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RESEARCH ARTICLE

How do antibiotic residues in manure affect grassland plants and soil nitrogen cycling?

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Abstract

Background and aims Animal manure is a valuable fertilizer, and its proper use is essential in circular agriculture. However, antibiotics are commonly administered to livestock and excreted in manure, thereby entering soil ecosystems. The effects of antibiotic-containing manure on soil nitrogen (N) cycling microbial guilds, plant productivity, and N turnover in grassland ecosystems remain unclear.

Methods In a two-factorial greenhouse pot experiment, we evaluated the impact of manure with different antibiotic residues in four plant communities: grass monoculture, clover monoculture, grass-clover culture, and a no-plant control. The fertilization treatments included antibiotic-free manure, manure containing oxytetracycline, and manure containing sulfadiazine. We measured soil N-cycling functional genes, aboveground and belowground plant biomass, clover symbiotic N fixation, soil mineral N pools, N_2O emissions, and antibiotic residues in plants and soil.

Results Oxytetracycline, but not sulfadiazine, significantly increased the relative abundance of ammonia-oxidizing archaea (AOA) and marginally increased the abundance of N-fixing microbes across all plant communities. In clover monoculture, both antibiotics reduced root biomass and root total N content. However, antibiotic residues in soil did not have significant impacts on N fixation of clover, soil mineral N pools, and soil N_2O emissions.

Conclusion At environmentally relevant concentrations, oxytetracycline residues in manure-amended soils could change the soil microbial community composition, favoring more tolerant or resistant groups such as AOA. Clover exhibited greater sensitivity to antibiotic exposure than grass. Further research is necessary to understand the long-term ecological consequences of persistent antibiotics like oxytetracycline in grasslands.

Keywords Antibiotics · Manure · Ryegrass · Clover · Nitrogen cycling

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Introduction

In circular agriculture, animal manure is a crucial soil fertilizer, improving soil fertility and increasing crop yields (Cai et al. 2019; Köninger et al. 2021; Hoogstra et al. 2024). However, livestock manure often contains antibiotic residues due to their frequent use in livestock production to prevent or treat bacterial infections (He et al. 2020; Van et al. 2020). In 2020, global veterinary antibiotic use was estimated at 99,502 tons and is expected to rise higher than 100,000 tons by 2030 (Mulchandani et al. 2023). A significant portion of the administered antibiotics is excreted in feces and urine, mainly as the intact parent compound and sometimes as bioactive metabolites (Sarmah et al. 2006). As a result, manure fertilization has been considered a major pathway for antibiotics to enter soil environments (Du and Liu 2012; Kuppusamy et al. 2018).

Various types of antibiotics have been detected in agricultural soils worldwide, sometimes even at mg kg^{-1} levels (Fang et al. 2023). Some compounds such as tetracyclines and fluoroquinolones are particularly persistent, raising concerns about their potential accumulation in soil (Cycoń et al. 2019). Once in the soil, they can disrupt microbial processes critical to biogeochemical cycles, particularly the nitrogen (N) cycle, which supports plant growth and food production (Eickhout et al. 2006; Ågren et al. 2012). Some key processes involved in the soil N cycle are nitrification, denitrification, and N fixation (Kuypers et al. 2018). Nitrification is a microbial process in which ammonia (NH_3) is oxidized to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB) or archaea (AOA), followed by the oxidation of NO_2^- to nitrate (NO_3^-) by nitrite-oxidizing bacteria (Li et al. 2018). Denitrification is the stepwise reduction of NO_3^- to nitrogen gas (N_2). This process begins with the reduction of nitrate to nitrite, followed by the production of nitric oxide (NO), nitrous oxide (N_2O), and finally N_2. However, the final step of denitrification is often incomplete, resulting in the release of N_2O, a potent greenhouse gas that contributes to global warming and ozone depletion (Domeignoz-Horta et al. 2016; Pan et al. 2022; Hiis et al. 2024). Plants can reduce N losses through gaseous pathways by competing with microbes for soil mineral N (Timilsina et al. 2024). In addition, symbiotic N fixation involves mutualistic relationships between certain plants, particularly legumes, and N-fixing bacteria, converting N_2 into NH_3, which plants can use for growth. This process has the potential to support sustainable agriculture by reducing reliance on synthetic N fertilizers (Boddey et al. 1997).

Microorganisms, as direct targets of antibiotics, have drawn significant research attention regarding the effects of antibiotic residues on soil N-cycling microbial guilds. Tetracyclines and sulfonamides, frequently found in animal manure, are the commonly studied antibiotic groups in this context (Marutescu et al. 2022; Jia et al. 2023; Fang et al. 2023). For nitrifying guilds, studies consistently show that soil AOB are more vulnerable to these two groups of antibiotics than AOA, possibly due to differences in membrane structure and metabolism between bacteria and archaea (Ollivier et al. 2010, 2013; Radl et al. 2015; Omirou et al. 2022; Yang et al. 2024). However, the impacts of antibiotics on denitrifying microbes are less consistent, with studies reporting either negative or negligible effects on the abundance of *nirK* and *nirS*, the genes catalyzing NO_2^- reduction (Kleineidam et al. 2010; Ma et al. 2014; Shan et al. 2018; Omirou et al. 2022). By quantifying the *nosZ* gene, tetracyclines have been shown to either decrease (Semedo et al. 2018; Shan et al. 2018) or increase (Omirou et al. 2022; Yang et al. 2024) the abundance of N_2O-reducing microbes. These conflicting findings may arise from the different soils used in these experiments, harboring distinct denitrifying microbial communities and antibiotic resistance profiles (Wei et al. 2015; Song et al. 2023; Pagaling et al. 2023). Furthermore, only a few existing studies investigate the effects of antibiotics on soil N-fixing bacteria. For instance, tetracyclines have been reported to reduce *Bradyrhizobiaceae* abundance in soybean root nodules (Zhang et al. 2024) and to shift *Bradyrhizobium* community composition after long-term manure exposure (14 years) in the field (Revellin et al. 2018).

Compared to soil microbial communities, the effects of soil antibiotic residues on plant physiology, productivity, and nutrient uptake are less studied. The limited body of research indicates that both sulfonamide and tetracycline antibiotics can inhibit root and shoot development, potentially affecting nutrient acquisition (Hillis et al. 2011; Michelini et al. 2013; Lu et al. 2016; Minden et al. 2017, 2018; Li et al. 2023). However, many studies have used hydroponic conditions and antibiotic concentrations far above soil

solution levels and in the absence of manure application, limiting their ecological relevance. Plants can take up the antibiotics present in soil solution (Kumar et al. 2005; Michelini et al. 2012; Bassil et al. 2013). The uptake of sulfonamides and tetracyclines has been shown to significantly alter the root metabolite profiles of garden peas (Tasho et al. 2018). Additionally, one study demonstrated that thale cress could detoxify sulfamethoxazole (a sulfonamide antibiotic) through oxidation and conjugation with other organic compounds within plant cells (Dudley et al. 2018). Rocha et al. (2021) proposed that antibiotic detoxification may compete with essential plant metabolic processes, potentially altering N use or increasing energy demands, thereby limiting growth.

Although we are gaining more insights into how antibiotics impact soil microorganisms and plant properties, significant research gaps remain in our understanding of how these veterinary compounds influence plant–microbe interactions and their subsequent impacts on soil N-cycling. Many studies have focused on either microorganisms or plants, overlooking their interactions. The presence of plants can shape soil microbial communities, altering their response to antibiotic exposure compared to bare soils (Lin et al. 2022). Notably, no studies have investigated the impact of antibiotics on soil N turnover and N_2O emissions in grassland ecosystems, even though grasslands account for a substantial portion of agricultural soils and contribute 54% of agricultural N_2O emissions (Dangal et al. 2019). Grass growth in these systems is often N-limited, and clover is commonly introduced for its ability to fix atmospheric N_2, reducing fertilizer needs (van Eekeren et al. 2009; Harris and Ratnieks 2022). Although there are a few studies examining the impact of antibiotics on symbiotic N fixation, the impacts on clover plants and their symbiotic microbes are still unclear.

This study aimed to address the following research questions: 1) How do antibiotic residues in manure affect soil N-cycling microbial communities in grasslands? 2) What are their impacts on above- and belowground biomass of grassland species? Consequently, 3) How do these changes influence N turnover in grassland systems? To answer these, we designed a two-factor greenhouse pot experiment. The plant factors included ryegrass monoculture, clover monoculture, grass-clover mixed culture, and no plants. The soil treatment factor involved the application of antibiotic-free manure, manure with oxytetracycline, and manure with sulfadiazine. We quantified the abundance of key N-cycling functional genes, plant aboveground and belowground yield, plant N content, N fixation of clover, soil N pools, N_2O emissions, and antibiotic residues of plants and soil. We hypothesize that: 1) Both antibiotics reduce the abundance of AOB and symbiotic N-fixing microbes across all plant communities. 2) Both antibiotics cause a reduction in shoot and root biomass across all plant communities. 3) The presence of antibiotic residues in manure can therefore decrease aboveground N uptake in ryegrass and impair the ability to fix atmospheric N of clover. Due to the complexity of pathways involved in soil N_2O production, the effects of antibiotic residues on soil N_2O emissions in the grassland system are unclear and will be further revealed in this study.

Materials and methods

Soil and manure collection

The soil used in this study was a sandy soil (2% clay, 17% silt, and 76% sand, collected from a depth of 0 ~ 20 cm in a perennial grassland, 51°99'N, 5°67'E, Wageningen, the Netherlands). This grassland had not been fertilized with organic or mineral fertilizers for seven years prior to this study. At the time of sampling, the soil had the following characteristics: a pH of 4.7 (0.01 M $CaCl_2$), organic carbon content of 2.5%, total N content of 1990 mg N kg^{-1}, and plant available phosphorus content of 0.5 mg kg^{-1}. After collection, the soil was air-dried and sieved through a 2 mm mesh to remove rocks and plant debris. Manure was collected from cows that had not recently received antibiotic treatments and were based at a research farm (Carus, Wageningen, the Netherlands). The manure had a dry matter content of 12% and a total N content of 4 g kg^{-1}, and it was stored at 4 °C until application. To ensure the starting materials were free of antibiotic contamination, we conducted an antibiotic residue analysis targeting 48 commonly found veterinary antibiotics, following the procedures developed by Berendsen et al. (2015). Chromatograms of the target compounds were visually examined to confirm the absence of antibiotics in both the soil and manure.

Experimental setup

In the greenhouse pot experiment, we applied a completely randomized block design with two factors: four plant communities and three soil treatments. The factors were arranged in five blocks, with each combination of plant community and soil treatment replicated once per block, resulting in a total of 60 mesocosms (Fig. 1A). More specifically, the four plant communities were: (1) ryegrass monoculture, (2) clover monoculture, (3) a mixed grass-clover culture, and (4) no plants. The three soil treatments applied were: (1) manure without antibiotics, (2) manure containing 10 mg kg^{-1} oxytetracycline (equivalent to 115 µg kg^{-1} in soil after manure application), and (3) manure containing 10 mg kg^{-1} sulfadiazine (equivalent to 115 µg kg^{-1} in soil after manure application). Antibiotic concentrations used in this study fall within ranges reported in other environmental studies, representing a relatively high level in cattle manure and soils (Marutescu et al. 2022; Fang et al. 2023).

As pots, we used polyvinyl containers without drainage holes, with a diameter of 22.0 cm at the top and 17.7 cm at the bottom, and with a depth of 20.4 cm. In each pot, we manually mixed 5 kg of air-dried soil with 0.75 kg of demineralized water. During the mixing process, we added triple superphosphate and potassium sulfate at rates equivalent to 30 kg P_2O_5 ha^{-1} and 100 kg K_2O ha^{-1}, respectively. The estimated bulk density of the soil without plants in the pots was 1.42 g cm^3, with a water-filled pore space (WFPS) of 55%. After filling the soil in the pots, we inserted a 5 cm diameter polyvinyl watering tube into the center of each pot to a depth of 7.5 cm. This setup helped to prevent disturbance of the soil surface and ensure an even soil moisture distribution during frequent soil moisture correction (Abalos et al. 2018; Yang et al. 2023). During the whole period, pots were weighed every two days, and water was added

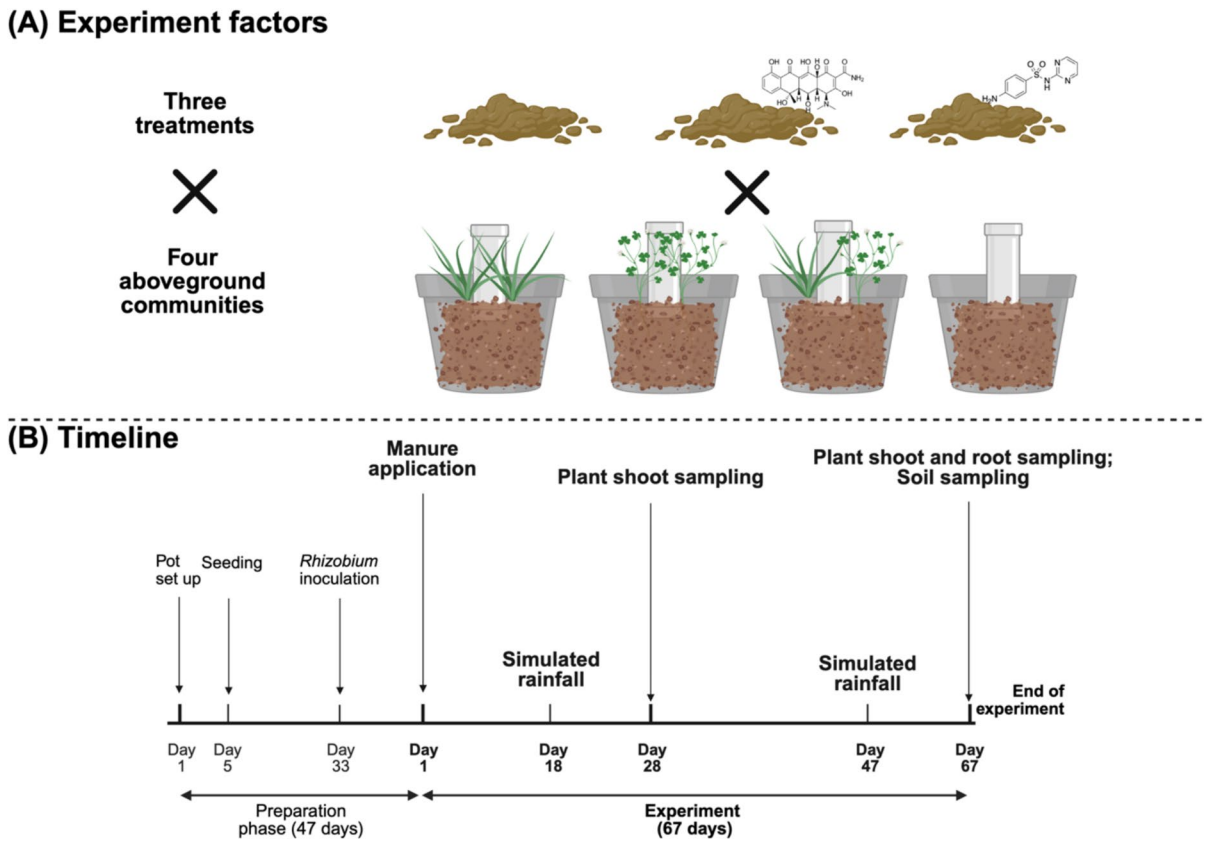

Fig. 1 Visualization of the factorial experiment (**A**) and the timeline of the experiment (**B**)

gravimetrically through the watering tube to maintain the target soil moisture.

Five days after filling the pots, seeds were sown. All seeds were obtained from Barenburg seed company in the Netherlands. To ensure good soil coverage, the seeding rates in the monocultures were set at eight times higher than the recommendations of the seed company. For the grass monoculture, perennial ryegrass (*Lolium perenne*) was sown at a rate of 16 g m^2. In the clover monoculture, white clover (*Trifolium repens*) was sown at a rate of 12 g m^2. In the grass-clover culture, ryegrass and clover were sown at rates of 8 g m^2 and 6 g m^2, respectively. The seeds were given six weeks to germinate and grow before manure application. Legumes were sparsely distributed and present in low abundance in the grassland where the soil was obtained. To ensure the presence of corresponding *Rhizobium* for symbiotic N fixation in this experiment, four weeks after seeding, each pot was inoculated with *Rhizobium trifolii* strain ANU843 by adding 7 ml of a diluted bacterial culture (30 mL of an OD600 = 0.1 culture in 600 ml demineralized water).

In the sixth week after seeding, manure was applied to all pots. Before the manure application, plants were cut to a height of 2 cm above the soil surface. Each pot received 66.6 g of manure, equivalent to an application rate of 70 kg N ha^{-1}. To simulate field conditions using a manure injector, the manure was applied via slit injection. First, manure was blended with demineralized water in a 1:1 ratio using an electric blender. The desired amount of antibiotic was also added to the blender to reach the target concentration. After blending, the mixture was transferred to a disposable squeeze bottle. Four slits, each 5 cm deep, were made in each pot using disposable wooden sticks, and the manure mixture was squeezed into the slits. Finally, the slits were covered with surface soil.

The manure application marked the start of the experiment, which lasted for 67 days. We harvested the aboveground plant biomass twice at a height of 2 cm on both days 28 and 67. The belowground biomass and soil samples were collected only at the end of the experiment, on day 67. Two rainfall events of 13 mm (equivalent to adding 500 mL of water directly to the soil surface) were simulated on days 18 and 47, increasing the soil moisture content to 100% water holding capacity. The rainfall events are designed to mimic field conditions where precipitation significantly stimulates denitrification, leading to increased soil N_2O emissions (Abalos et al. 2018). After each simulated rain event, no additional water was added until the soil moisture content dropped below its initial level. The timeline of the greenhouse experiment is shown in Fig. 1B.

Soil N-cycling communities

Soil samples were collected at the end of the experiment using a 1 cm diameter auger, sampling from four random locations in each pot at a depth of 0–20 cm. The collected soil was sieved to 2 mm and stored at −80 °C until DNA extraction. DNA was extracted from the soils using DNeasy PowerSoil Pro Kits (Qiagen). The DNA yield was quantified with a NanoDrop 2000 spectrophotometer. The abundance of key N-cycling functional genes was measured in the soil DNA extracts using quantitative PCR (qPCR) with a Bio-Rad opus CFX 96. Each qPCR reaction contained 7.5 µL of KAPA SYBR FAST master mix (Roche), 400 nM of each primer, 2 µL of soil DNA template (diluted to 0.5 ng DNA μL^{-1}), and sterilized Milli-Q water added to a total volume of 15 µL. The 16S rRNA gene was used to quantify total prokaryotic abundance in the soil (Takai and Horikoshi 2000). The abundance of ammonia-oxidizing bacteria and archaea was quantified using the *amoA* gene (Leininger et al. 2006; Tourna et al. 2008), while soil denitrifiers were quantified using two nitrite reductase genes (*nirK* and *nirS*) (Henry et al. 2004; Kandeler et al. 2006). N_2O-reducing microbes were quantified using the *nosZI* and *nosZII* genes (Jones et al. 2013). Also, N-fixing bacteria were quantified using the *nifH* gene (Poly et al. 2001). Unfortunately, quantification of the *nosZII* gene was unsuccessful due to unspecific amplification from the soil samples by the primer pair. The amplification efficiency for the other genes ranged between 90 and 100%. We examined the results of N cycling functional genes with the copy number per gram of dry soil to evaluate the corresponding N transformation potential per unit of soil. To examine changes in the N functional groups within the bacterial communities, the ratio between the abundance of each functional gene and the abundance of 16S rRNA gene was calculated (expressed as relative abundance).

Plant analysis

The plant shoots were harvested on days 28 and 67, dried at 70 °C for 48 h, and weighed to determine the aboveground dry biomass. For belowground biomass, roots were harvested on day 67, washed with demineralized water, dried at 70 °C for 72 h, and then weighed. In the grass-clover pots, we were unable to separate the clover roots from the grass roots because they were tightly intertwined and difficult to disentangle. The dried plant materials were subsequently ground using a ball mill with stainless steel balls, and approximately 3.5 mg was weighed into tin cups for analysis. Total N content and natural abundance δ15 N were analyzed at the UC Davis Stable Isotope Facility (California, USA) using an Elementar vario MICRO cube elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) interfaced with a Sercon Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, United Kingdom). The aboveground and belowground plant N content in each pot was calculated by multiplying the N concentration in shoots or roots by the respective dry biomass.

The N derived from the atmosphere (Ndfa) in clover plants was estimated using the ^{15}N natural abundance method (Unkovich et al. 2008). The percentage of Ndfa was calculated using the following equation:

$$\%N_{dfa} = \frac{(\delta 15N_{reference\_} - \delta 15N_{clover})}{(\delta 15N_{reference\_} - B)} \times 100$$

The term $\delta^{15}N_{reference}$ refers to the mean $\delta^{15}N$ value calculated from the plant materials in the grass monoculture, while $\delta^{15}N_{clover}$ represents the $\delta^{15}N$ value of the clover plant materials. The value B is the $\delta^{15}N$ of the clover that obtains all its N entirely through atmospheric N_2. The total Ndfa in shoots or roots was determined by multiplying the N content in shoots or roots by the %Ndfa. To obtain the B value precisely, we planted white clover (*T. repens*) in quartz sand (in duplicate) in the same greenhouse next to the main experiment. The sand-grown clover was sown and inoculated with *Rhizobium trifolii* on the same day as the main pot experiment and was frequently watered with McKnight's solution (N-free complete nutrient solution) as recommended by Unkovich et al. (2008). We did not apply any manure or antibiotics to the sand-grown clover to ensure all the N present in these plants were derived from the atmospheric N_2 in the greenhouse. The shoots and roots of the sand-grown clover were harvested on the same dates as in the main experiment.

Soil mineral N pools

Soil samples collected on day 67 were dried at 40 °C and sieved to 2 mm. To assess the readily available mineral N, we extracted the soil using 0.01 M $CaCl_2$ (Houba et al. 2000). Specifically, 3 g of soil was shaken with 30 mL of $CaCl_2$ solution for two hours. After centrifugation, the supernatant was filtered through a 0.45 µm filter (Aqua 30, Whatman). The concentrations of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ in the extracts were then measured using a segmented flow analyzer (Skalar, SAN + +).

Soil N_2O emissions

The soil N_2O fluxes were measured at least twice a week throughout the experiment. We used the closed chamber method to quantify the N_2O flux from the soil (Charteris et al. 2020). During measurements, a custom-made polyvinyl chamber was sealed over a mesocosm, with the watering tube covered by parafilm. The chamber remained on the mesocosm for approximately 30 min. Following this period, gas samples were taken from the chamber using a gas analyzer (Gasera One Pulse) via Teflon tubes, and the concentration of N_2O was measured. The ambient N_2O concentration in the greenhouse was used to estimate the initial headspace concentration (Chadwick et al. 2014). N_2O emission rates were calculated by assuming a linear increase in concentration during the chamber closure. Cumulative N_2O emissions were determined through trapezoidal integration over time, with the assumption that flux rates changed linearly between measurement events (Abalos et al. 2014; Oram et al. 2020).

Antibiotic residue analysis

We quantified the residual oxytetracycline and sulfadiazine in plant shoots (from both harvests) and soils (at the end). The wet soil samples collected from the pots were sieved using a 2 mm sieve to remove any plant debris. At each harvest, about 3 g of plant shoots from each pot were cryogenically ground. The

soil and plant materials were then stored at −20 °C before further processing.

The quantification procedures of antibiotic residues in plant and soil materials were identical to those in previous studies (Jansen et al. 2019; Berendsen et al. 2021). In short, 1.5 g materials were weighted into 50 ml polypropylene tubes. Internal standards were added to each tube (sulfadiazine-d4 for sulfadiazine and demeclocycline for oxytetracycline). The materials were extracted with 4 mL of acetonitrile (ACN) containing 0.125% trifluoroacetic acid, along with 4 mL of McIlvain-EDTA buffer (made from 0.1 M citric acid and 0.2 M disodium hydrogen phosphate, pH 4.0). The mixture was shaken using a head-over-head rotator for 15 min. Following this, 2 mL of 200 g L^{-1} lead acetate was added before centrifugation. The supernatant was transferred into a new glass tube, and the ACN added during the extraction was evaporated under a gentle nitrogen flow at 40 °C. After the evaporation of ACN, 13 mL of 0.2 M EDTA solution was added, and the entire extract was passed through a preconditioned solid-phase extraction cartridge (Phenomenex, Strata-X RP 200 mg, 6 mL) for clean-up. The cartridge was rinsed with 5 mL of water and vacuum dried. The antibiotic residues were then eluted from the cartridge with 5 mL of methanol (MeOH) into a glass tube. After evaporating the MeOH at 40 °C under nitrogen gas, the remaining residues were redissolved in 100 µL of MeOH and 400 µL of water. The amounts of oxytetracycline and sulfadiazine in the extracts were quantified with liquid chromatography coupled with tandem mass spectrometry, following the method used by Berendsen et al. (2015). The chromatograms were analyzed, and the results were calculated using SCIEX OS software (version 2.2.0).

The detection limit (LOD) for oxytetracycline and sulfadiazine residues in soil and plant materials was estimated using the five-point calibration curves. The LOD was calculated by dividing the standard deviation of the y-intercept by the slope and multiplying by three. For soil samples, with calibration ranges of 0–100 µg kg^{-1} for oxytetracycline and 0–25 µg kg^{-1} for sulfadiazine, the calculated LODs were 5.02 µg kg^{-1} and 0.33 µg kg^{-1}, respectively. In plant materials (grass and clover), the calibration range was 0–16 µg kg^{-1} for oxytetracycline and 0–4 µg kg^{-1} for sulfadiazine. The calculated LODs for sulfadiazine were 0.80 µg kg^{-1} in grass and 0.08 µg kg^{-1} in clover. However, the oxytetracycline calibration curves for grass and clover yielded R^2 values of 0.97, suggesting the relatively poor sensitivity of oxytetracycline in plant materials within the calibration range. Still, the calculated oxytetracycline LOD for grass and clover was 5.66 µg kg^{-1} and 5.61 µg kg^{-1}, respectively. Furthermore, since the LOD derived from the calibration curve might be deviated from the instrumental LOD (Saadati et al. 2013; Şengül 2016), we calculated the signal-to-noise ratio (S/N) of the plant samples to examine the sensitivity of the antibiotics in the plant materials related to the empirical values: S/N = 3 for the LOD and S/N = 10 for the quantification limit (LOQ).

Statistical analysis

All statistical analyses were conducted using R version 4.4.1. Linear mixed-effect models (LME4 package) were used, fitted with Type III analysis of variance (ANOVA) to assess the effects of experimental factors on the response variables. In all models, the block was treated as a random effect. We checked the distribution of model residuals using the Shapiro–Wilk normality test and visually inspected them through Q-Q plots and histograms. Homoscedasticity was examined by plotting residuals against fitted model values. If parametric assumptions were violated, Box-Cox transformations were applied to improve the residual distribution. Statistical significance was determined using p-values, with values less than 0.05 considered significant.

For soil N-cycling functional genes, we used two-way ANOVA to examine the effects of plant communities, antibiotic treatments, and their interaction on gene abundance, both in the abundance per unit of soil and relative to 16S rRNA gene abundance. Using the same model structure, we also analyzed the impacts of antibiotic treatments and plant communities on cumulative soil N_2O emissions and soil N pools.

For plant data, each plant community was analyzed separately to meet the assumptions of parametric tests. A two-way ANOVA was used to assess the effects of harvesting time, antibiotic treatments, and their interaction on aboveground dry yield, aboveground N content, and aboveground Ndfa. For belowground dry mass, N content, and Ndfa, a one-way ANOVA was used to examine the effects of antibiotic

treatments. All figures were generated using Matplotlib and Seaborn.

Results

Soil N-cycling communities

The N transformation potential of soil was assessed by the abundance of the corresponding functional genes per unit of soil. The presence of both oxytetracycline and sulfadiazine in soil did not significantly affect the abundance of targeted nitrifying genes (*amo*AOA and *amo*AOB) and denitrifying genes (*nirK*, *nirS*, *nosZI*) across all plant communities (Supplementary Fig. 1B, C, D, E, and F). The presence of plants significantly increased the abundance of the *nifH* gene per unit of soil (Supplementary Fig. 2B).

The relative abundance of a functional group within the soil microbial community was calculated as the ratio between the abundance of the functional gene and the 16S rRNA gene. The application of manure containing oxytetracycline significantly increased the relative abundance of AOA compared to antibiotic-free manure (Fig. 2). However, the relative abundance of AOB was not significantly affected by antibiotic treatments, although plant presence showed a trend to reduce AOB relative abundance within the soil microbial community (Supplementary Fig. 3B). For the denitrifying functional genes, neither antibiotics nor plant communities had a significant effect on their relative abundance (Supplementary Fig. 4). Interestingly, two-way ANOVA results indicated that antibiotic treatments had a significant impact on the relative abundance of *nifH* (Fig. 3A). Post-hoc analysis revealed only a marginally significant increase in *nifH* relative abundance in soil with the application of manure containing oxytetracycline compared to antibiotic-free manure (Fig. 3B). Additionally, the presence of clover significantly increased the relative abundance of *nifH* compared to soils without plants (Fig. 3C).

Plant biomass

Significant effects of antibiotic residues in manure were detected only on root biomass (Fig. 4), with no significant impact on shoot biomass (Supplementary Fig. 5 and 6). More specifically, both oxytetracycline and sulfadiazine in manure significantly

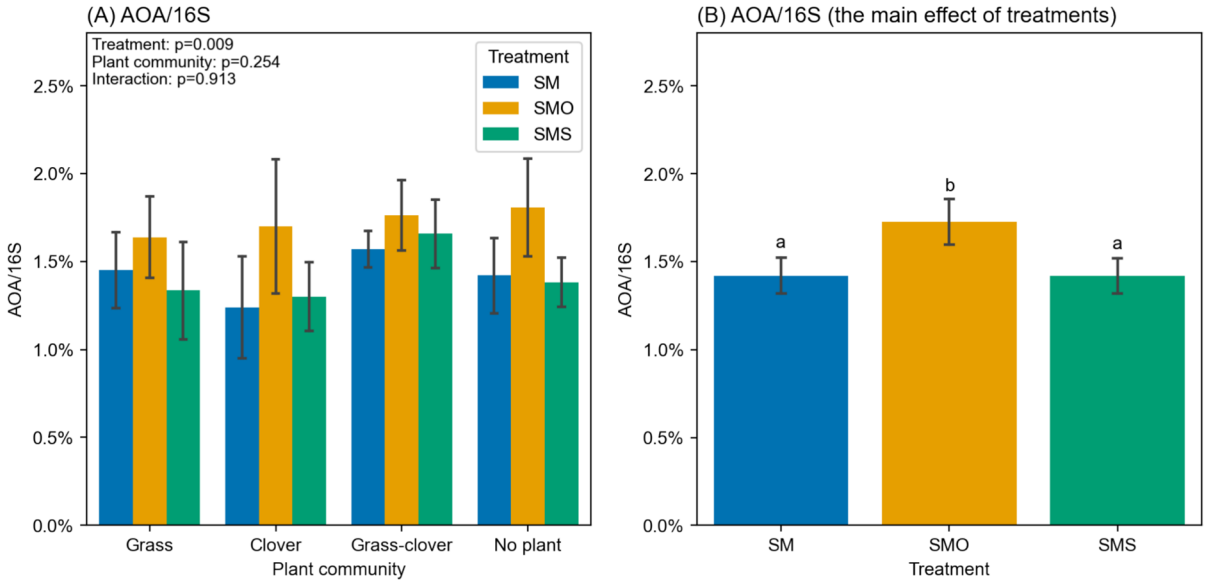

Fig. 2 The relative abundance of AOA in soil in relation to plant communities and antibiotic treatments. Error bars represent the standard error of the mean (Panel A: n = 5, Panel B: n = 20). Results from the two-way ANOVA are displayed in the upper-left corner of Panel A. Significant differences between treatments are indicated by different letters in Panel B. The three antibiotic treatments are: SM (soils receiving antibiotic-free manure), SMO (soils receiving manure containing oxytetracycline), and SMS (soils receiving manure containing sulfadiazine)

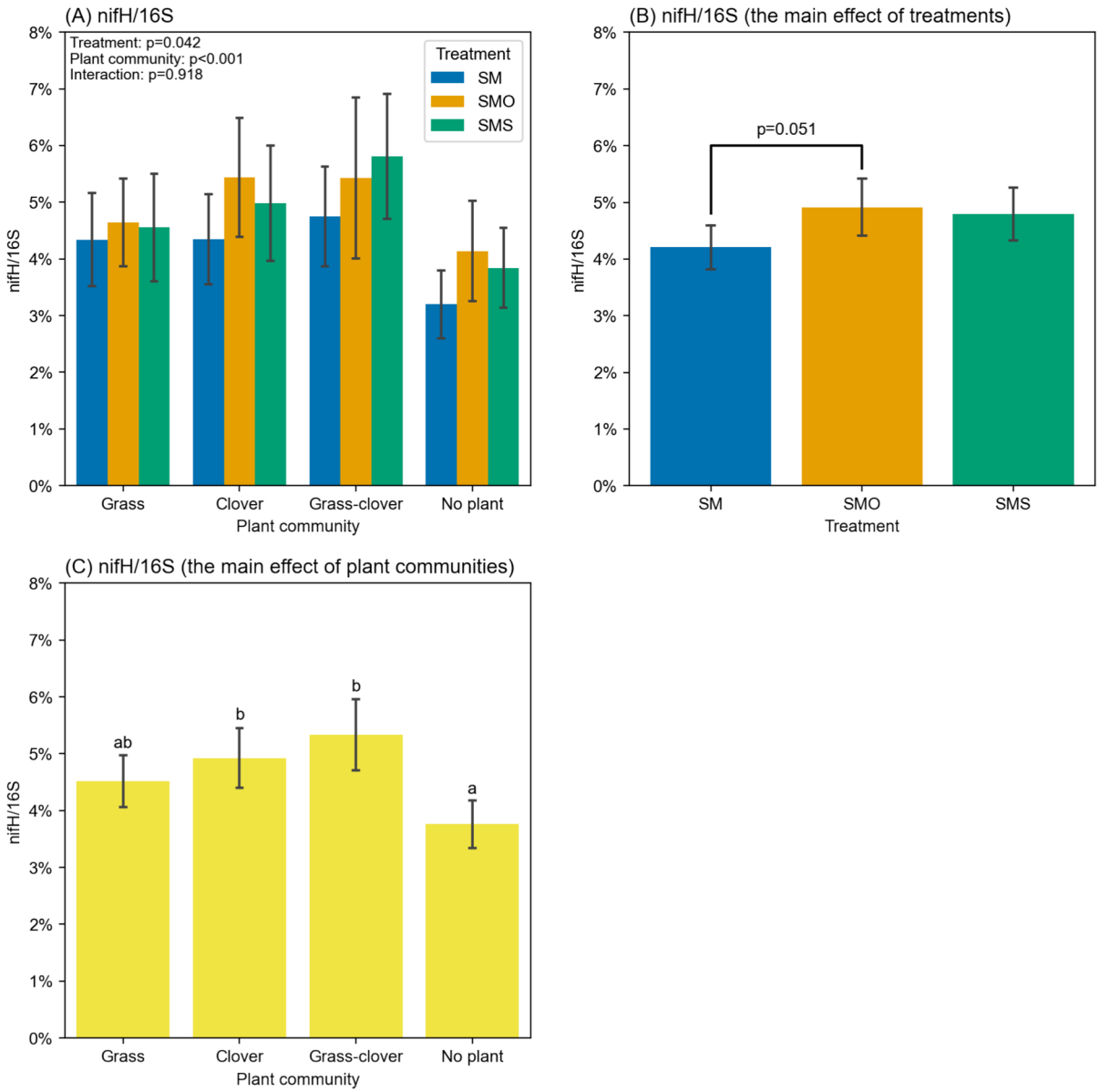

Fig. 3 The relative abundance of *nifH* gene in soils in relation to plant communities and antibiotic treatments. Error bars represent the standard error of the mean (Panel A: n = 5, Panel B: n = 20, Panel C: n = 15). Results from the two-way ANOVA are displayed in the upper-left corner of Panel A. Significant differences between treatments and plant communities are indicated by different letters in Panel B and Panel C, respectively. The three antibiotic treatments are: SM (soils receiving antibiotic-free manure), SMO (soils receiving manure containing oxytetracycline), and SMS (soils receiving manure containing sulfadiazine)

decreased root biomass in the clover monoculture compared to antibiotic-free manure (Fig. 4B). Although a similar reduction was observed in the grass monoculture, the difference was not statistically significant (Fig. 4A).

Plant N content and symbiotic N fixation

Similar to the plant biomass, in the clover monoculture, the application of manure containing oxytetracycline or sulfadiazine led to a significant reduction

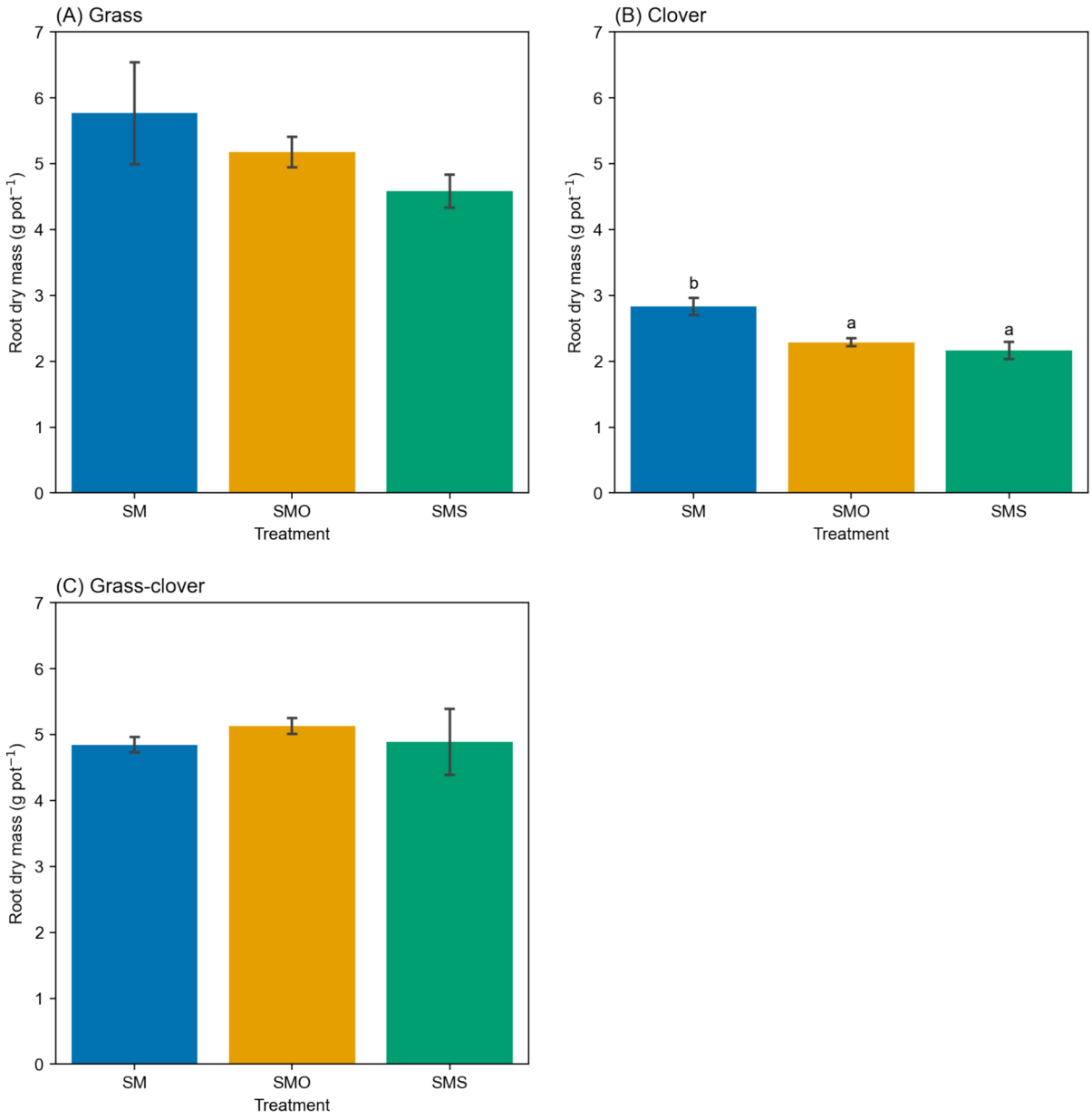

Fig. 4 Belowground dry yield from the second harvest in relation to the treatments in different communities, A) grass monoculture, B) clover monoculture, and C) grass-clover mixed culture. Error bars represent the standard error of the mean (n = 5). Significant differences between treatments are indicated by different letters. The three treatments are: SM (soils receiving antibiotic-free manure), SMO (soils receiving manure containing oxytetracycline), and SMS (soils receiving manure containing sulfadiazine)

in root N content compared to that of the application of antibiotic-free manure (Fig. 5B). The effects of antibiotics in manure on shoot N content were absent in all plant communities (Supplementary Fig. 7 and 8).

Regarding the symbiotic N fixation of clover plants, in the clover monoculture, the presence of either oxytetracycline or sulfadiazine in the manure did not affect Ndfa levels in the shoots or roots

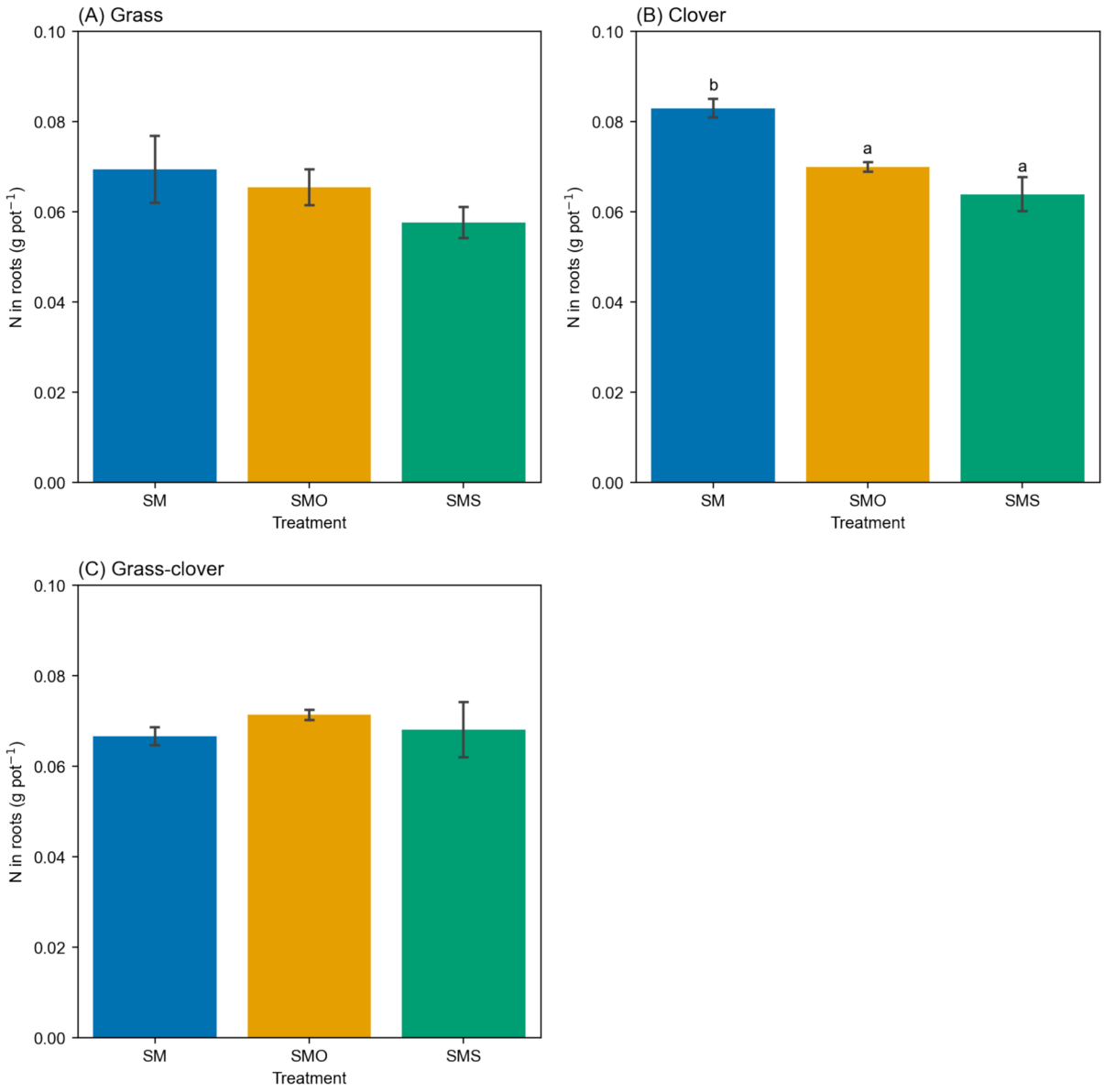

Fig. 5 Belowground total N in roots from the second harvest in relation to antibiotic treatments in different plant communities, **A** grass monoculture, **B** clover monoculture, and **C** grass-clover mixed culture. Error bars represent the standard error of the mean (n = 5). Significant differences between treatments are indicated by different letters. The three antibiotic treatments are: SM (soils receiving antibiotic-free manure), SMO (soils receiving manure containing oxytetracycline), and SMS (soils receiving manure containing sulfadiazine)

(Fig. 6). Similarly, no significant effects of antibiotics in manure on shoot Ndfa were observed in the clover grown within the grass-clover mixture (Supplementary Fig. 9).

Soil N pools and N_2O emissions

We observed significantly higher levels of readily available NH_4^+ in bare soils compared to soils with plants (Supplementary Fig. 10 A). However,

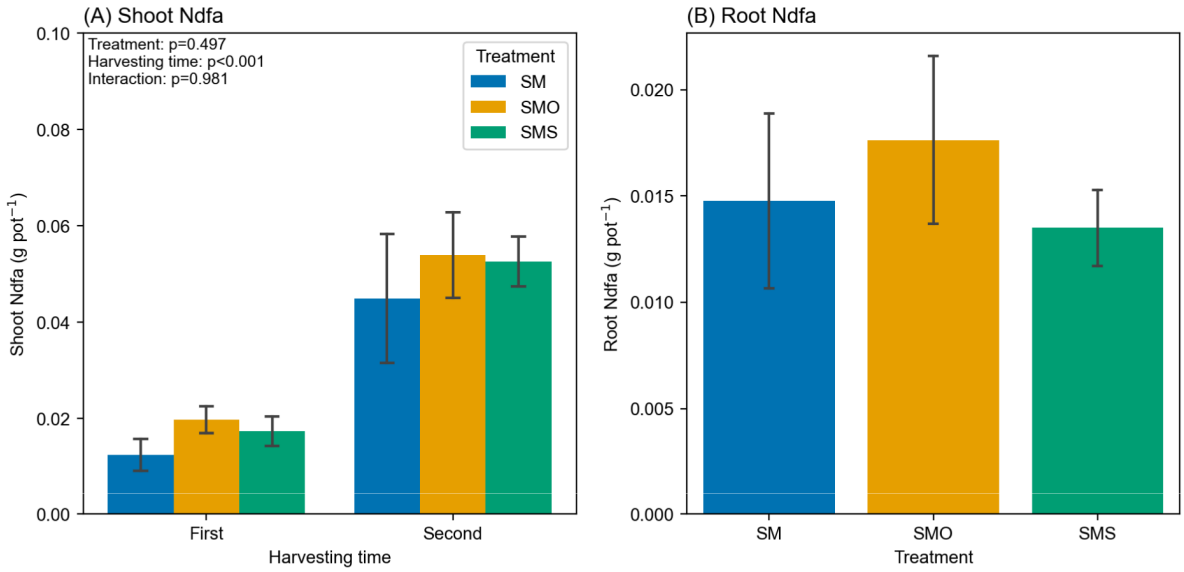

Fig. 6 Ndfa in the clover monoculture in relation to antibiotic treatments. Panel A shows Ndfa in shoots from the clover monoculture on the two harvest times. Results from the two-way ANOVA are displayed in the upper-left corner of Panel A (n = 5). Panel B shows Ndfa in roots from the clover monoculture at the final harvest (n = 5). The three antibiotic treatments are: SM (soils receiving antibiotic-free manure), SMO (soils receiving manure containing oxytetracycline), and SMS (soils receiving manure containing sulfadiazine)

the presence of antibiotics in the manure did not have a significant effect on soil NH_4^+ content. For soil NO_3^- in the pots with plants (Supplementary Fig. 10B), statistical analysis could not be performed because many data points were below the detection limit (0.3 mg kg^{-1}). In contrast, a substantial amount of available NO_3^- was found in the soils without plants, showing a trend of reduced NO_3^- in the presence of antibiotics (Supplementary Fig. 10 C).

Overall, soil N_2O emissions remained relatively low across all soils throughout the experimental period. Emissions of N_2O were not affected by the antibiotics but were lower in soils with plants compared to bare soil (Supplementary Fig. 11E). A temporary increase in N_2O fluxes was observed after the first rain event, though this pattern was not observed following the second rain event (Supplementary Fig. 11 A, B, C, and D).

Antibiotic residues

After 67 days of manure application, less than 10% of the applied sulfadiazine from manure remained in the soil across all plant communities, a percentage much lower than that of oxytetracycline (about 20% to 40% remained) (Supplementary Fig. 12). The highest concentrations of both antibiotics were found in soils without plants, while the lowest concentrations were observed in grass-clover mixed cultures albeit statistical insignificance. Notably, soils without plants had significantly more sulfadiazine residues compared to soils of the grass monoculture or the grass-clover mixture (Supplementary Fig. 12B).

The uptake of antibiotics in plant shoots was minimal. Oxytetracycline residues were not detectable in grass and clover shoots from both harvests, as all samples were below the detection limit (S/N = 3). In contrast, plants were able to take up small amounts of sulfadiazine from the soil. Sulfadiazine uptake was more noticeable in the first harvest than in the second (Supplementary Fig. 13). However, only five plant samples from the first harvest had sulfadiazine concentrations above the quantification limit (S/N > 10) (Supplementary Fig. 13 A). Even with the sample showing the highest sulfadiazine uptake from the first harvest (3.61 μg kg^{-1} sulfadiazine in grass), it only accounted for 10^{-4}% of the amount in manure. In the second harvest,

sulfadiazine was detectable in five plant samples (S/N > 3), but all concentrations were below the quantification limit (S/N < 10) (Supplementary Fig. 13B).

Discussion

Impacts of antibiotic residues on soil N cycling communities

We hypothesized that both sulfadiazine and oxytetracycline would reduce the abundance of AOB and symbiotic N-fixing microbes (*nifH*) across all plant communities. However, our findings did not support this hypothesis. Although we applied antibiotic concentrations representative of environmentally relevant exposure, they were at the high end of the reported environmental range (Fang et al. 2023). Under these conditions, rather than suppressing susceptible microbial groups, the antibiotics can selectively favor more resistant or tolerant taxa (Gullberg et al. 2011; Murray et al. 2018), resulting in their enrichment within the soil microbial community, such as AOA and N-fixing microbes in this experiment (Fig. 2 and 3).

Similarly, Omirou et al. (2022) found that tetracycline at a concentration of 2 mg kg^{-1} of soil significantly reduced soil AOB abundance but did not affect AOA, while lower concentrations had negligible impacts on both AOA and AOB. Another study suggests that the EC50 (half-maximal effective concentration) of sulfathiazole (a related sulfonamide antibiotic) for soil AOA may be more than 200 times higher than for AOB (Shen et al. 2013). This difference in the susceptibility likely originates from variations in cell membrane structure, with bacterial cell membranes being more permeable to antibiotics (Dridi et al. 2011; Villanueva et al. 2021). Additionally, the mode of action of certain antibiotics, such as sulfonamides, specifically disrupts folic acid synthesis, a pathway that may differ in archaea from that of bacteria, reducing their vulnerability (Brown et al. 2011). The ecological implications of AOA enrichment in grasslands by oxytetracycline exposure are still unclear and need further investigation considering the different physiology of soil AOA and AOB (Sarkar et al. 2025). The soil with relatively higher AOA abundance may have a lower nitrification rate and N_2O emissions after N fertilization (Prosser et al. 2020; Rütting et al. 2021).

In addition to soil nitrifying guilds, our findings of increased *nifH* relative abundance (Fig. 2) contrast with Zhang et al. (2024) who observed that tetracycline significantly reduced *Bradyrhizobiaceae* abundance in soybean root nodules. A possible explanation lies in methodological differences. Zhang et al. (2024) used genus-specific primers (*Bradyrhizobiaceae*), potentially overlooking N-fixing bacteria from other genera. Additionally, Zhang et al. (2024) applied tetracycline concentrations of 10 to 25 mg kg^{-1} in soils, far higher than the concentration used in our study. At more realistic concentrations (0.1 mg kg^{-1}), Revellin et al. (2018) observed that applying manure with multiple antibiotics could change the community composition of microbes occupying soybean nodules, and many root-nodulating isolates are resistant to a mixture of three antibiotics (a mix of sulfonamide, tetracycline, and macrolide). Similarly, a metagenomic study on wild legumes suggests that antibiotic resistance genes are most abundant in root nodules, followed by rhizosphere and bulk soils, indicating that *Rhizobium* and related microbes may be reservoirs of antibiotic resistance (Liu et al. 2022). Given that we inoculated all soils with *Rhizobium trifolii*, this strain may have a higher tolerance or resistance to oxytetracycline than other N-cycling microbes.

Our findings also suggest that antibiotic persistence in soil potentially modulates the antimicrobial impacts on soil N-cycling microbes. Oxytetracycline, which persisted at higher concentrations than sulfadiazine (Supplementary Fig. 12), was associated with increased abundance of the *amo*AOA and *nifH* gene (Fig. 2 and Fig. 3), indicating a comparably stronger selection for tolerant or resistant taxa. After manure application, sulfadiazine could dissipate rapidly in soils (Berendsen et al. 2021; Yang et al. 2024), and its antimicrobial effects likely attenuated quickly after the initial exposure. However, rapid dissipation of sulfadiazine does not imply environmental safety. For instance, a field study with repeated sulfadiazine applications revealed that while the first application showed no impact on N-cycling microbial communities, a second application significantly increased the AOA to AOB ratio (Ollivier et al. 2013). Also, sulfadiazine has higher mobility in soil than oxytetracycline and is prone to leach from the soil into aquatic systems (Luo et al. 2011; Spielmeyer et al. 2020). On the other hand, persistent antibiotics in soil such

as oxytetracycline deserve further investigation due to the risk of accumulation in agricultural soils with repeated manure applications, potentially leading to prolonged selection of soil microorganisms.

One limitation of this experiment is that we spiked both antibiotics into antibiotic-free manure rather than using manure collected from animals that had been administered the antibiotics. This approach was chosen due to the distinct physicochemical properties of oxytetracycline and sulfadiazine. These two antibiotics differ in their excretion rates from livestock and in their persistence in manure (Berendsen et al. 2018; Kuppusamy et al. 2018). By spiking the manure, we ensured equal initial concentrations of both antibiotics in the soil across treatments, thereby improving comparability. However, this method may slightly overestimate the antimicrobial disturbance to soil microbial communities. A previous study reported the greatest reduction in soil microbial biomass carbon occurred when oxytetracycline was directly added to soil, followed by manure spiked with oxytetracycline, and finally manure from antibiotic-treated animals (Chen et al. 2014). This gradient likely reflects the role of the livestock gut as a reservoir of antibiotic-resistant bacteria and genes (Xie et al. 2018). Selection for resistant species begins within the animal host, and many resistance genes are associated with mobile genetic elements (Checcucci et al. 2020; Jadeja and Worrich 2022). Therefore, manure from antibiotic-treated animals may introduce a broader array of resistance genes into the soil, potentially mitigating the disruptive effects of antibiotics by increasing microbial adaptability. Future experimental designs should address this issue to more accurately reflect the ecological impacts of antibiotic residues on soil microbial communities.

Impacts of antibiotic residues on plant yield in grassland

We hypothesized that the presence of either oxytetracycline or sulfadiazine in soil would reduce shoot and root biomass across plant communities. This hypothesis can only partly be confirmed. While aboveground biomass remained unaffected (Supplementary Fig. 5 and 6), clover exhibited a significant root biomass reduction by both antibiotics (Fig. 4). Root systems are typically more sensitive than shoots to antibiotic exposure due to their direct interaction with antimicrobial compounds in the soil (Liu et al. 2009; Minden et al. 2018). Many previous studies reporting reductions in aboveground yield have relied on unrealistically high antibiotic concentrations (Liu et al. 2009; Tasho et al. 2018; Mukhtar et al. 2020; Li et al. 2023). In contrast, a systematic review estimated that the average EC10 (effective concentration) of antibiotics on overplant biomass was at 3 mg kg^{-1} in soils for most crops, which is at the very upper-end level reported in the agricultural soils (Carballo et al. 2022; Fang et al. 2023).

A possible explanation for the observed resilience in ryegrass lies in species-specific detoxification capacities (Arslan et al. 2017). Ryegrass (*Lolium perenne* L.) could increase the expression of certain defensive genes, degrading the sulfadiazine in plant cells (Wang et al. 2024). In addition, through network analysis, the researchers also found that the sulfadiazine-degraded bacteria in soil were closely associated with plant detoxification pathways, indicating a symbiotic relationship between ryegrass and soil microbes to degrade sulfadiazine (Wang et al. 2024). Such mechanisms may buffer ryegrass against antibiotic stress. However, no comparable detoxification mechanisms might exist in clover, potentially explaining its impaired root development in this study. We highlight the importance of considering species identity when evaluating the ecological risks of antibiotic residues in grassland ecosystems. Importantly, the selective reduction in clover root biomass may have long-term implications for nutrient cycling. Long-term field-based research will be essential to fully understand these ecological consequences.

Impacts of antibiotics residues on soil N turnover of grassland

We hypothesized that antibiotic residues would reduce N uptake in ryegrass, while for clover, they would lower symbiotic N fixation. However, our findings contradict these expectations. The absence of effects on ryegrass is likely due to insufficient antibiotic concentrations to inhibit growth or N uptake (Supplementary Fig. 5, 6, 7, and 8). In clover, reduced root biomass under realistic antibiotic exposure appears to limit mineral N uptake without compromising N fixation (Fig. 5 and 6).

Among the existing studies, there are relatively few studies on how antibiotics affect symbiotic N fixation,

especially the application of antibiotic-containing manure. In only one other study, researchers found that alfalfa fixed more atmospheric N when receiving manure containing oxytetracycline than with antibiotic-free manure, with no mechanistic pathway for this effect provided by the researchers (Ostermann et al. 2019). While we did not observe increased N fixation, we did find marginally increased abundances of N-fixing microbes, leading us to hypothesize that *Rhizobium* may be resilient or resistant, maintaining N-fixing capacity as long as root biomass remains sufficient for colonization. This hypothesis, however, is based on a single application of oxytetracycline-containing manure in the current experimental set up. Given the high persistence of oxytetracycline, repeated applications could lead to rising soil antibiotic levels. Under such conditions, whether clover could maintain its N-fixing capacity remains unknown and warrants further study.

We investigated the effects of antibiotic-containing manure on soil available N levels and N_2O emissions in absence and presence of grass and clover. Although antibiotics are known to disrupt microbial N transformation processes (DeVries and Zhang 2016; Zhou et al. 2024), plant presence is often overlooked. Plants play a crucial role in soil N cycling by taking up N or interacting with soil microbes (Abalos et al. 2019). In our study, the presence of plants significantly reduced mineral N levels in the soil, leaving minimal N available for soil microorganisms (Supplementary Fig. 10). Manure was applied only at the start of the experiment to introduce N into the system without oversupplying it, thereby preserving the capacity of clover for N fixation. This approach led to a very N-limited system (Supplementary Fig. 10). As a result, N scarcity restricted microbial denitrification, leading to very low N_2O emissions (Supplementary Fig. 11). In a similar experimental setup, a study found that the absence of plants allowed enrofloxacin to substantially increase microbial N_2O emissions, while the presence of vegetables such as radish and pak choi altered microbial community structure and removed significant amounts of soil N, reducing resources available for N_2O production (Lin et al. 2022). Our study is the first to assess the effects of antibiotic-containing manure on N_2O emissions in grassland systems. Notably, our results reflect an N-limited grassland system. In agricultural systems with higher N availability or excessive fertilization, antibiotics may exert different impacts on soil N dynamics.

Conclusion

The persistence of oxytetracycline in soil raised the abundance of certain microbial guilds across all plant communities, such as AOA and microbes possessing *nifH* genes, suggesting that some N-cycling guilds are more tolerant or resistant than others. In monoculture communities, we observed that both oxytetracycline and sulfadiazine significantly reduced root biomass in clover while leaving ryegrass unaffected, highlighting a species-specific sensitivity to these antibiotics. Despite the reduction in clover root biomass, its capacity to fix atmospheric N remained comparable to that of clover grown in soil treated with antibiotic-free manure, indicating the resilience or adaptability of this function to antibiotic contaminants in soil. The long-term implications of the shifts in microbial N-cycling guilds and plant properties caused by antibiotics remain intriguing research topics.

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Author contributions All authors contributed to the study conception and experimental design. Zhongchen Yang and Youssef C. Wang-Touri performed the experiment, data collection, data analysis, and data visualization. The first draft of the manuscript was written by Zhongchen Yang and Youssef C. Wang-Touri. All the authors reviewed and edited the manuscript considerably. The project supervision was performed by Jan Willem van Groenigen, Bjorn J.A. Berendsen, Milou G.M. van de Schans, and Gerlinde B. De Deyn. The funding administration was performed by Milou G.M. van de Schans and Gerlinde B. De Deyn.

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Data availability The datasets generated from the current study are available from the corresponding author upon request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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