcriptional modifications. Although slow growth rates of ammonia-oxidizing microorganisms could potentially obscure PPP-related effects, we observed significant increases in AOA and AOB gene abundances between 8 and 29 days of exposure – suggesting that a measurable response could be possible despite slower turnover. Nevertheless, relic DNA may confound these observations (Lennon et al., 2018), and longer exposure times could be necessary to detect such effects.

To contextualise these findings within broader agricultural and environmental implications, it is essential to consider how the observed decrease in nitrification rates might translate to field-scale consequences. On one hand, reduced nitrification may decrease NO_3^- leaching, leading to better N retention in the soil and gradual release for plant uptake (Beeckman et al., 2024; Wang et al., 2024). On the other hand, increased NH_4^+ retention while enhancing N availability can result in N loss through volatilisation as ammonia gas. Insufficient nitrification may also impair plant nutrient uptake, leading to reduced crop yields and nutrient imbalance.

4.2. Impact on denitrification

Denitrifying enzyme activity (DEA) also showed significant treatment effects, but the temporal dynamics were more complex. While low-intensity treatments initially stimulated DEA, higher intensity and prolonged exposure led to marked reductions. The increased inhibition after 29 days cannot be explained by a lack of NO_3^--substrate availability due to the intensity-dependent NO_3^- accumulation at that time point. This pattern therefore suggests a potential compensatory, hormetic microbial response, where low-dose exposure stimulates microbial activity before inhibition occurs at higher concentrations (Stebbing, 1982). Hormetic responses in microbiota to toxicants have been shown to depend on both concentration and exposure duration. For example, Sun et al. (2020) demonstrated hormetic effects to antimicrobial mixtures on soil microbial activity over a 24-h period. Hormetic responses have also been observed over longer time frames, e.g., in arbuscular mycorrhizal fungi under stress from the fungicides mancozeb and carbendazim (Jakobsen et al., 2021). However, time-dependent hormetic effects in soil microbial communities in response to PPPs remain little researched and to confirm whether the stimulatory effect of the low-intensity PPP application aligns with hormesis, further detailed dose- and exposure-time-response experiment would be necessary. Here, the hormetic response is then overridden by stress-induced suppression at higher intensities and longer durations.

Gene abundances generally exhibited limited sensitivity to PPP treatments compared to the functional assay DEA. The exception being *nirK* gene abundance showing a slight increase under low PPP intensity, followed by a decline under medium and high-intensity treatments. This pattern, consistent with its significant correlation to DEA, suggesting *nirK*'s potential as a molecular indicator of denitrification. Nonetheless, the significant treatment effect may not be visually apparent or large enough to reveal consistent trends, particularly when results are averaged or compared across treatment intensities. These findings partly align with those of Su et al. (2020), who reported that the PPP chlorothalonil caused a more pronounced decrease in nitrate reductase enzyme activity than the changes observed in gene abundance, identifying enzyme activity as a crucial factor in determining denitrification rates. However, they also detected a significant downregulation of certain denitrifying genes, while our study observed only weaker effects.

N_2O reduction capacity (NRC) demonstrated strong reductions under medium and high-intensity treatments, raising concerns about increased N_2O emissions from the soil, thereby contributing to greenhouse gas accumulation. The diminishing effects after prolonged exposure in our study may reflect PPP degradation or microbial adaptation. Overall, while reductions in DEA with higher PPP intensities might initially seem promising, given N_2O's global warming potential of approximately 300 times that of CO_2 (Griffis et al., 2017). However, the pronounced reduction in NRC under PPP influence leading to increases in N_2O accumulation could ultimately exacerbate greenhouse gas emissions. This aligns with findings by Su et al. (2020) and Yang et al. (2023), who reported that PPPs like chlorothalonil inhibited denitrification while significantly promoting bacterial N_2O emissions due to shifts in microbial pathways. Notably, Su et al. (2020) demonstrated that chlorothalonil significantly inhibited N_2O reductase activity without affecting *nosZ* abundance, further elucidating the mechanism behind increased N_2O emissions in the presence of certain PPPs.

A noteworthy observation was the significant, intensity-dependent accumulation of NO_3^- following prolonged exposure to medium- and high-intensity PPP applications compared to the control. Several factors may contribute to this observation. The accumulation suggests that PPPs may also serve as N sources, undergoing ammonification followed by nitrification to NO_3^-, a phenomenon documented in previous research (Joly et al., 2015). Additionally, differential oxidation rates may play a role, with NH_4^+ oxidation to NO_2^- being less efficient (as indicated by the PN assay), while NO_2^- oxidation to NO_3^- may proceed more effectively (Papadopoulou et al., 2020). Interestingly, despite the increased NO_3^- availability, which typically promotes the growth of denitrifying microbes (Joly et al., 2015) our findings indicate the opposite effect of higher treatment intensities. This further supports a disruption in the denitrification process, wherein NO_3^- is not efficiently converted into other N forms. This finding is consistent with previous research by Su et al. (2019), which reported decreased denitrification accompanied by increased NO_3^- accumulation and elevated N_2O emissions after 72 h of chlorothalonil exposure. These observations collectively suggest a complex interaction between PPP exposure and N cycling processes in the studied environment, highlighting the necessity for further investigation to fully elucidate the underlying mechanisms and potential

ecological implications. The elevated NO_3^- levels associated with higher PPP application intensities pose significant environmental risks. While NO_3^- is a critical N source for plant growth and soil fertility, excessive accumulation can result in leaching, groundwater contamination, and eutrophication of aquatic ecosystems, leading to severe ecological consequences (Mahmud et al., 2021).

4.3. Consolidation of findings and path forward

Our findings highlight the complex interactions between PPP exposure and key N cycling processes within soil ecosystems. Functional assays such as PN, DEA, and NRC revealed intensity- and time-dependent effects of PPPs, while gene abundances like *amoA* and *nirK* provided limited yet complementary insights into microbial responses. The significant disruptions observed in nitrification and denitrification pathways, along with potential increases in greenhouse gas emissions and nitrate accumulation, underscore the extensive implications of PPP usage for soil health.

The temporal dynamics identified in this laboratory-based study, where certain effects intensified over time, emphasise the importance of considering the long-term impacts of PPP applications. Future research should extend exposure periods to determine whether these effects persist, escalate, or if microbial communities can adapt and recover functionally over extended durations. Additionally, it is crucial to explore how these impacts vary across different soil types, field conditions, plant interactions, and management practices. Such investigations should focus on both the direct toxicity to microbial groups and the indirect effects resulting from alterations in soil properties or substrate availability.

Some limitations of our microcosm design deserve mention. First, the test soil was an organic grassland Cambisol and thus lacked crop roots, pests and the pesticide legacy typical of an oat/potato cropping system. This clean background minimised confounding and let us attribute responses directly to the applied spray plans, yet field soils may react differently once rhizosphere inputs and historical residues come into play. Second, molecular endpoints often trail functional shifts in the field, especially when sprays recur through a season. For instance, Karas et al. (2018) observed significant *amoA* shifts by day 7 in lab soils and day 14 in field plots, while Sim et al. (2023) found *amoA* changes within 14 days of each spray in both lab and field trials. These studies confirm that gene-level responses can emerge within weeks, although they may lag functional changes. Longer incubations and in-situ trials that apply the same oat and potato spray regimes are required to track the full trajectory of both functional and molecular markers and to test the transferability of our findings. Finally, our molecular panel did not include markers for complete ammonia oxidizers (comammox). Given recent evidence that comammox can contribute substantially to both ammonia oxidation and N_2O emissions in arable soils (Bi et al., 2024), future studies should incorporate comammox-specific assays to fully resolve PPP impacts on the entire ammonia-oxidizing guild.

Our findings advocate for an integrated approach to soil ecotoxicology that combines functional and molecular indicators to capture the multifaceted impacts of PPPs on soil ecosystems. Such a comprehensive strategy is vital for informing agricultural practices and shaping environmental policies aimed at mitigating the adverse effects of PPPs while preserving soil fertility and maintaining essential ecosystem services.

4.4. Implications for risk assessment frameworks

Building on these insights, it is essential to examine how current risk assessment frameworks can be adapted to more effectively evaluate the complex impacts of PPPs on soil ecosystems.

4.4.1. Current risk assessment limitations

Although PPPs are rigorously tested prior to their use, current risk assessment frameworks, such as OECD Guideline No. 216 (2000), fail to address the broader impacts of PPPs on soil microorganisms, which provide critical ecosystem services such as nutrient cycling. These assessments focus narrowly on N mineralisation, relying on outdated and simplistic tests that do not reflect real-world agricultural conditions. Furthermore, existing PPP risk assessments in the European Union have primarily focused on single-compound or formulation applications (Europäisches Parlament und Rat der Europäischen Union, 2009), overlooking the repeated use and combinations of different compounds into mixtures. Such mixtures can exert concerted effects on soil microbial communities and nutrient cycling, complicating the evaluation of their true environmental impact. The European Food Safety Authority (EFSA PPR Panel et al., 2017) has identified significant regulatory gaps in assessing PPP toxicity on soil microbes, advocating for updates to the N transformation test and the integration of advanced molecular methods, such as direct soil DNA extraction and qPCR.

A revised framework is urgently needed—one that combines molecular and functional indicators, considers realistic application scenarios, and evaluates the cumulative effects of PPP mixtures over time. Functional microbial endpoints, such as ammonia-oxidizing microbes, have been suggested as sensitive bioindicators to enhance the protection of soil health and its ecosystem services (Karpouzas et al., 2022). The findings of our study highlight the need for a multimethod, tiered risk assessment framework that integrates molecular and functional approaches to enhance sensitivity and accuracy. Such a framework would provide a cost-effective method for evaluating PPP impacts at multiple levels of biological organisation while addressing the complexities of soil ecosystems.

4.4.2. Tier 1: Scalable qPCR-based gene quantification

Quantifying gene abundance through qPCR offers a reliable and high-throughput method suitable for large-scale soil monitoring. It provides valuable insights into the potential of microbial communities to perform key ecosystem functions, making it an efficient screening tool for identifying shifts caused by PPPs. While gene abundance measured through qPCR is considered a good proxy for enzyme activity (Pérez-Guzmán et al., 2021), this study and others highlight its limited sensitivity in detecting functional changes resulting from PPP stress. The observed discrepancies between gene abundance and functional outputs in soil N cycling processes underscore the need for complementary methods to capture broader impacts. Therefore, even subtle trends observed through qPCR should be regarded as potential indicators of PPP-related disruptions, warranting further evaluation through Tier 2 functional assays to ensure comprehensive risk assessment.

4.4.3. Tier 2: Functional assays

Functional assays, such as PN, DEA, and NRC, directly measure microbial activity, capturing real-time biochemical transformations that underpin soil health. These assays provide a more accurate and sensitive reflection of microbial function than molecular techniques, effectively detecting immediate and dynamic responses to PPP-stressors. In this study, functional assays revealed more pronounced effects of PPP treatments on soil N cycling compared to gene quantification, demonstrating their value as sensitive indicators for processes such as nitrification and denitrification.

However, functional assays are resource-intensive and less scalable than molecular methods, which limits their feasibility for large-scale monitoring (EFSA PPR Panel et al., 2017). For this reason, functional assays should be employed as Tier 2, following preselection through Tier 1 molecular screening. This approach ensures that resources are focused on cases where molecular methods suggest potential disruptions, allowing for a more targeted and efficient evaluation of PPP impacts.

4.4.4. Post-registration monitoring

Post-registration monitoring has been proposed as an essential component of risk assessment to address the limitations of pre-registration tests, particularly in capturing the long-term and

cumulative effects of PPPs under field conditions (Vijver et al., 2017). Building on this, we propose that post-registration monitoring should evaluate PPPs not only as individual compounds but also as the mixtures in which they are applied according to industry-recommended spray plans.

Testing PPPs in realistic scenarios would reflect the cumulative and interactive effects of repeated applications on soil microbial communities and nutrient cycling, better aligning risk assessments with real-world agricultural practices. This approach ensures that potential impacts on soil health from mixtures and frequently repeated applications – overlooked in single-compound studies – are systematically evaluated, offering critical insights into their ecological consequences.

4.5. Conclusion

This study shows that real-world PPP spray plans, as hypothesised, can markedly disrupt soil N cycling, and that functional assays such as PN, DEA and NRC are more sensitive than gene counts.

Across treatments, PN declined steeply, with Cohen's d values of −6.32, −4.27 and − 4.96 at low, medium and high PPP-intensity after 29 days. DEA transiently rose at low intensity (d = +5.27) but fell at medium and high intensity (d = −3.19, −3.36), and a build-up of unreduced N_2O under heavier PPP loads (d = +3.33, +3.47) also showed a decrease of NRC. Soil NO_3^- levels rose markedly with PPP intensity (d = +2.22 to + 7.80), indicating accumulation of oxidised N pools. Among gene markers, only *nirK* showed a marginal treatment effect ($p = 0.053$) and correlated with DEA (rho = 0.387); *amoA* tracked PN (rho = 0.308–0.443) without significant differences between treatments.

Inhibition generally grew from day 8 to day 29, confirming time-dependent toxicity. The most pronounced deviation from this pattern was NRC, exhibiting milder yet still substantial reduction later on.

These intensity- and time-dependent impacts underline the importance of integrating functional and molecular indicators for a comprehensive assessment of their effects. We therefore recommend a tiered risk-assessment framework that combines high-throughput qPCR screening with targeted functional assays, followed by post-registration field monitoring of PPP mixtures applied under realistic field conditions is essential for addressing cumulative and long-term effects on soil microbial communities and ecosystem services. These findings highlight the need for updated risk assessment practices that better reflect real-world agricultural scenarios, ensuring sustainable soil health management and improved regulatory frameworks.

CRediT authorship contribution statement

Laura Å. Medici: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pascal A. Niklaus:** Writing – review & editing, Validation, Methodology, Formal analysis. **Florian Walder:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Miriam Langer:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

AI tools, including ChatGPT and Paperpal, were used to help formulate sentences, with ChatGPT and DataCamp assisting in writing R scripts. Scispace and ResearchRabbit were utilised for literature searches. All AI-suggested revisions were carefully reviewed, and the authors take full responsibility for the final content of the published article.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106297.

Data availability

Data will be made available on request.

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Glossary

PPPs: plant protection products
N: nitrogen
PN: potential nitrification
DEA: denitrifying enzyme activity
NRC: N_2O reduction capacity
amoA: ammonia monooxygenase
nirK: nitrite reductase
ES: ecosystem services
EFSA: European Food Safety Authority
NH_4^+: ammonium
NO_2^-: nitrite
NO_3^-: nitrate
nap / nar: nitrate reductase
nir: nitrite reductase
nor: nitric oxide reductase
nos: nitrous oxide reductase
RT: room temperature
WHC: water-holding capacity
ITS: internal transcribed spacer
qPCR: quantitative polymerase chain reaction
LME: linear mixed-effects model
ANOVA: analysis of variance
AOA: archaeal amoA
AOB: bacterial amoA
TPs: transformation products